MULTIPLE MYELOMA: A STUDY OF THE FACTORS THAT CONTROL DEATH AND CELL SURVIVAL IN NEOPLASTIC PLASMA CELLS.

by

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Abstract

Since malignant plasma cells in myeloma patients home to the bone marrow, a study was undertaken firstly to define the phenotypic profile of a panel of myeloma cell lines and then to investigate their ability to adhere to various extracellular matrix molecules. The extracellular matrix molecules studied were- collagen, fibronectin and FN-RGD, a synthetic peptide consisting of multiple arginine-glycine-aspartic acid (RGD) repeats which mimics the part of the fibronectin molecule that binds to the integrin VLA-5, expressed on some myeloma cells. Adhesion blockade was attempted using monoclonal antibodies directed against various myeloma surface antigens in order to ascertain the importance or otherwise of these molecules in the binding of myeloma cells to members of the extracellular matrix. It was found that, although the panel of eight myeloma cell lines tested all expressed VLA-4 and showed a varying expression of VLA-5, their ability to adhere to fibronectin differed. None of the cell lines tested adhered to FN-RGD or collagen. Adhesion blockade using anti-VLA-4 and anti-VLA-5 antibodies was only successful in half of the lines tested with a combination of the two antibodies resulting in improved blockade compared with either antibody alone.

It has been demonstrated that CD40 crosslinking on the surface of B cells and myeloma cells rescues them from apoptosis and induces IL-6 secretion. This is usually, but not always, accompanied by up-regulation of the *bcl-2* gene. Since the Fas antigen has homology with CD40 and has been reported to act in opposition to *bcl-2*, it was decided to study these genes for mRNA and protein expression in myeloma cells in an effort to elucidate any potential link between expression of these antigens and the onset of multiple myeloma. A more complete knowledge of the interactions between IL-6, CD40 and its ligand, and the expression of *bcl-2* or Fas and its ligand is necessary in order to determine possible improvements in treatment strategies for this disease.

The panel of eight myeloma cell lines used in this study was examined for CD40 and Fas antigen expression by flow cytometric analysis. All the myeloma cell lines tested positive for CD40 and Fas albeit at different intensities. It has been shown that resting tonsil B cells, expressing either low or absent levels of Fas, are induced to express Fas after ligation of CD40 using the CD40Lig-L culture system. Anti-Fas monoclonal antibody was shown to

inhibit the later phases of CD40-induced B cell growth due to apoptosis. Fas ligation was shown to inhibit proliferation and immunoglobulin secretion of CD40 activated B cells in response to recombinant cytokines. This implies that engagement of CD40 antigen on B cells induces Fas expression and sensitises them to Fas-mediated apoptosis. It was decided, therefore, to investigate whether myeloma plasma cells could be stimulated to proliferate in the same manner and whether they, too, would be sensitive to Fas-mediated apoptosis.

It was noted from the literature that the myeloma cell lines resistant to apoptosis were mostly dependent on IL-6 and it was therefore proposed to investigate this further by broadening the study by looking at several myeloma cell lines and comparing IL-6 dependent and independent passages of the same myeloma cell line (ANBL-6) for their susceptibility to Fas-mediated apoptosis. The fact that fresh myeloma samples were also resistant to activation induced cell death (AICD) is interesting since these are likely to be dependent on IL-6 for their growth also. It has previously been reported that Fas antigen is not expressed on normal plasma cells. Two culture systems originally established in an effort to generate factor-dependent B cell lines were adapted for the study of myeloma cell activation of proliferation via CD40 (and the cytokines IL-4 and IL-6) and induction of apoptosis via Fas activation. Although myeloma cell lines are obviously no longer dependent on stromal layers for their propogation it was decided that the use of the CD40 and CD40Lig-L culture systems may potentiate the response to CD40 activation. Cross-linkage of this antigen on the surface of myeloma cells using mouse fibroblasts transfected with either the human immunoglobulin Fc receptor (FcγRII/CDw32) or with the human CD40 ligand constitute the CD40 and CD40Lig-L culture systems respectively. Interleukin-4 was used to test for proliferation as a comparison to interleukin-6 in these studies since it is a potent B cell stimulation factor.

It was initially attempted to establish a fast, reliable, cost-effective and informative method of assessing apoptosis and then to develop an effective assay system for Fas and CD40 activation in myeloma cell lines. Finally, a comprehensive study was undertaken to examine, in these cell lines, the role of activation of CD40 and/or Fas with or without co-stimulatory cytokines (IL-6 and IL-4). Changes in the regulation of CD40, Fas, Bcl-2, IL-6, IL-6R protein or mRNA expression under different culture conditions were assessed using a combination of flow cytometric analysis, immunocytochemistry, reverse transcription

polymerase chain reaction (RT-PCR) and *in situ* hybridisation. It was also attempted to establish whether myeloma cell lines co-expressed the ligands for CD40 or Fas antigen by RT-PCR and by *in situ* hybridisation using sequence-specific riboprobes (none of the lines tested were found positive for transcription or translation of these ligands). It was found that, although all of the myeloma cell lines tested expressed CD40 and Fas antigen, their intensity of expression varied, as did their susceptibility to Fas-mediated apoptosis. All the interleukin-6 dependent cell lines tested (as well as two IL-6 independent cell lines) proved to be resistant to AICD mediated by anti-Fas monoclonal antibody. The addition of IL-6 to these cultures had no effect on this resistance. In contrast, the remaining five myeloma cell lines tested (all IL-6 independent) were killed by Fas activation which was potentiated by CD40 stimulation. IL-6 together with CD40 ligation however, afforded a substantial protective effect on Fas-mediated cell death in these cell lines (50-90% of the cells were still viable following a 72 hour incubation with anti-Fas and IL-6 in the CD40Lig-L culture system). These results have important implications for the direction of future therapy regimes since, if myeloma plasma cells could be rendered insensitive to IL-6 and the Fas antigen on their surface activated via Fas ligand (e.g. as a targeted transgene) *in vivo* this may lead to extensive tumour cell death via apoptosis. Bcl-2 protein expression following a three day incubation with anti-Fas was found to be down-regulated in four out of five of the myeloma lines tested which were susceptible to Fas-mediated apoptosis. The fifth cell line was negative for Bcl-2 expression throughout whilst those cell lines non-susceptible to Fas-mediated cell death showed no change in Bcl-2 expression when incubated with IL-4, IL-6 or anti-Fas with or without CD40 ligation. It will be crucial to further investigate these important findings and to study the possible role of some of the other rapidly emerging Bcl-2 family members as well as the recently discovered interleukin-1beta converting enzyme (ICE) protease cascade with a view to discovering future treatment strategies for this disease.

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I dedicate this thesis to my father Andrew and my sister Judi both of whom I adored and are both lost but never forgotten.

ABBREVIATIONS

AICD	Activation induced cell death
ALL	Acute Lymphoblastic Leukaemia
AP	Alkaline Phosphatase
BCIP	Bromochloro-Indolylphosphate
bp	base pairs
BSA	Bovine serum albumin
Ca^{2+}	calcium
$CHCl_3$	Chloroform
CML	Chronic Myelogenous Leukaemia
cpm	Counts per minute
CTL	Cytotoxic lymphocytes
ddH_2O	Distilled water
DEPC	Diethyl Pyrocarbonate
DMF	Dimethyl formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPX	Dibutyl Phthalate Xylene
DTT	Dithiothreitol
EBV	Epstein Barr virus
EDTA	Ethylene Diamine Trichloroacetic acid
EtBr	Ethidium Bromide
FACS™	Fluorescence activated cell sorting (Becton Dickinson Trademark)
FCS	Foetal calf serum
FITC	Fluorescein Isothyocyanate
GAM	Goat anti-mouse
HAT	Hypoxanthine-Aminopterin-Thymidine
HCl	Hydrochloric acid
ICC	Immunocytochemistry
Ig	Immunoglobulin
ISH	In situ Hybridisation
IVT	In vitro transcription
kb	Kilobase pairs

LMP	Latent membrane protein
mBq	Megabecquerel
ml	Millilitre
MoAb	Monoclonal antibody
$MgCl_2$	Magnesium chloride
MNC	Mononuclear cells
µg	microgram
NaAc	Sodium acetate
NBT	Nitroblue Tetrazolium
NHS	Normal human serum
dNTP	Deoxynucleoside triphosphate
ng	nanogram
OD	Optical Density
PBS	Phosphate buffered saline
PCD	Programmed cell death
PE	Phycoerythrin
PK	Proteinase K
PVP	Polyvinylpyrollidine
RNA	Ribonucleic acid
RT	Reverse Transcriptase
SDS	Sodium dodecyl sulphate
SSC	Standard Sodium Citrate
STWS	Scottish Tap Water Substitute
Ta	Annealing temperature
TBE	Tris Borate EDTA
TBS	Tris buffered saline
TE	10mM Tris Hcl, 1mM EDTA
Tm	Melting temperature
U	units
UV	Ultraviolet
vol.	volume
v/v	volume:volume
X-Gal	5-Bromo-4-chloro-3-indolyl-βD-galactoside

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CHAPTER 1

INTRODUCTION

MULTIPLE MYELOMA: A STUDY OF THE FACTORS THAT CONTROL DEATH AND CELL SUVIVAL IN NEOPLASTIC PLASMA CELLS

Multiple myeloma is a malignancy of bone marrow plasma cells, with an incidence in the United Kingdom 4:100,000 (1). It is characterised by the proliferation of malignant plasma cells in the bone marrow (BM), an abnormal clonal immunoglobulin in the blood and lytic bone lesions. Plasma cells are terminally differentiated B lymphocytes responsible for secreting normal immunoglobulin, and in myeloma, the mutation of a single B lymphocyte leads to the proliferation of a clone of identical cells which will all secrete the same immunoglobulin molecule and which have the morphology of plasma cells (2). Malignant plasma cells from myeloma patients are rarely found in the circulation but monoclonal immunoglobulin (paraprotein) is present in the serum and/or urine in 98% of cases (monoclonal free light chain in the urine is termed Bence-Jones protein). The remaining 2% have non-secretory disease (1). The serum paraprotein may be of any other immunoglobulin class other than IgM, which, in multiple myeloma, occurs only as an extreme rarity.

The disease can present with a variable spectrum of features and at different stages of development. Patients initially diagnosed with monoclonal gammopathy of unknown significance (MGUS) can remain clinically stable for many years, whereas others may proceed rapidly from stage I (smouldering or indolent myeloma) to stage II disease. Anaemia, hypercalcaemia, and/or renal insufficiency are frequent in stage III myeloma (3).

The incidence of the disease varies. It is most rare amongst the Chinese population (1:100 000). The incidence in white Americans is around 4:100 000, whilst it is most common amongst black American males, 10:100 000. The present incidence of myeloma is between 1500 and 2000 cases per year in England and Wales with around 250 cases per annum in Scotland (1).

Myeloma is predominantly a disease of the elderly with the mean age at diagnosis around 60 years (4). With the exception of chronic lymphocytic leukaemia, the disease shows the strongest age dependency of any neoplasm (5). Peak age of onset is between 65 and 75 years of age with a male preponderance of 70-80% in the earliest reported cases (6). Since

then, however, there has been a gradual shift towards an almost equal sex incidence (only 5-10% excess in male incidence) in newly diagnosed cases.

The longterm prognosis with this disease is not good - a median of around 30-36 months is typical although patients who respond well to therapy survive longer (7). Current therapy regimes consist of cytotoxic therapy or combination chemotherapy, the most commonly used drugs being Melphalan, dexamethasone, prednisolone, carmustine, doxorubicin and cyclophosphamide (1,8).

The vast majority of patients (80-90%) respond well to initial therapy with the majority of these showing a 75% reduction in tumour mass. Those responding to therapy generally manifest a rapid improvement in bone pain and reversal of symptoms of hypercalcaemia. Anaemia recovers gradually but bone lesions rarely heal (9). Depressed levels of normal immunoglobulins rarely improve and complete disappearance either of paraprotein or the monoclonal plasma cell infiltration of the bone marrow is very unusual. Stable patients have a low paraprotein level and remain in the "plateau phase" for long periods even when therapy is discontinued. Plateau phase is usually defined as 3-6 months of clinical stability, stable paraprotein levels and transfusion independence (8,10). It is either present at diagnosis or acheived by chemotherapy (smouldering myeloma).

All patients eventually relapse due to residual tumour cells. This can initially be treated with alkylating agents but myeloma becomes progressively drug resistant and patients die from renal failure, anaemia and infection. Myeloma cells from the bone marrow may spill over into the blood stream resulting in a plasma cell leukaemia at this stage.

Ultrastructurally, most myeloma cells ressemble normal or reactive plasma cells in that they are round or oval with a nucleus, small in relation to cell size and eccentrically placed. Myeloma plasma cells contain more nucloeli than normal plasma cells and bi- or multi-nucleate forms are frequently present. The cytoplasm is characterised by abundant rough endoplasmic reticulum and a highly developed Golgi apparatus in the paranuclear area. It has been noted that the cytoplasm of myeloma cells is disproportionately mature in comparison to the degree of nuclear differentiation and that this degree of asynchrony is proportional to the extent of the disease (11).

The precursor cell in multiple myeloma is still a matter of debate but evidence suggests that it may be a plasmablast. Bakkus et al (12) have shown that the pre-myeloma cell is situated in the pre-switched but somatically mutated B cell compartment and that heavy chain switching can occur without further somatic mutation. They demonstrated that the VDJ (variable, diversity and joining) region of the myeloma immunoglobulin heavy chain gene is somatically hypermutated and antigen-selected without intraclonal variation or evolution in time supporting the theory that myeloma precursors develop from a late stage in B lymphocyte development. A number of workers have also found lymphoid cells in the marrow and blood of patients with myelomatosis which can be induced in vitro to differentiate into plasmablasts (13).

Multiple myeloma and interleukin-6

Studies of patients with plasma cell leukaemia and *in vitro* studies with long-term myeloma cell lines have shown that multiple myeloma is dependent on the cytokine, interleukin-6 (IL-6), for growth and proliferation (14,15). Most myeloma cells proliferate very slowly, however and the importance of IL-6 in these cases is not clear. Whether this growth factor is produced by an autocrine or paracrine loop is also a matter of debate. It may be that, in the initial stages of the disease, the myeloma cells require paracrine IL-6 production produced by the stromal cells of the bone marrow but as the disease becomes established, an autocrine loop takes over (16,17).

Interleukin-6 is a pleiotropic cytokine with a wide range of biological activities. It acts on B cells inducing terminal differentiation into antibody secreting cells (18-24). IL-6 is also an essential accessory factor for T-cell activation and proliferation (25,26). It stimulates production of acute phase proteins by hepatocytes (27-29) and has colony stimulating activity on haematopoietic stem cells (30-32), fibroblasts (33) and cells of the neural system (34). It is produced by many cell types including monocytes (35), fibroblasts (36), T cells and B cells (37), endothelial cells (38), and various tumour cells (39). Interleukin 6 is also a potent growth factor for murine plasmacytomas and B cell hybridomas (40) and for human multiple myeloma both *in vivo* and *in vitro* (41-45,52).

Interleukin-6 initiates it's biological effects through binding to a high affinity receptor complex consisting of two membrane glycoproteins: an 80 kDa membrane bound receptor which binds IL-6 with low affinity (IL-6R,CD126) and a component with a molecular size of 130 kDa (gp130,CDw130) which is required for high-affinity binding and for signal transduction (46,47).

The signal is generated into the cell through gp130 which acts as a transducer, not only for IL-6, but also for Oncostatin M (Kaposi's sarcoma growth factor), Leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and IL-11 (47-52). This indicates that activation of the gp130 IL6 transducer is a key signal for inducing myeloma cell proliferation. Increased and high levels of (soluble) sIL-6R are found in the plasma of myeloma patients as compared with age-related healthy controls (53). As a result of this sIL-6R is a powerful prognostic factor in multiple myeloma during the course of the disease, patients with high levels having the worst prognosis (54). Inhibition of IL-6R or gp130 transducer chain by antibodies or other antagonists as a treatment regime would appear to be difficult since there are very high levels of soluble IL-6R and soluble gp130 circulating in plasma, and IL-6R and gp130 are present on many cells.

In patients with multiple myeloma, malignant plasma cells proliferate and accumulate in the bone marrow in close proximity to stromal and haematopoietic cells (myeloid, monocytic, fibroblastic). It has been demonstrated that short-term in vitro cultures of bone marrow stromal and myeloma cells results in spontaneous myeloma cell proliferation (52). These short term cultures produce a variety of cytokines including IL-6, G-CSF (granulocyte colony stimulating factor), IL-11, IL-1, TNF and others. The addition of anti-IL-6 antibodies almost completely inhibits the proliferation of these cultures indicating that IL-6 is an essential myeloma growth factor in vitro.

In favour of the paracrine theory of initial IL-6 stimulation in myeloma patients is the observation that freshly isolated myeloma cells in culture without the addition of exogenous cytokines fail to proliferate (55). Furthermore, it has been shown that IL-6 production by stromal cells can be up-regulated by cytokines secreted by the myeloma cells, particularly IL-1 and TNF-α (56). Also, myeloma cell adhesion triggers IL-6 secretion by normal or myelomatous bone marrow stromal cells (BMSC) and related IL-6-mediated tumour cell

growth (57,58). It would appear that this process requires cell to cell contact between tumour and BMSC's since paraformaldehyde fixation of the BMSC's prior to tumour cell adhesion was found to abrogate IL-6 secretion. This implies that BMSC's are the major source of IL-6-mediating paracrine myeloma cell growth with the adhesion of myeloma cells to BMSC's leading to the induction of IL-6 transcription in BMSC's. In contrast, there are data from *in vitro* studies of myeloma cell lines to suggest that myeloma cells themselves contribute to the production of excess IL-6 which implies an autocrine growth loop for tumour expansion. IL-6 has been detected in cell culture supernatants (17) and myeloma cell growth has been shown to be inhibited by anti-IL-6 monoclonal antibody and IL-6 antisense oligonucleotides (17,59).

Adhesion molecule expression in Multiple Myeloma

During differentiation, leucocytes show a changing pattern of adhesive interactions with extracellular matrix proteins as well as with other cells. These adhesive properties are mediated by specific surface proteins designated as cellular adhesion molecules (CAM). Cellular adhesion molecules in general are widely distributed amongst many cell types and play an important role in guiding cell migration and localisation in embryonic development.

Adhesive interactions between neoplastic cells, endothelial cells and extracellular matrix (ECM) proteins are crucial for the localisation of tumours as well as their egress from sites of origin. The proteins of the extracellular matrix (ECM), fibronectin, collagen, laminin are components of the bone marrow microenvironment and tumour cells may adhere to these proteins via various adhesion molecules on their surface.

It is now recognised that adhesion between cellular elements and the intercellular matrix is mediated through molecules which belong to a number of major groups or families of molecules. These include the integrins, the immunoglobulin superfamily, the selectins, cadherins and cell surface proteoglycan (60). It may also be that surface adherent growth factors may contribute to cellular adhesion and subsequent activation.

Integrins are heterodimeric membrane glycoproteins expressed on diverse cell types and function as the major receptors for extracellular matrix and cell-cell adhesion molecules. As

adhesion molecules, they play an important role in numerous biological processes such as platelet aggregation, inflammation, immune function, wound healing, tumour metastasis and tissue migration during embryogenisis. There is increasing evidence to implicate integrins in signalling pathways, transmitting signals both into and out of cells.

Three subfamilies of integrins can be distinguished by their beta subunits: these are known as the β1 (CD29), β2 (CD18) and β3 (CD61) integrins (61). Members of the β1 integrin subfamily (VLA proteins) contain the beta1 subunit in association with at least 6 different alpha subunits and include receptors that bind to the ECMs fibronectin, laminin and collagen.

Fresh myeloma plasma cells have been found to strongly express the intercellular adhesion molecule, ICAM-1, the fibronectin receptor, VLA-4 (Beta1/alpha4 integrin) and the lymphocyte homing receptor, CD44 on their surface (60,62,63). A similar phenotype was found in normal plasma cells, suggesting that these adhesion molecules, or at least some of them, are involved in the normal homing process of plasma cells in the bone marrow. The binding of the fibronectin receptor, VLA-4, to fibronectin has been shown to be an important event in IL-6-mediated proliferation of plasma cells in normal bone marrow (64). The integrin VLA-5, which also binds to fibronectin, has also been shown to be expressed in myeloma cell lines (65,66). VLA-5 binds to an RGD- (arginine-glycine-aspartic acid) peptide on the fibronectin molecule (66,67). Expression of surface fibronectin and fibronectin mRNA (detected by Northern blot analysis) has also been shown in these myeloma cell lines (66).

Initial investigation of marrow myeloma cells suggest that they lack alpha2/beta1, alpha3/beta1 or alpha6/beta1 and have only variable expression of alpha4/beta1 integrins (68,69). The lack of alpha2/beta1 (VLA-2) and alpha6/beta1 (VLA-6) expression is consistent with their inability to invade endothelial basement membranes, which are composed of collagen type IV and laminin (65). Preliminary studies suggest that myeloma cells cannot bind to rat high endothelial venules (HEV), consistent with their lack of migration into peripheral blood (70). In contrast, plasma cell leukaemias do adhere to rat HEV, facilitating their exit into the circulation. Since some myeloma cells and cell lines

express CD44 which binds to high endothelial venules these observations are somewhat contradictory however and require clarification.

Expression of CD44 which is a broadly distributed cell surface protein which is thought to mediate cell attachment to extracellular matrix components or specific cell surface ligands, has been correlated with lateral movement on the endothelium in VLA-4 positive cell lines (71). CD44 is the principal cell surface receptor for hyaluronate which is an important component of the extracellular matrix. A large fraction of newly diagnosed myeloma patients have been demonstrated to have elevated levels of serum hyaluronan (72) and CD44 is widely expressed on myeloma cell lines (65), and fresh marrow plasma cells (62).

Normal peripheral blood B cells express VLA-4 but the level of alpha4 subunit is almost twice the level of beta1, suggesting that not all of the alpha4 subunit is associated with beta1 (73). Studies on myeloma cell lines (65) revealed that the levels of alpha4 and beta1 subunits are equivalent in contrast with normal B cells.

The adherence of VLA-4 to fibronectin can be partially blocked by anti-β1 integrin or anti-α4 integrin monoclonal antibodies (65). Also, myeloma cell lines expressing VLA-4 are partially blocked from binding to fibronectin by the addition of RGD peptide, suggesting that their binding to fibronectin is mediated, not only by VLA-4 protein, but also by other receptors with fibronectin binding properties (eg VLA-5, vitronectin receptor or gpIIbIIIa), (60,65).

These data suggest that the ability of myeloma cells to bind to fibronectin through VLA-4 and RGD-dependent mechanisms (perhaps through VLA-5 although this integrin is not universally expressed on myeloma cell lines), (65), may contribute to their localisation in the bone marrow and, conversely, the loss of fibronectin receptors may lead to extravasation into the circulation. Recent migration studies of leukaemic cells suggest that VLA-4 may regulate adhesion whilst VLA-5 controls the motility of these cells (74).

Mature B and T cells do not express much fibronectin on their surface, so it is possible that adundant fibronectin expression on myeloma cells (66) is a specific and important phenomenon in the pathogenesis of the disease. Mature B cells circulating in the peripheral

blood may not be able to bind to stromal cells or to the microenvironment because of a lack of fibronectin on their surface. The presence of fibronectin receptors on stromal cells (75) indicates that there may be an interaction between fibronectin on myeloma cells and FN receptors on stromal cells, allowing myeloma cells to localise in the bone marrow. It has also been reported that fibronectin itself may mediate FN-FN adhesion (76). Alternatively heparan sulphate may mediate binding to fibronectin, since heparan sulphate proteoglycans reportedly attach to fibronectin (66) and it has been suggested that heparan sulphate may mediate specific interactions between cells and their respective environments (77).

Kawano et al (78) reported that myeloma cells, freshly isolated from bone marrow could be divided into two sub-populations. $CD38^{+}VLA\text{-}5^{+}$ and $CD38^{+}VLA\text{-}5^{-}$ cells were distinguished by their response to interleukin-6 induced proliferation. The $VLA5^{-}$ population consisted of mostly immature, IL-6 responsive cells whereas the $VLA\text{-}5^{+}$ population were mature plasma cells which were not responsive to IL-6 stimulation in vitro.

Proteoglycans which also form part of the extracellular matrix, are named according to their glycosaminoglycan (GAG) side-chain and are also invoved in cell-matrix adhesion. The GAG side chains of proteoglycans in stromal cultures include heparan sulphate, heparin and chondroitin sulphate. Hyaluronic acid, another GAG present in the ECM, is not covalently bound to protein to form a proteoglycan (79). The chondroitin sulphates may play a part in the release of mature cells into the blood stream and in the premature release of leukaemic cells (80-82).

N-CAM (CD56), the neural cell adhesion molecule has been found to be strongly expressed by myeloma plasma cells in the majority of patients and is absent from normal plasma cells (62,83,84). The absence of N-CAM expression in some myeloma cell lines revealed N-CAM mRNA expression on subsequent analysis by northern blot analysis (85). It looks as if N-CAM expression in myeloma is regulated at the transcriptional level.

It is interesting to note that N-CAM is not usually expressed in circulating cells in patients with end stage myeloma and plasma cell leukaemia (63) and neither is it expressed in refractory myeloma patients. Patients positive for N-CAM prove to be more responsive to chemotherapy (62). It appears from these studies that this adhesion molecule is probably

mostly downregulated with progressive disease and peripheral involvement, perhaps indicating that downregulation may facilitate detachment of tumour cells from the marrow environment into the circulation. This is in contrast to VLA-5 expression which appears to be upregulated during prolonged cell culture and in fresh myeloma bone marrow as the disease progresses and mature myeloma cells come into contact with bone marrow stroma (69,78).

In summary, multiple adhesive interactions are undoubtedly occurring as a dynamic process controlling the localisation and adhesion of myeloma plasma cells in the bone marrow. Adhesive interactions, in turn, appear to regulate cytokine production either from the plasma cells or the stromal cells providing a favourable environment for tumour cell expansion. Further investigation should explain why, in the minority of patients who proceed to develop plasma cell leukaemia, these malignant plasma cells eventually extravasate back into the general circulation.

Adhesive interactions of myeloma cells with bone marrow stroma - effect of CD40 activation

Since it has been demonstrated that myeloma plasma cells express adhesion molecules (60,62,65,83,86,87) and that these are almost certainly linked to localisation of myeloma cells in the bone marrow, the ability of tumour cell adhesion-induced IL6 secretion was examined by blocking cell surface-associated molecules with monoclonal antibodies (57). Antibodies to CD29, VLA4, VLA5, CD18, CD11a, ICAM-2 ICAM-3 and CD44 failed to prevent IL-6 secretion. Activation of the pan-B cell marker CD40, which has also been demonstrated as being present on malignant plasma cells (88) but absent from normal plasma cells (89) on the other hand, has been demonstrated to result in IL-6 secretion by B cells (90) and myeloma cells (88). Cross-linking of cell surface CD40 on human B cells with a monoclonal antibody (MoAb) to CD40 (90,91,92), or cross-linking of CD40 with its ligand (CD40L) which is normally found on activated T cells, (90,93-96) results in B cell activation. Similar CD40 activation via anti-CD40 MoAb or through its ligand results in IL-6 secretion in myeloma cells, myeloma cell lines (88) bone marrow myeloma cells, bone marrow stromal cells (BMSC) and BMSC-derived cell lines (57). CD40 ligand has only been shown to be transiently expressed on activated T cells, especially the $CD4^+$ T cell

population to date (96). It may be either that myeloma cells or BMSC express CD40L or accessory cells within the marrow microenvironment (ie, activated T cells) mediate triggering of IL6 secretion by these cells.

CD40, a 50kDa surface glycoprotein, is a member of a family of surface molecules with homology to the low affinity nerve growth factor receptor, the two TNF-α receptors and the antigen *Fas* (89,97-104). It is crucial in normal B cell differentiation and activation with one of it's functions being to rescue germinal centre B cells from apoptosis when activated (105) and another (already mentioned) being induction of low-level IL-6 secretion by cross-linkage of CD40 in normal B cells with mAb (90).

Activation of CD40 results in the induction of homotypic adhesion amongst B cells mediated primarily through LFA-1 and ICAM-1 (106,107), proliferation (91,108), immunoglobulin heavy chain isotype switching - in conjunction with interleukin-4, isotype switches to IgE (109), immunoglobulin secretion (109-111) and rescue of B cells from apoptosis after somatic hypermutation in the germinal centre (105,112,113). Patients with the X-chromosome-linked disease Hyper-IgM syndrome who have an inability to synthesise IgG, IgA or IgE have been demonstrated to have a mutation in the gene encoding the CD40 ligand, (114). Human CD40L, expressed transiently on activated T cells, is a 39kDa type II integral membrane protein with sequence homology to TNF (89). CD40L has mitogenic activity on human B cells comparable to that seen using MoAb to CD40. This effect is considerably enhanced in the presence of additional cytokines including IL-2, IL-4 and IL-10 (115). Since the adhesion molecules LFA-1 and ICAM-1 are also expressed on myeloma cells CD40 activation on the surface of myeloma cells could result, at the very least, in their localisation and aggregation in the bone marrow.

Urashima et al. (57) have recently reported that activation of CD40 on the surface of myeloma cell lines using NIH3T3 CD40 ligand transfectant (CD40LT) cells also resulted in increased surface expression of CD80, CD18, CD11a, CD11b, and CD11c. CD40 stimulation is known to upregulate CD80 (B7-1) expression on normal and leukaemic B cells (116-118). CD80 is a ligand for CD28 expressed on T cells and it s activation results in cytokine secretion by T cells, eg IL-2. Since activated T cells also stimulate B cell

differentiation and proliferation it appears from these results that T and B cells exhibit a reciprocal amplification mechanism.

The intracellular signal transduction pathways utilised by CD40 to generate its diverse and potent effects on B cells are not yet clearly defined. It is likely that CD40 may be connected to different signalling pathways at different stages of B-cell differentiation. For example, whereas CD40 delivers a rescue signal to germinal centre B cells about to undergo apoptosis, it can actually promote programmed cell death in some B cell lymphoma lines (119). Also, CD40, generally thought to mediate signalling for B cell viability and growth, has been shown to induce surface expression of Fas and to promote susceptibility to Fas-mediated apoptosis (120-122).

The Cell Surface Antigen - Fas (APO-1).

The human cell surface antigen Fas (CD95) is a 50kD transmembrane protein expressed in many neoplastic and normal cells including lymphocytes, myeloid cells, liver, heart and ovary, myeloma cells, lymphoma cells and other tumour cells (101). Fas belongs to the tumour necrosis factor receptor (TNFR), nerve growth factor receptor (NGFR) and CD40 antigen families (47).

The human Fas ligand (FasL) is a member of the TNF family and is expressed in activated splenocytes and thymocytes (123). It is involved in T cell mediated cytotoxicity (death by apoptosis or activation induced cell death). Fas antigen (APO-1) is also expressed on germinal centre cells which are IgD negative, but not on follicular mantle zone (surface) sIgD positive lymphocytes (103). This is in complete contrast to Bcl-2 protein expression (102). An inverse relationship between the expression of Bcl-2 protein and Fas antigen in germinal centre cells and lymphoblasts has been demonstrated leading to the postulation that upregulated Fas antigen expression concommitant with downregulated *bcl-2* expression may help to control apoptosis *in vivo* (102). The cytoplasmic domains of members of the NGFR/TNFR family consist of 36-221 amino acids and in the case of the CD40 system, this region is essential for signal transduction (104). The cytoplasmic domain of the Fas antigen is 41% homologous with the corresponding region of the CD40 antigen.

The intracellular domain of Fas (145 amino acids) contains a region ("death domain") which shows homology with the p55 TNF receptor (TNFR1) which is reported to be essential for transmission of the apoptotic signal (298). Signalling by the p55 tumour necrosis factor receptor and by Fas antigen is initiated by receptor clustering and self interaction via their structurally related death domains (298,299). TNF and Fas ligands occur as homotrimeric molecules and can therefore induce clustering of receptors merely by binding to them (300). Activation via the death domains of TNFR1 and Fas triggers apoptosis and activation of the transcription factor NF-κB (298,299). The death domain seems to modulate multimeric interactions between receptor-associated proteins that can induce apoptosis (300-305).

Two distinct classes of receptor-associated proteins have been described which appear to couple the TNF and Fas receptors to downstream signalling cascades. Three intracellular proteins have been described which contain death domains which interact with the death domains of Fas and TNFR1. TRADD (TNFR associated Death Domain) is a 34kD protein which interacts specifically with TNFR1 (301), whereas the 23kD Fas associated death domain, FADD (303,304) and the 74kD receptor interacting protein, RIP (305) interact with Fas. Death domains have been shown to be capable of both homotypic and heterotypic associations (301-304) suggesting that they may function as adaptors to couple some members of the TNFR superfamily to other signalling proteins. Overexpression of TRADD activates TNFR1-like signalling pathways for both apoptosis and activation of the transcription factor NF-κB (301). Similarly, overexpression of FADD (303,304) or RIP (305) mimics Fas activation leading to activation induced cell death. The second family of signal transducing proteins utilised by the TNFR family are the TNFR-associated factors (TRAFs). TRAF1 (45kD) and TRAF2 (56kD) exist as a multimeric complex that interacts via TRAF2 with the signalling domains of both TNFR2 and CD40 (306,307). TRAF3 was identified by a yeast two-hybrid interaction cloning system as a CD40 associated protein of 64kD (308,309). The three known TRAFs share a highly conserved C-terminal "TRAF domain" of about 150 amino acids, which is involved in oligomerisation and receptor association (306,307,309). Overexpression of TRAF2 but not TRAF1 or TRAF3, activates NF-κB and TRAF2 has been shown to be a common mediator of signal transduction by TNFR2 (p75) and CD40 (307). TRAF3 has also been shown to interact with the

cytoplasmic C-terminus of EBV-LMP1 (310), suggesting that some of the pleiotropic effects of the LMP1 on cell phenotype and growth may be mediated through TRAF3.

Although ligation of CD40 expressed on the surface of B lymphocytes provides a potent survival signal, this molecule is also expressed in basal epithelial cells and in a number of different carcinomas where its function is unknown (311-313). It has been reported that, contrary to studies in normal B cells, CD40 ligation in some carcinoma cell lines and in normal primary epithelial cells results in growth inhibition and enhanced susceptibility to apoptosis induced by the anti-neoplastic drugs, TNF-α, Fas and ceramide (314). The expression of Bcl-2 did not affect growth inhibition induced by CD40 ligation in epithelial cells but its homologue EBV-LMP1 blocked the effect. It has been postulated from these experiments that CD40 regulates epithelial cell growth in a manner mimicked by LMP1 and TRAF3 has been implicated as a common mediator in the transduction of the growth inhibitory signals generated via the CD40 and LMP1 pathways.

The role of Fas in the immune system is perhaps best exemplified in *lpr* and *gld* mice. These mice have either a point mutation in the gene encoding Fas ligand (mice homozygous for *gld* - generalised lymphoproliferative disease), (124) or in the gene encoding the Fas antigen (mice homozygous for *lpr* - lymphoproliferation). Both phenotypes lead to the development of lymphadenopathy and autoimmune disease (125). This may be explained by a failure of autoimmune cells to undergo apoptosis, ie Fas antigen has an important role in the negative selection of autoreactive T cells in the thymus.

In addition to the massive accumulation of abnormal T cells, *lpr* and *gld* mice produce large amounts of polyclonal IgG and IgM, indicating that B lymphocytes are also abnormally activated. Since activated, mature B cells express Fas, it seems that collaboration between T cells expressing FasL and activated B cells expressing Fas results in removal of the activated B cells. It could be postulated that myeloma plasma cells have escaped normal homeostatic control due to a defect in the Fas activation network. This could be manifested either by non-functional Fas antigen expression (rather than no expression since most myeloma cells and cell lines are strongly positive for Fas antigen expression as demonstrated by flow cytometry), (126 and personal findings), or possibly due to a mutation in Fas ligand expression as in *gld* mice. Studies on the T cells of myeloma patients however, (126a), have shown that activated ($HLA\text{-}DR^+$) T cells found in these patients co-expressed Fas but lacked Bcl-2. They also reported that there was a higher incidence of Fas positive T cells and a lower percentage of Bcl-2 positive T cells amongst myeloma patients as opposed to controls. The $HLA\text{-}DR^+$ restricted T cells were sensitive to Fas-mediated apoptosis as opposed to other T cell subsets in these patients. They concluded that myeloma T cells have a dysregulated expression of Fas and bcl-2 antigens and that this is associated with an enhanced susceptibility to apoptosis amongst the T cell population. This could mean that T cells from myeloma patients are weakened in their ability to exert an anti-tumour response *in vivo*.

Normal B lymphocyte development

Since myeloma is a tumour of the B cell lineage a review of normal B cell lymphopoesis was undertaken with the view that there may be some similarities in the processes of cell activation and induction of, or protection from, activation-induced cell death (apoptosis).

Germinal centres (GC) develop in secondary lymphoid tissue from primary lymphoid follicles as B cells respond to T-dependent antigenic stimulation (127). Analysis of the phenotype of human tonsillar germinal centre cells in situ identifies four distinct compartments - the inner dark zone, the basal light zone, the apical light zone and the outer zone, all of which are encompassed by the follicular mantle (128).

Upon antigenic stimulation, rapid B cell proliferation gives rise to the dark zone which contains centroblasts and is relatively devoid of T cells. These centroblasts undergo somatic mutation of their immunoglobulin variable (V) region genes. Maturing B cells then move into the basal light zone which also contains abundant follicular dendritic cells and some T cells. These centrocytes are then selected on the basis of their affinity for antigen presented by follicular dendritic cells, those with lower affinity being eliminated by apoptosis (death by neglect or programmed cell death). The high affinity centrocytes expressing IgM surface antibody then undergo isotype switching of their immunoglobulin heavy chain (129). Those cells, which would otherwise die (centrocytes and centroblasts, not selected by antigen), can be rescued from apoptosis by cross-linking surface CD40 with recombinant cell-bound CD40L (CV1-EBNA-CD40L),(112), by treatment with MoAb to CD40 or cross-linked anti-immunoglobulin (anti-Ig bound to sheep red blood cells),(130).

In the apical light zone the centrocytes (B blasts) can then follow one of two terminal differentiation pathways, becoming either a memory B lymphocyte or a plasmablast precursor to a plasma cell. Those cells selected via CD40 cross-linking (with subsequent induction of Bcl-2 protein expression) becoming memory cells whilst those selected via CD23 (low affinity receptor for IgE) + interleukin-1α become plasmablasts (105,128,130-134). The B cells in the follicular mantle are long-lived resting cells which are resistant to apoptosis (133,135,136). It has been demonstrated that rescue from apoptosis by CD40 cross-linking in germinal centre B cells is usually (105,130), but not always (112) accompanied by up-regulation of the *bcl-2* gene.

Fas ligation induces apoptosis of CD40-activated human B lymphocytes.

Fas is expressed at high levels on B cells in the germinal centres of secondary lymphoid follicles where massive B cell proliferation, somatic mutations within Ig variable region genes and antigen-driven selection of high affinity B cells occur (102,129,137,140,142). Germinal centre B cells are characterised by their rapid entry into spontaneous apoptosis, a process that can be prevented by CD40 ligation but accelerated by Fas triggering (121,130). Since resting B cells are virtually devoid of Fas and are not prone to apoptosis (102) and CD40-CD40L interaction appears to be necessary to induce germinal centre formation (138,139) it was investigated whether CD40 activation of B lymphocytes could modulate Fas expression and function (120). It was shown that CD40 engagement, using either a recombinant human CD40L or a cross-linked anti-CD40 MoAb, induces resting B cells to express high levels of Fas antigen and that ligation of such, using a monoclonal anti-Fas antibody (clone, CH-11), leads to apoptotic cell death (120,121). This resulted in inhibition of CD40-induced B cell growth and differentiation in response to recombinant cytokines (IL-2, IL-4 and IL-10) indicating that the function of Fas on B cells prevails over that of CD40 and is therefore likely to control the expansion of antigen-specific B cell clones and that none of these B cell trophic factors are able to inhibit Fas-induced death. It has been shown that the activation state of B cells is critical for their sensitivity to Fas (140,141). Although germinal centre B cells express high levels of the apoptosis-inducing antigen Fas this does not appear to be involved in antigen-specific selection (142).

Bcl-2 expression in Germinal centres and myeloma cells

Bcl-2 is a proto-oncogene expressed in normal and malignant cells of the lymphoid and myeloid lineages. The Bcl-2 protein acts by protecting cells against programmed cell death (apoptosis), and maintenance of B cell memory since there is a high level of Bcl-2 expression in long-lived, recirculating, memory B cells (105,136,143). Bcl-2 is now known to inhibit apoptosis induced by a wide range of agents. Enhanced survival in the absence of cytokine (144) has been demonstrated for both normal and immortalised cells of several lineages (lymphoid, myeloid, neuronal). Substantial protection is also afforded against irradiation, genotoxic drugs, glucocorticoids, sodium azide, calcium influx, heat shock and reactive oxygen

species (145). Thus, multiple pathways to cell death must converge on a step that can be regulated by Bcl-2.

The subcellular localisation of the Bcl-2 protein has been found, by subcellular fractionation studies, to be within the inner mitochondrial membrane (146) although some groups have demonstrated the Bcl-2 protein to be in the separated membrane fractions containing the nuclear envelope, plasma membrane and endoplasmic reticulum of t(14;18) cells from patients with follicular lymphoma (147-150). These data suggest that Bcl-2 may belong to a group of proteins involved in import of precursors across membranes.

The *bcl-2* gene was initially identified at the chromosome breakpoint of the t(14;18) translocation which is present in more than 70% of Follicular Lymphomas and involves a reciprocal translocation between chromosomes 14 and 18 (151). This brings part of the *bcl-2* gene on chromosome 18 into juxtaposition with the immunoglobulin heavy chain gene locus on chromosome 14. Most breakpoints on chromosome 18 occur in the untranslated region of the *bcl-2* gene, with the consequence that the portion of the gene encoding this protein remains intact (148,152-154).

Detailed studies by Korsmeyer of the B cells within the different compartments of germinal centres have revealed that the expression of the protein product of the *bcl-2* proto-oncogene (by immunohistochemical assessment) reflects the degree of apoptosis within these compartments (136). There is no Bcl-2 protein expressed in the centroblasts of the dark zone or centrocytes of the light zone, where these cells are dying by apoptosis. There is a very low level of Bcl-2 protein expression in the B blasts of the apical light zone where B cells differentiate into plasma cells or memory cells but there is marked Bcl-2 expression in cells of the follicular mantle which comprise long-lived, recirculating $IgM^{+}IgD^{+}$ B cells (see Figure 2.2.10a).

Comparison of *bcl-2* mRNA and protein expression in germinal centres and in myeloma cell lines, by *in situ* hybridisation, immunohistochemistry and flow cytometry techniques indicates that *bcl-2* mRNA is expressed throughout the germinal centre albeit in varying amounts, and in most myeloma cell lines tested but the protein product is only manifested in the follicular mantle, in normal plasma cells and in some myeloma cell lines

(136,155,156,157 and personal findings). The *bcl-2* gene has been reported to be downregulated at later stages of B lymphocyte differentiation (158, 159), although it has been demonstrated as present in normal plasma cells (156). It is of interest that the gene is consistently expressed in myeloma cell lines although not always translated (155, 156 and personal findings). In keeping with this, it has been reported that, whilst normal and neoplastic lymphoid cells express messenger RNA transcripts, translation does not necessarily follow implying that *bcl-2* gene regulation is post-transcriptional (155,160).

It has been proposed that Bcl-2 plays a critical role in the growth and prevention of spontaneous or Dexamethosone-induced apoptosis in myeloma cell lines (161). It has also been reported that Bcl-2 is upregulated in fresh myeloma plasma cells with normal levels of expression in patients with monoclonal gammopathy of unknown significance (MGUS), (162). The role of *bcl-2* in conferring resistance to drug-induced apoptosis in B cell malignancies, especially in Follicular Lymphoma has been examined (163). The frequency of t(14;18) in myeloma is only 10-15% but several groups (156,161) have found that the majority of myeloma cell lines and freshly isolated myeloma cells express Bcl-2 protein. This suggests that Bcl-2 in myeloma may play a similar role to Bcl-2 in follicular lymphoma but in the absence of t(14;18) expression. Dexamethasone is one of the most effective drugs for the treatment of myeloma and has been shown to induce apoptosis in various myeloma cell lines (164), the extent of which was inversely correlated with intracellular levels of Bcl-2 (161,165).

Apoptosis and T cell-mediated cytotoxicity

Apoptosis is a widespread biological phenomenon in which cells are deleted, during the course of their normal development, through a programmed response, rather than as a result of accidental injury (166). Apoptosis is an active process that provides an additional means of precisely regulating cell numbers and biological activities (167). It is a highly ordered process characterised by nuclear changes such as chromatin condensation, fragmentation and margination as well as internucleosomal DNA cleavage into approximately 180 base pair segments (168). Ultrastructural changes also occur that include cytoskeletal disruption, cell shrinkage, cytoplasmic organelle compaction and membrane blebbing leading to fragmentation of the dying cell into numerous membrane-bound apoptotic bodies. These are

subsequently engulfed by neighbouring cells or professional macrophages in the final resolution of the suicide process. Apoptotic suicide has many advantages over other forms of cell death, owing principally to the membrane integrity that is maintained throughout the entire process. There is no leakage of intracellular components, inflammation, or scar formation. The process is remarkably fast and can proceed to completion within a few hours. Necrotic cells, for example, leak their constituents into the surrounding extracellular space usually resulting in an inflammatory response.

Lymphocytes appear to be more susceptible to programmed cell death than most cell types, particularly at early stages of their development (169,170). Apoptosis of lymphocytes is likely to be regulated in multiple ways, most of which are still poorly understood. Death of mature lymphocytes by apoptosis frequently involves signalling through Fas antigen (170). Both Fas and the 55kDa TNF receptor share a distinctive cytoplasmic region (the "death box") essential for inducing apoptosis.

While not all cell types undergoing physiological cell death display every cardinal feature of apoptosis (171), the underlying chemical pathway may prove to be universal. The demonstration that enucleated cells can undergo cytoplasmic changes strongly ressembling those of apoptotic cells and that *bcl-2* blocks this process (172) suggests that apoptosis may be orchestrated from the cytoplasm rather than the nucleus (see below,173).

Recent studies have suggested that there are two distinct pathways of cell death: PCD which is a result of inadequate activation and is independent of Fas, and activation-induced cell death (AICD), which is induced by TCR engagement and is mediated by Fas-FasL interaction (174,175). It has been proposed that the survival genes *bcl-2* and *bcl-x*$_L$ play a crucial role in protecting cells from PCD but not from AICD (174-179) but there are conflicting views on this however (102,180,181).

The Fas pathway is one of two lytic mechanisms utilised by cytotoxic T lymphocytes to kill their targets, perforin being the other (182,183). Fas-mediated cytotoxicity, which is calcium independent for its execution, requires the expression of Fas at the target cell surface (184) and of Fas ligand at the effector cell surface (185). The second mechanism, dependent on calcium for its execution, requires perforin and serine esterases (granzymes or fragmentins), (186,187). Fas-

based T cell-mediated cytotoxicity is the main lytic mechanism in $CD4^+$ MHC class II restricted T cell killing whereas $CD8^+$ MHC class I restricted cytotoxic T lymphocytes use both the Fas and perforin pathways (188,189).

In the method of killing using cytotoxic granules the killing process is initiated by recognition and binding of the target cell to the cytotoxic T lymphocyte (CTL) and the transmission of signals, including calcium fluxes in the CTL. These signals result in rapid reorientation of the secretory apparatus of the CTL towards the target cell and the subsequent secretion of intracellular granules in the direction of the target contact area. Exocytosis of granules into the confined space of the contact area is the next event in the ensuing target-cell death (190).

Perforin is the critical and essential component for the initiation of target cell lysis. It is a pore-forming protein and belongs to the perforin family, together with the complement proteins C6, C7, C8 and C9 which may have arisen from a common ancestor. The presence of perforin-induced pores in the target cell membrane results in an immediate attempt at repair by endocytosis of the membrane patch together with the pore. This results in the uptake of a small amount of liquid from the surrounding medium (pinocytosis) including granzymes which are secreted proteins which may be associated with perforin via the proteoglycan complex or may be present in solution as a result of granule secretion. The internalisation of granzymes during the repair process leads to the initiation of nuclear disintegration and the combination of granzyme- and perforin-mediated lysis results in an unavoidable killing mechanism (190).

Fas based cell death is mediated by receptor-counter receptor interaction. CTLs express the Fas ligand which, upon interaction with Fas antigen on a target cell, mediates apoptosis (184,191). Paradoxically, Fas receptor triggering can also provide a proliferative versus an apoptotic signal (140,181,192) which suggests that *in vivo* Fas may play different roles depending on the context of its cellular interactions.

The ability of *bcl-2* to block these two independent pathways has been studied (180,193) with conflicting results. Chiu et al. (193) suggest that *bcl-2* may block apoptotic lysis induced by perforin plus granzymes, but not apoptotic lysis induced by the Fas-Fas ligand pathway, whereas Lee et al. (180) state that *bcl-2* protects against Fas-based, but not perforin-based T cell mediated cytolysis. This would explain why over-expression of *bcl-2* does not always protect

cells from apoptosis. The role of Bcl-2 in Fas-mediated lysis triggered by Fas antibody is also controversial. An inverse relationship between levels of Fas and Bcl-2 is believed to regulate the ability of Fas to induce apoptosis (102,181) but others have reported that no such relationship exists in many non-haematopoeitic cells and tumour lines (175,179,193).

These controversial results may suggest differences in the mode of action and regulation of *bcl-2* and Fas in different cells. In addition, Fas-antibody may not have the same effects on Fas-signalling as membrane-bound Fas ligand expressed by cytotoxic lymphocytes. Since the Fas ligand acts as a trimer (194) and trimerises the Fas receptor for signalling, antibodies would be unable to act analogously. There is also the possibility that apoptosis is controlled by one of the other members of the *bcl-2* family such as *bcl-x* (195), or Bax (196) although Strasser et al. (175) have recently reported that Fas signalling does not alter expression of Bcl-2, Bax or Bcl-x in a human lymphoblastoid cell line. These results don't exclude the possibility that Fas signalling may interfere with *bcl-2* function by other mechanisms such as post translational modification of Bcl-2 or Bax or by induction of other inhibitors but they raise the possibility that Fas signalling triggers death by a mechanism that bypasses *bcl-2*.

The increasing number of members of the *bcl-2* family which are involved in the control of apoptosis in a range of different cell types include Bax (196), Bak (Bcl-2 antagonist/killer) which functions as an inducer of apoptosis (197), Bcl-x_L, Bcl-x_S (195), A1 (198), Mcl-1 (199), Bad (200). Bag (201) is a Bcl-2-interacting protein which protects against AICD. Bcl-2 forms heterodimers with Bax and this is essential for Bcl-2 to exert its protective effect since mutations in the BH1 and BH2 binding domains of Bcl-2 result in a protein product which fails to dimerize with Bax with subsequent loss of its protective effect on apoptosis (202). Bcl-2 can also form heterodimers with Bcl-x_L, Bcl-x_S, Bak and Mcl-1 (203,204). In contrast, Bax appears to only interact with Bcl-2 and Bcl-x_L. Bcl-x_L and Bcl-x_S have opposing functions, with the former (47% homologous to Bcl-2) long form of Bcl-x acting as a cell death blocker, and the latter, short form of Bcl-x acting as a Bcl-2 and Bcl-x_L antagonist by promoting apoptosis (195). Bcl-x_L increases the cellular apoptotic threshold and is able to form stable complexes with Bax *in vitro* and *in vivo* (205). Overexpression of Bcl-x_S does not alter the ability of Bax to heterodimerise with Bcl-x_L *in vivo*. In other words, Bcl-x_S does not appear to function by competitively disrupting the formation of dimers composed of other Bcl-2 family members.

In addition to Bcl-x_S, the Bax, Bad and Bak proteins function as promoters of cell death, whilst the Mcl-1 and A1 proteins appear to be suppressors of cell death like Bcl-2 and Bcl-x_L. Several homologues of Bcl-2 have also been discoverd in viruses, including the E1B-19-kD protein of adenovirus and the BHRF-1 protein of Epstein-Barr virus (EBV), both of which function as suppressors of cell death (206,207). The complexity of these interactions among Bcl-2 family proteins has undoubtedly evolved to provide multiple opportunities for fine-tuning the relative sensitivity or resistance of cells to apoptotic stimuli through differential regulation of the expression of various Bcl-2 family genes (200,208).

The ability of Bcl-2 to block programmed cell death by diverse cytotoxic agents (144,145) implies that it acts at a step common to many pathways. Genetic analyses of the programmed cell death of the nematode *Caenorhabditis elegans* revealed that the *ced-3* and *ced-4* genes were both required for programmed cell death to occur (209,210). This could be blocked by the *ced-9* gene (which is homologous to both *bcl-2* and *bcl-x*). *ced-3* is related to the mammalian interleukin-1β (IL-1β) converting enzyme (ICE), a cytoplasmic cysteine protease that cleaves the cytokine precursor at specific aspartate (Asp) residues (211). ICE is involved in apoptosis induced by various stimuli, including Fas-mediated apoptosis (212-216). The activity of ICE can be inhibited by the product of *crmA*, a cytokine-response modifier gene encoded by the cowpox virus (212,214). Several homologues of ICE have recently been identified which are sub-divided into three groups (ICE-, CPP32- and Ich-1-like proteases), (217-226). It has recently been shown that specific inhibitors of ICE- or CPP32-like proteases (including *crmA*, and *bcl-2*) can inhibit Fas-mediated apoptosis (213,227,228). Enari et al. (227) have shown that Fas sequentially activates ICE- and CPP32-like proteases, both of which have different substrate specificities (224,225,229) and that downstream, CPP32 is sufficient to cause apoptotic DNA degradation in nuclei together with a component (or components) in the cytoplasm. One of CPP32's substrates is PARP (poly (ADP-ribose) polymerase) which has been implicated in a variety of apoptotic events and has been claimed as the relevant "death substrate" in Fas-mediated apoptosis (230,231). It has been proposed that proteolytic cleavage of PARP inhibits most of its DNA repair activity leading to the demise of the cell.

Fas-mediated apoptosis proceeds without RNA or protein synthesis implying that the appearance of ICE- or CPP32-like activity is a post translational activation of these proteases. The fact that the typical features of apoptosis can be induced in enucleated cells suggests that nuclear

Activation of CD95 recruits the Fas-associated death domain-containing molecule FADD (MORT1) which in turn binds and presumably activates the FADD-like ICE (FLICE/MACH1/Mch5), a member of the ICE family of proapoptotic proteases (303,304,315). FLICE has homology to both FADD and the ICE family of cysteine proteases. It binds to the death effector domain of FADD and upon overexpression induces apoptosis that is blocked by the ICE family inhibitors, *crm*A and z-VAD-fmk. This provides evidence to link a death receptor physically to the proapoptotic proteases of the ICE/Ced-3 family (315). FLICE is believed to be the enzyme responsible for activating a protease cascade after Fas receptor ligation, leading to cell death.

signalling and DNA fragmentation are not the critical events for Fas-mediated cell death. This would support the concept of an orchestrator of cell death localised to the cytosol (173). Shimizu et al. (228) have also shown that overexpression of Bcl-2 or Bcl-x_L can inhibit ICE- and CPP32-like protease-mediated cell death, indicating that Bcl-2 and Bcl-x_L act upstream of these proteases. These results strongly indicate that a death signalling pathway exists *in vivo* in which various death signals activate a protease cascade which may be blocked by members of the Bcl-2 family of proteins. The presence of multiple mammalian ICE-proteases with partially overlapping but distinct activities suggests that this is a complex protease cascade which is induced upon Fas ligation. The precise role of single members of the ICE family in Fas-mediated apoptosis is still unclear (232). The regulation of a cell's apoptotic threshold is likely to result from a complex set of interactions among Bcl-2 family members and other regulators of apoptosis.

One of the proteases in the perforin-mediated mechanism of cytotoxic lymphocyte killing is granzyme B. This protease has an unusual substrate site preference for Asp residues, a property which it shares with members of the ICE family of proteases (233,234). It has been shown that granzyme B is sufficient to produce all the key features of apoptosis, including the degradation of several protein substrates, when introduced into Jurkat cell-free extracts (235). This granzyme B-induced apoptosis could be neutralised by a tetrapeptide inhibitor of the ICE family protease CPP32 although a similar inhibitor of ICE had no effect. Granzyme B was found to convert CPP32, but not ICE, to its active form. The cowpox virus protein CrmA was found to inhibit granzyme B-mediated CPP32 processing and apoptosis demonstrating that CPP32 activation is a key event during apoptosis initiated by granzyme B. This indicates that both the Fas-mediated and perforin/granzyme-mediated cell death pathways could be linked through a common, final pathway of protease cascade activation.

Fas expression in myeloma cells and cell lines-correlation with IL-6 dependency and Bcl-2 expression

Studies of Fas expression in fresh myeloma plasma cells as well as established myeloma cell lines has revealed that Fas is constitutively expressed in both although the intensity of expression is variable (126,236,236a) which is typical of antigens whose expression varies with cellular activation status such as Fas expression on normal B cells (103,237). Normal plasma cells have been reported to be negative for Fas expression (103,238). Susceptibility

to Fas-mediated apoptosis in freshly isolated myeloma samples and some myeloma cell lines was also variable but the majority (4/5 and 6/7 patient samples, Refs.236, 236a respectively) of those cell lines dependent on interleukin-6 were not killed by activation of Fas. The IL-6 dependent myeloma cell line shown to be killed by Fas activation was not protected by addition of exogenous IL-6, nor did IL-6 treatment of these lines have any effect on Fas expression. No correlation between Bcl-2 expression and the susceptibility of myeloma cell lines to Fas-mediated apoptosis was observed (126,236), despite the fact that some groups have found that the intracellular levels of Bcl-2 may influence the susceptibility of cells to such Fas-induced cell death (136,181). An interesting observation made by Jelinek's group (236) was that malignant plasma cells in freshly isolated bone marrow MNC cultures were resistant to Fas-mediated apoptosis but isolated plasma cells from the same patient were sensitive. This suggests that there may be a protective factor or signal derived from bone marrow MNC which prevents Fas-mediated cell death in fresh myeloma samples.

Research aims

Since malignant plasma cells in myeloma patients home to the bone marrow, a study was undertaken firstly to define the phenotypic profile of a panel of myeloma cell lines and then to investigate their ability to adhere to various extracellular matrix molecules. The extracellular matrix molecules studied were- collagen, fibronectin and FN-RGD, a synthetic peptide consisisting of multiple arginine-glycine-aspartic acid (RGD) repeats which mimics the part of the fibronectin molecule that binds to the integrin VLA-5, expressed on some myeloma cells. Adhesion blockade was attempted using monoclonal antibodies directed against various myeloma surface antigens in order to ascertain the importance or otherwise of these molecules in the binding of myeloma cells to members of the extracellular matrix (Chapter 3).

It has been demonstrated that CD40 crosslinking on the surface of B cells and myeloma cells rescues them from apoptosis (88,105) and induces IL-6 secretion (88,90). This is usually (105,130), but not always (112,239), accompanied by up-regulation of the *bcl-2* gene. Since the Fas antigen has homology with CD40 and has been reported to act in opposition to *bcl-2* (102), it was decided to study these genes for mRNA and protein expression in myeloma cells in an effort to elucidate any potential link between expression

of these antigens and the onset of multiple myeloma. A more complete knowledge of the interactions between IL-6, CD40 and it's ligand, and the expression of *bcl-2* or Fas and it's ligand is necessary in order to determine possible improvements in treatment strategies for this disease.

Jelinek's group at the Mayo Clinic described a new myeloma cell line (ANBL-6) which is IL-6 dependent, expresses CD40 on its surface and is sensitive to monoclonal antibodies to both CD40 and IL-6 (88). Activation of CD40, via a monoclonal anti-CD40 antibody or ligation with its ligand, was found to induce proliferation of this myeloma cell line with a resultant increase both in IL-6 secretion and in expression of IL-6 mRNA by the cells. This CD40-mediated proliferation was substantially inhibited by a neutralising monoclonal antibody to IL-6. These results indicate that the primary mechanism of anti-CD40 stimulated proliferation of the ANBL-6 cells is the induction of autocrine IL-6 production and that perhaps CD40 expression in myeloma cells may play an important role in tumour cell expansion by induction of an autocrine IL-6 loop.

CD40 expression on fresh myeloma bone marrow samples has previously been reported as negative (86,240,241), or low (242) but it is not clear how the malignant plasma cells in these studies were defined and the number of patient samples were small. It is possible that previous failures to detect CD40 in myeloma cells may be due to differences in the monoclonal antibodies used for phenotyping and/or in staining or analysis protocols. Another possible explanation for these discrepancies could be that CD40 expression may have been lost by long term in vitro culture and that loss of CD40 expression may be associated with loss of IL-6 dependency in some cell lines. Loss of IL-6 dependency is common in long term myeloma cell lines.

The panel of eight myeloma cell lines used in this study was examined for CD40 and Fas antigen expression by flow cytometric analysis. All the myeloma cell lines tested positive for CD40 and Fas albeit at different intensities. Garrone et al. (120) have shown that resting tonsil B cells, expressing either low or absent levels of Fas, were induced to express Fas after ligation of CD40 using the CD40Lig-L culture system (see below). Engagement of B cell antigen receptor by immobilised anti-κ and -λ antibodies did not turn on Fas expression. Anti-Fas monoclonal antibody was shown to inhibit the later phases of CD40-induced B cell

growth due to apoptosis. Fas ligation was shown to inhibit proliferation and immunoglobulin secretion of CD40 activated B cells in response to recombinant cytokines. This implies that engagement of CD40 antigen on B cells induces Fas expression and sensitises them to Fas-mediated apoptosis. It was decided, therefore, to investigate whether myeloma plasma cells could be stimulated to proliferate in the same manner and whether they, too, would be sensitive to Fas-mediated apoptosis.

It was noted from the literature that the myeloma cells lines resistant to apoptosis were mostly dependent on IL-6 (126,236,236a) and it was therefore proposed to investigate this further by broadening the study by looking at several myeloma cell lines and comparing IL-6 dependent and independent passages of the same myeloma cell line (ANBL-6) for susceptibility to Fas-mediated apoptosis. The fact that fresh myeloma samples were also resistant to AICD is interesting since these are likely to be dependent on IL-6 for their growth also (55). It has previously been reported that Fas antigen is not expressed on normal plasma cells (103,238). Jelinek's group have reported ANBL-6 (IL-6 dependent) to be negative for Fas antigen expression (236) and that this cell line was non-susceptible to Fas-mediated apoptosis.

Two culture systems originally established in an effort to generate factor-dependent B cell lines (108,120) were adapted for the study of myeloma cell activation of proliferation via CD40 (and the cytokines IL-4 and IL-6) and induction of apoptosis via Fas activation. Although myeloma cell lines are obviously no longer dependent on stromal layers for their propagation it was decided that the use of the CD40 (108) and CD40Lig-L (120) culture systems may potentiate the response to CD40 activation. Cross-linkage of this antigen on the surface of myeloma cells using mouse fibroblasts transfected with either the human immunoglobulin Fc receptor (FcγRII/CDw32) or with the human CD40 ligand constitute the CD40 and CD40Lig-L culture systems respectively. Interleukin-4 was used to test for proliferation as a comparison to interleukin-6 in these studies since it is a potent B cell stimulation factor (108).

The observation that the CD40 ligand also stimulates ANBL-6 cells into IL-6 mediated proliferation indicates that the natural ligand for CD40, which is expressed mainly on activated helper T cells (93-96), but has been reported as present on monocytes, natural

killer cells, small intestine and fetal thymocytes (243), may play a role in the bone marrow microenvironment. Either $CD40L^+$ activated T cells in the bone marrow or other stromal cells, yet to be identified, may provide the stimulus needed to activate autocrine IL-6 production in myeloma cells. Alternatively, the CD40L-expressing cell may be stimulated by interaction with myeloma cells resulting in production of other cytokines increasing paracrine IL-6 secretion in myeloma patients. It was therefore deemed necessary to examine whether the ligands for CD40 and/or Fas antigen were expressed on the myeloma cells used in this study.

Although anti-CD40-driven proliferation of the ANBL-6 cell line primarily results from autocrine secretion of IL-6, treatment of the cells with a neutralising antibody to IL-6 did not completely inhibit CD40 stimulation of the cells. This suggests that there may be other mechanisms involved in the CD40 stimulated proliferation of myeloma cells, perhaps by activation of tyrosine- and serine- protein specific kinases or phospholipase- C (244,245).

In summary, due to the paucity of studies of human myeloma cells with relation to their expression of CD40 and Fas antigen and their susceptibility or otherwise to Fas-mediated apoptosis, it was decided to investigate these factors in a panel of myeloma cell lines. It was initially attempted to establish a rapid, reliable, cost-effective and informative method of assessing apoptosis (Chapter 4) and then to develop an effective assay system for Fas and CD40 activation in myeloma cell lines (Chapter 5). Finally, a comprehensive study was undertaken to examine, in these cell lines, the role of activation of CD40 and/or Fas with or without co-stimulatory cytokines (IL-6 and IL-4). Changes in the regulation of CD40, Fas, Bcl-2, IL-6, IL-6R protein or mRNA expression under different culture conditions were assessed. It was also attempted to establish whether myeloma cell lines co-expressed the ligands for CD40 or Fas antigen by reverse transcription polymerase chain reaction and by *in situ* hybridisation using sequence-specific riboprobes (Chapter 6).

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 TISSUE CULTURE

All cell lines were obtained from their original source or, with the permission of their authors, from other researchers. The lines were all proved to be routinely Epstein Barr virus (EBV) and mycoplasma (2.2.23) negative following testing every 6 months. Cells were maintained in a humidifed atmosphere at 37°C with 5% CO_2 in RPMI-1640 medium containing 10-20% foetal calf serum, 1% Penicillin/Streptomycin and 2% L-Glutamine as described .

Cell lines:

JIM-1, JIM-3 (Ref: 246) Description: IgA-*lambda* myeloma cell line.

JJN-3 (Ref: 247) Description: IgA-*kappa* myeloma cell line.

EJM (Ref: 248) Description: IgG *lambda* myeloma cell line.

U266 (Ref:249) Description IgEλ myeloma cell line.

RPMI-8226 (Ref: 250) Description - IgGλ myeloma cell line.

ANBL-6 (Ref: 251) Description - IgAλ myeloma cell line

DoHH2 (Ref: 252) Description - Non-Hodgkin's B cell line with a chromosomal translocation t(14;18) (q32;q21).

RK Epstein Barr virus negative myeloma cell line, monoclonal *kappa* light chain positve. Developed by MML, January 1994, unpublished.

JT EBV negative, *kappa* positive myeloma cell line established January 1995 by MML, unpublished.

IM9 (Refs: 253,254) Description - Monoclonal *kappa* light chain positive myeloma cell line.

L3055 (Ref: 255) Description - EBV negative ALL cell line.

LCDw32 (Ref: 108) Description - mouse fibroblastic Ltk$^-$ cells stably transfected with the human Fc receptor (FcγRII/CDw32).

CD40LigL (Ref: 120) Description - mouse fibroblastic Ltk$^-$ cells stably transfected with the human CD40 ligand (CD40Lig-L cells).

Media for propogation of cell lines:

1. JIM-1, JIM-3, JJN-3, EJM, U266, IM9, ANBL-6 (IL-6 independent passages 17/2, 27/2, 6/3, 22/5), RPMI-8226, DoHH2, CD40Lig-L, L3055

RPMI 1640 containing: 2% L-Glutamine (200mM)
1% Penicillin/Streptomycin (5000IU-5000μg/ml)
10% heat inactivated Foetal Calf Serum (Hyclone)

Cells seeded at approximately 10^5/ml and fed 2-3 times per week.
JJN-3 and EJM have a 48-72 hour doubling time, the JIM cell lines have a 24 hour doubling time.

2. RK, JT, ANBL-6 (IL-6 dependent passages 16/1, 26/12, P32)

As above but supplemented with 1ng/ml of recombinant human IL-6 (generously donated by Serono Labs.)

3. LCDw32 cells

As above but supplemented with Hypoxanthine-Aminopterin-Thymidine (HAT) media supplement (Sigma) diluted 1:50.

2.1.2 Plasmids

pBluescript (Stratagene)	phagemid cloning and sequencing vector (Ref. 256)
CDM8 (Invitrogen)	transient mammalian expression vector (Ref. 257)
pCRII (Invitrogen)	linearised plasmid vector providing 3' T-overhangs ready for insertion of PCR products (Invitrogen)
pUC18 (Invitrogen)	supercoiled (positive control) plasmid cloning vector derived from pBR322 (Ref. 258)

2.1.3 Bacterial strains

The bacterial strains used were derivatives of *Escherishia coli*

1. XL1-Blue (Stratagene), Source: Ref. 259

Genotype: *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F' proAB $lac^{q}Z\Delta M15$ Tn10 (Tet^{r})]

2. MC1061/P3 (Invitrogen), Source: Ref. 257

Genotype: F^{-},*ara*D139 Δ(*ara*ABC-*leu*)7679 *gal*U *gal*K Δ*lac*X74 *hsd*R2 (r_K^{-},m_K^{+}) *rps*L(Str^{R}) *thi*-1 *mcr*B {P3: Kan^{R},amp^{R} (amber), tet^{R} (amber)}

3. INVαF' (Invitrogen), Source: Ref. 260

Genotype: F',*end*A1 *rec*A1 *hsd*R17 (r_K^{-},m_K^{+}) *sup*E44 *thi*-1 *gyr*A96 *rel*A1ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169

2.1.4 Selection of bacterial transformants

The following antibiotics were used:

Kanamycin (50μg/ml), Tetracycline (7.5-10μg/ml), Ampicillin (30-100μg/ml)

XGal (40mg/ml in DMF), was added at 25μl/plate

XGal (Gibco BRL) is the chromogenic substrate for beta galactosidase (βGal). E.Coli contains a segment of DNA encoding the βGal gene. When XGal is added to plates where plasmids containing the LacZ promotor (eg pBluescript) are growing, the plaques formed are blue. If the LacZ gene is interupted, ie if pBluescript contains an insert, the colonies formed are white.

2.1.5 dNTP stock solutions (100mM)

Stock solutions for dATP, dTTP, dGTP and dCTP were made by dissolving 60mg dNTP in 0.8ml distilled water The pH was adjusted to 7.0 with 0.1M NaOH and the solutions were then made up to 1ml with distilled water and stored at -20°C.

2.1.6 Agarose gel loading buffer

Loading buffer consisted of 15% Ficoll, 0.25% Xylene cyanol, 0.25% Bromophenol Blue, 0.25% Orange G in distilled water. For PCR gel loading buffer the Bromophenol blue was omitted.

2.1.6a Agarose gel electrophoresis

Agarose gels (1-2%, according to the size of the expected product), containing ethidium bromide at 0.25μg/ml, were prepared in 1xTBE buffer and electrophoresed in 1xTBE buffer for around 90 minutes at 100 volts. Samples were visualised by illuminating the gel under ultraviolet light and photographing as a record.

2.1.7 Autoradiography

For detection of radioactive signals, filters were partially dried, sealed in a heat-sealed bag and exposed to a Kodak XAR film at -80°C overnight or longer before developing flim.

2.1.8 Removal of probes from blots

In order to re-probe blots, the membrane was placed in a tray and covered with 1 litre of 0.1x SSC, 0.1% SDS which had been brought to boiling point. This was shaken gently until the solution had cooled to room temperature. The blot was rinsed with 2xSSC, blotted dry on 3MM paper and stored at -20°C in a heat-sealed bag until ready for reprobing.

2.1.9 Phenol and Phenol/chloroform

Water-saturated phenol was purchased from Rathburn chemicals, buffered against 1M Tris/HCl, pH 8.0 and then stored under TE buffer. Phenol/Chloroform was prepared as a 50% v/v mixture.

2.1.10 Buffers

1. TBE buffer, 0.045M Tris-borate, 1mM EDTA
2. TBS, 0.05M Tris/HCl, 0.15M NaCl pH7.6
3. TE, 10mM Tris, 1mM EDTA, pH8.0
4. PBS, 20 PBS tablets (Oxoid Pharmaceuticals) added to 2 litres distilled water

2.1.11 Solutions

1. DEPC treated water (DEPC.H_2O); 1ml DEPC added per litre of distilled water, left overnight with cap loose and then autoclaved.
2. DEPC.PBS; 1ml DEPC added per litre of PBS, solution autoclaved.
3. 20 x SSC; 3M NaCl, 3M Na_3Citrate
4. 3M Sodium Acetate, pH7.0
5. Detection Buffer 1; 0.1M Tris.HCl, pH7.5, 0.15M NaCl
6. Detection Buffer2; buffer1 + 3% BSA
7. Detection Buffer 3; 0.1M Tris.HCl, 0.1M NaCl, 50mM $MgCl_2$, pH9.0
8. NBT/BCIP; 7.5% NBT in 70% DMF, 5% BCIP in 100% DMF and 0.02% levamnisole (Inhibits endogenous Alkaline Phosphatase activity).
9. Anti-Digoxygenin/AP conjugate (Boehringer Mannheim), 1:500 dilution in detection buffer 1 containing 20% NHS

Normal human serum obtained from "SAPU" (Scottish Antibody Production Unit). Filter sterilised through a 0.45μM filter and stored at -20°C in 500μl aliquots.

10. $T_{10}E_1$, 0.1% SDS buffer
11. 1M Tris.HCl, pH8.0
12 0.5M EDTA, pH8.0
13. 10% SDS
14. Salmon sperm DNA (100mg/ml)

2.1.12 Materials

All materials were purchased from the following companies except where stated otherwise.

General chemicals and organic solvents	Sigma, BDH, May & Baker
Agar	Life Technologies
Agarose	Flowgen
Biochemicals	Sigma
Antibiotics	Sigma
Agarose	Sigma
Radio-isotopes	Amersham Life Sciences
Nitrocellulose membranes	Sartorius,Amersham Life Sciences
Nylon membranes	PALL
Antibodies	DAKO, Serotec, Becton Dickinson Immunotech
Restriction enzymes	Life Technologies, NBL
Tissue Culture reagents	Gibco BRL
Cytokines	R&D Systems
DNA preparation	Scotlab
DPX mountant for microscopy	BDH

2.2 METHODS

2.2.1 DNA PREPARATION

DNA was prepared using the "Nucleon II", DNA extraction kit (Scotlab) according to the protocol supplied. Briefly: cells were lysed, deproteinised with sodium perchlorate, the DNA extracted with chloroform, separated by centrifuging through a silica suspension so that the DNA was left in the aqueous phase and it was then precipitated with ethanol. DNA samples were stored at 4°C.

2.2.2 RNA PREPARATION

All RNA samples prepared consisted of total cellular RNA.

Cultured cells:

At least 2 x 10^6 cells were required for each RNA preparation. Cells were washed in 15ml PBS/Dulbecco's and resuspended in 1ml RNAZOL (Biogenesis Ltd.)

Fresh tissue:

Fresh tissue sections, eg human tonsil, were snap frozen in liquid nitrogen and pulverised in a mortar dish previously chilled with liquid nitrogen. The sample was kept frozen by topping up the mortar dish with liquid nitrogen. The powdered sample, still frozen, was scraped into a test tube containing 10-20ml RNAZOL.

Fresh tissue and cultured cells:

Samples were vortexed vigorously for at least 30 seconds. A 1/10th volume of chloroform ($CHCl_3$) was added and the samples vortexed again (this results in shearing of residual DNA which is an important step in reducing DNA contamination). Samples were left on ice for 15 minutes and then centrifuged at 10,000g for 10 minutes. The upper aqueous phase was removed into a fresh tube and an equal volume of propan-2-ol added. This was precipitated for 1 hour at -20°C then centrifuged at 4000rpm for 30 minutes to collect the RNA. The pellet was washed with 70% ethanol and then absolute ethanol. After air drying, the pellet was resuspended in DEPC treated water (50-400µl) and precipitated by adding a 1/10th volume of 3M Sodium acetate, pH7.0 and 3 volumes of ethanol. Samples were left at -20°C

for at least 1 hour but could be stored indefinately at this stage. When continuing, the RNA was collected by spinning at full speed in the microfuge for 15 minutes. The pellet was washed with 70% ethanol and then absolute ethanol as above. The final pellet was air dried and resuspended in DEPC H_2O at approximately 1mg/ml. Samples were stored at -20°C.

2.2.3 PHENOL EXTRACTION OF NUCLEIC ACIDS

This procedure was used to purify RNA and DNA and remove residual enzyme activity following restriction digestion or *in vitro* transcription procedures. A 1/10th volume of 3M sodium acetate was added to digests with an equal volume of phenol:chloroform ($CHCl_3$). This was mixed for 5 minutes on a rotating mixer then spun in a microfuge for 5 minutes. The upper aqueous phase was extracted into a fresh microfuge tube and an equal volume of $CHCl_3$ added. This was mixed for 2 minutes then spun for 2 minutes to dissolve any residual phenol. The top phase was precipitated with 3 volumes of ethanol at -20°C overnight. The following day the sample was spun for 5 minutes, washed in 70% ethanol and then in 100% ethanol. The pellet was resuspended in 20µl fresh, ribonuclease free DEPC-treated sterile distilled water.

2.2.4a PREPARATION OF SILANISED SLIDES AND COVERSLIPS

Silane, (3-Aminopropyltriethoxysilane), (Sigma Pharmaceuticals), Coated Slides were prepared by washing slides in 2% "Decon" detergent overnight. They were then washed thoroughly in running water (for approximately 2 hours), dried overnight, immersed in 2% Silane/Acetone for 5 minutes, rinsed in running water for 5 minutes and dried overnight. Sigmacoated (Sigma) coverslips were prepared by soaking coverslips in Sigmacote for 30 minutes, air drying, rinsing in water x3 and air drying.

2.2.4 PREPARATION OF CYTOSPINS

Cytospins were prepared using previously coated silanised glass slides. Cell suspensions containing approximately 10^6 cells in 100-200µl medium were spun at 500rpm for 5 minutes using a Shandon cytocentrifuge.

2.2.5 STAINING WITH WRIGHTS STAIN

For simple staining, cytospins were stained in an "Ames Haematek" slide stainer with modified "Wrights" stain (type of Romanowsky Dye) containing methylene blue and eosin. The slides were then mounted under glass coverslips with DPX mountant.

2.2.6 IN SITU HYBRIDISATION

In situ hybridisation techniques (ISH) allow specific nucleic acid sequences to be detected in morphologically preserved tissue sections, cells or chromosome preparations. Four types of probe may be employed: Oligonucleotide probes, single- or double- stranded DNA probes or riboprobes. Oligonucleotide probes are prepared from deoxynucleotides by automated synthesis and are commercially available. They are resistant to RNases which eliminates some of the technical difficulties of the procedure and are small (20-40 bases) so they penetrate cells easily. Riboprobes are complementary RNAs prepared by RNA polymerase-catalysed transcription of mRNA in the 3' to 5' direction. They have the advantage that RNA-RNA hybrids are very thermostable and resistant to attack by RNases. This allows post-hybridisation digestion by RNases to remove non-hybridised RNA, thereby reducing background. Penetration of the probe into cells can be enhanced by controlled alkaline hydrolysis to produce probes of 50-150 bases.

In this study, commercial FITC-labeled oligonucleotide (DNA) probes were used to detect *kappa* (κ) and *lambda* (λ) immunoglobulin light chain mRNA in tonsil controls and myeloma cell lines. Light chain monoclonality as described in section 2.1.1 was confirmed using this method. Results are not shown. Digoxygenin-labeled sense and antisense riboprobes were synthesised (2.2.19) and used to attempt the detection of *bcl-2*, *fas*, Fas ligand, CD40, CD40 ligand and IL-6R mRNA in cytospins of cell lines (2.2.8).

An FITC-labeled Epstein Barr virus latent membrane protein (EBV-LMP) oligoprobe cocktail (DAKO) was also used to analyse the panel of myeloma cell lines used in this study for their EBV status by *in situ* hybridisation. An EBV positive human tonsil section was used as positive control. The method was as described in section 2.2.7. All myeloma cell

lines were found to be negative for EBV-LMP as expected. A representative result showing the ANBL-6 cell line is shown in Figure 2.2.6.

ISH testing for *bcl-2* transcription. using the B cell line DoHH2 (2.1.1) as a positive control showed that all myeloma lines tested were positive for *bcl-2* mRNA expression although expression in JIM-3, IL-6 independent passages of ANBL-6 and RPMI-8226 was weak. Representative experiments showing *bcl-2* positivity for the DoHH2 and JIM-1 cell lines are shown in Figures 2.2.6a and 2.2.6b.

No positive hybridisation indicating transcription of IL-6R, CD40L and FasL was detected in any of the myeloma cell lines tested despite repeated attempts at *in situ* hybridisation using specific Digoxygenin-labeled riboprobes. IL-6R mRNA was detected by RT-PCR however (Figure 2.2.11a), in JIM-1, JIM-3, U266, RPMI-8226 and the IL-6 independent passages of ANBL-6 but not in JJN-3 or IM-9. See discussion (chapter 7).

ISH results for *fas* and CD40 mRNA expression in myeloma cell lines cultured in the CD40 system with and without culture additives are shown in section 6.5.

2.2.7 ISH for κ and λ Oligonucleotide FITC-Labelled Probes (DAKO kits: K045, probes and K046, detection)

This is an adaptation of the supplied protocol.

Pretreatments:

A. Paraffin sections (eg Tonsil control)

These sections were immersed in wax and had to resume their water solubility in order to perform ISH. Slides were immersed in xylene for 2x5 minutes, in alcohol for 2x5 minutes, in methylated spirits for 5 minutes and then into DEPC-treated water for 5 minutes. Slides were then immersed in 0.2N HCl/DEPC for 20 minutes (this breaks the methylene bridges formed during the paraffin fixation step) and rinsed in DEPC/H_2O with shaking.

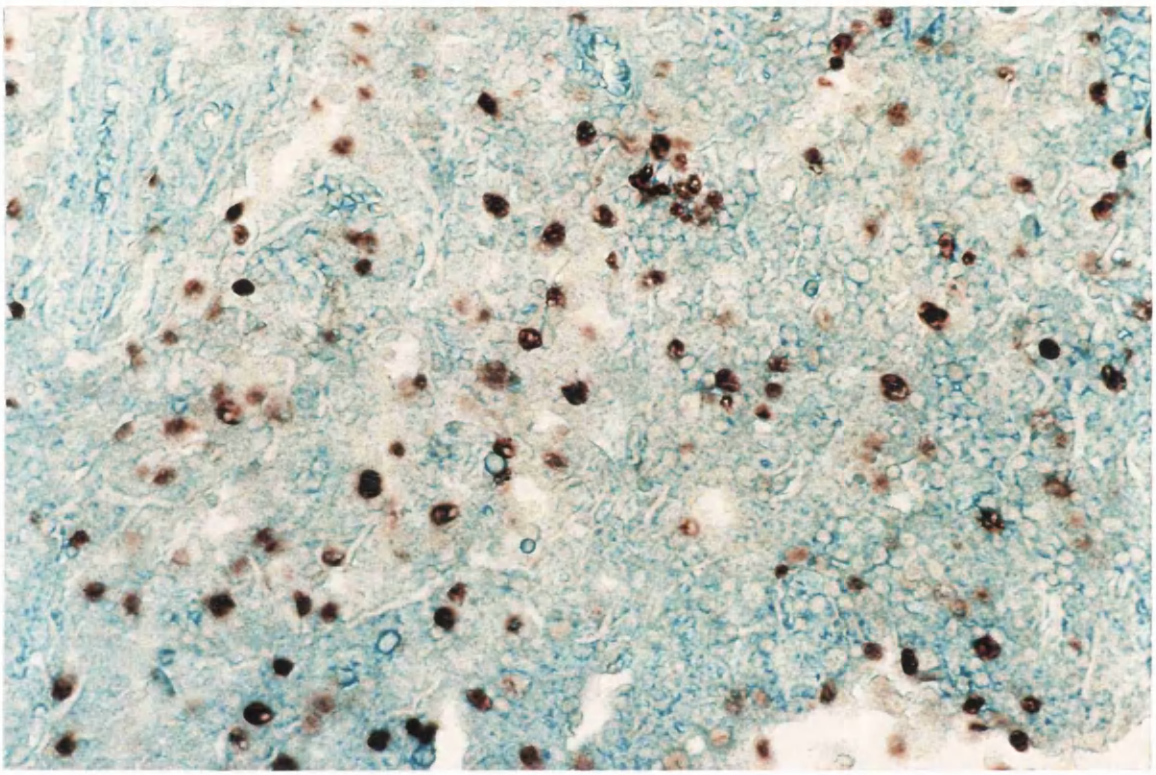
B Cytospins

Cytospins (2.2.4) were immersed immediately in buffered formalin overnight to fix them, after which they were rinsed for 2x5 minutes in DEPC/H_2O.

Figure 2.2.6

In situ hybridisation using an FITC-labeled LMP (EBV) oligoprobe cocktail (DAKO) to test for EBV status in a) positive tonsil control and b) myeloma cell lines (ANBL-6 22/5 in this example) - EBV negative. Counterstaining was with Light Green. Cytospins were subsequently incubated with a rabbit anti-FITC-AP conjugate with colourimetric detection using NBT/BCIP. Slides were viewed under light microscopy (magnification x 40).

a)



b)

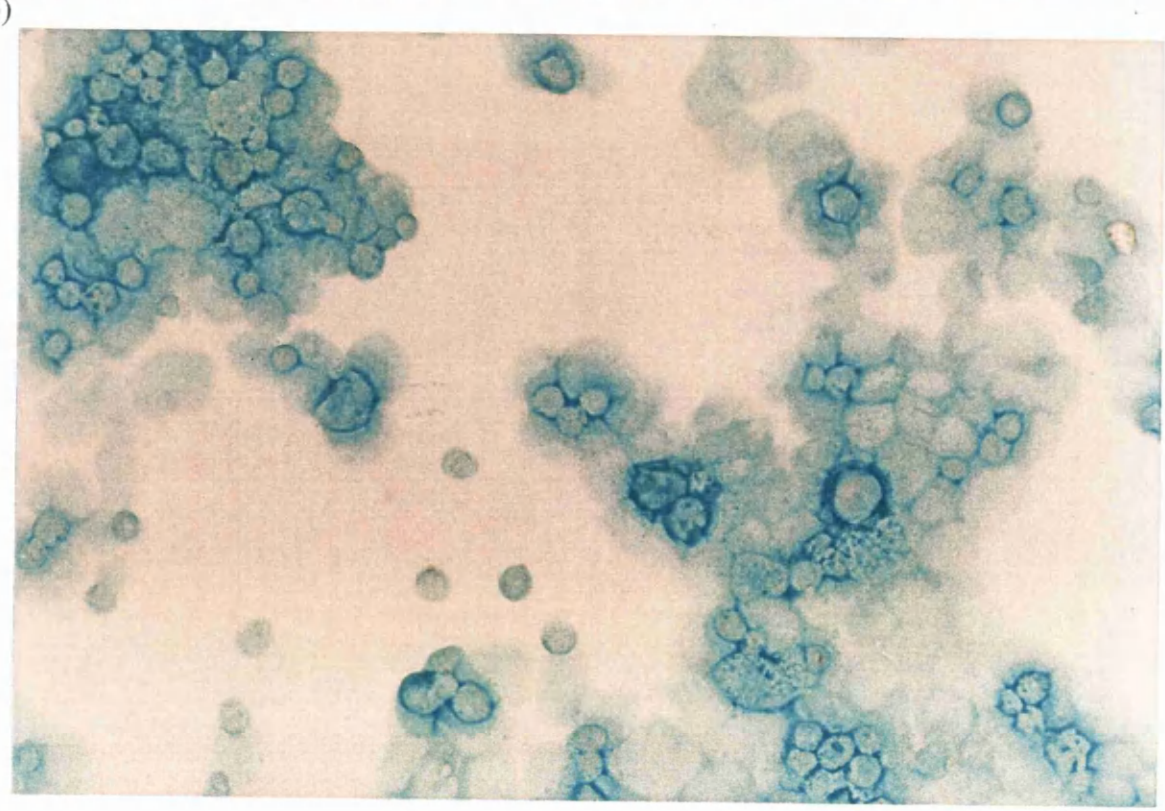


Figure 2.2.6a

In situ hybridisation showing *bcl-2* mRNA expression in the B cell line DoHH2 using Digoxygenin-labeled sense (a) and antisense (b) riboprobes. Counterstaining was with Light Green. Cytospins were further labeled with anti-Dig-AP conjugate with colorimetric detection using NBT/BCIP. Slides were viewed under light microscopy (magnification x 10).

a)

b)

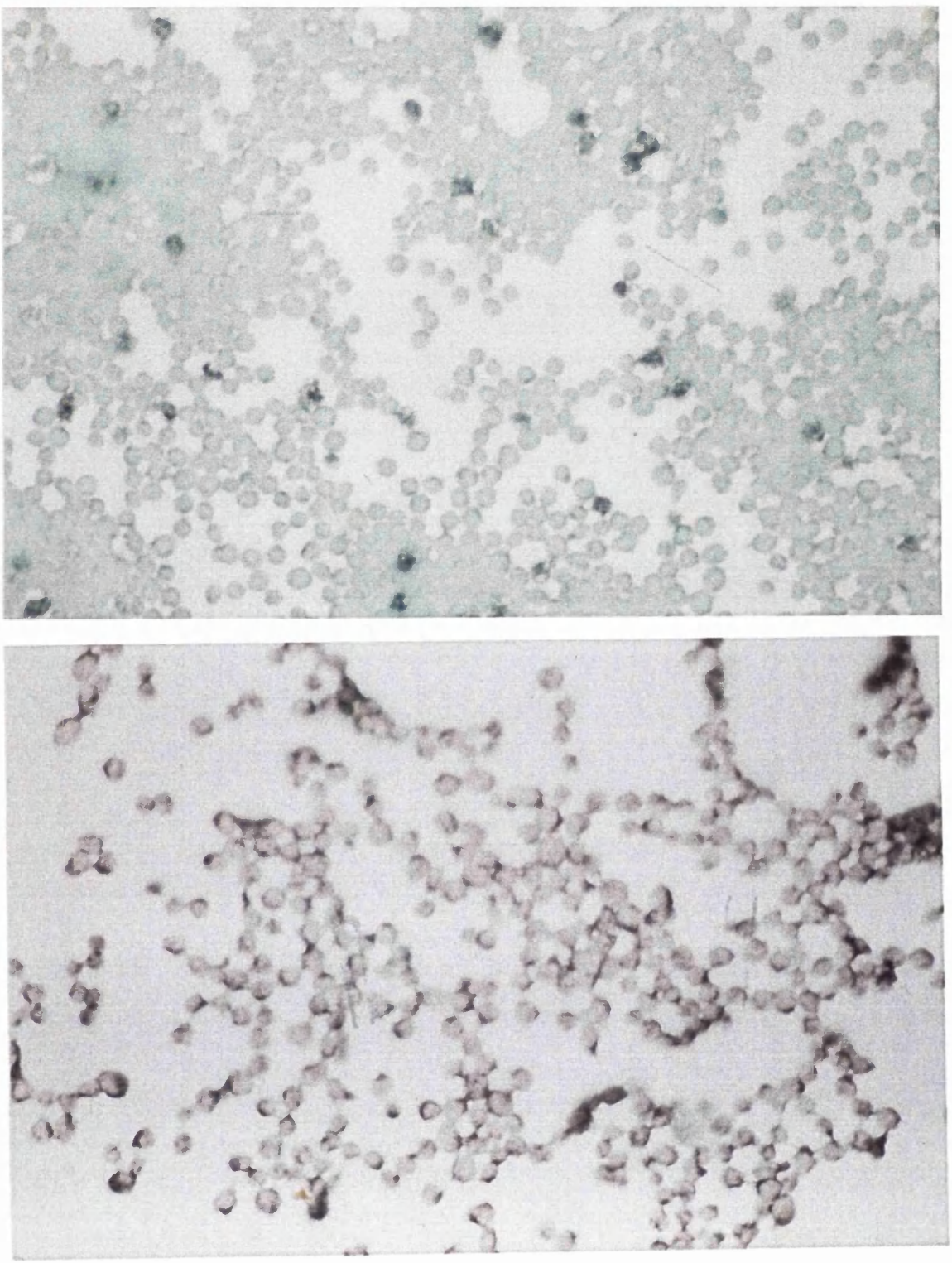
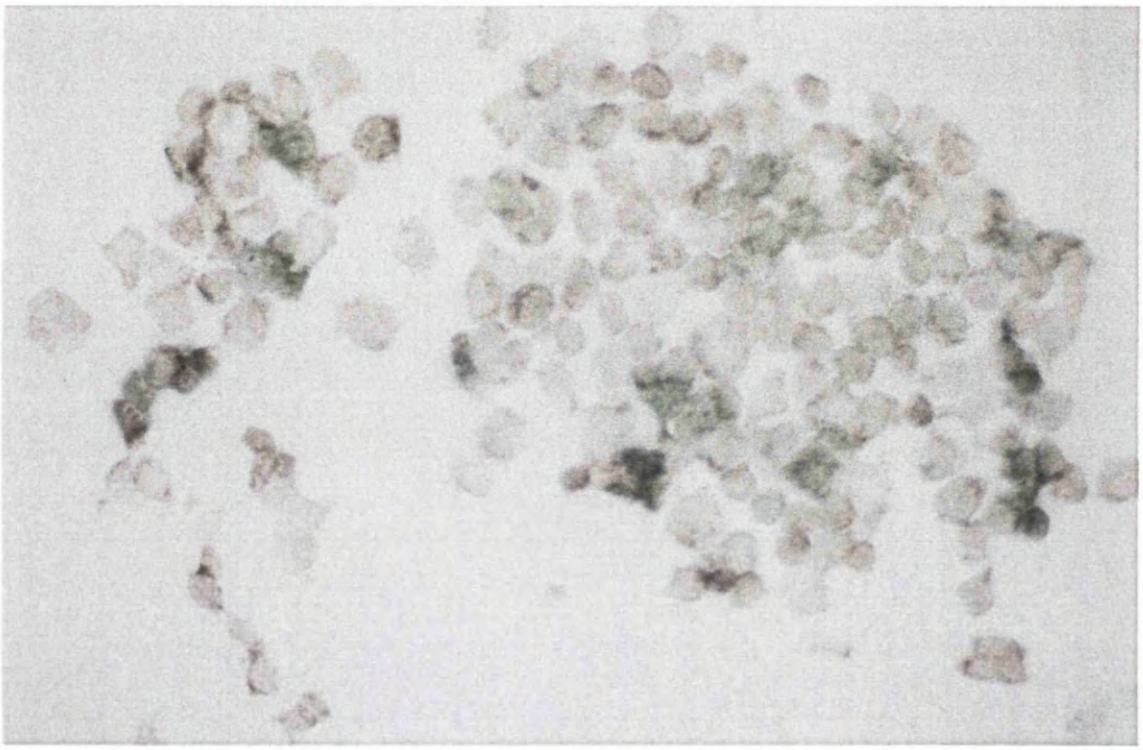


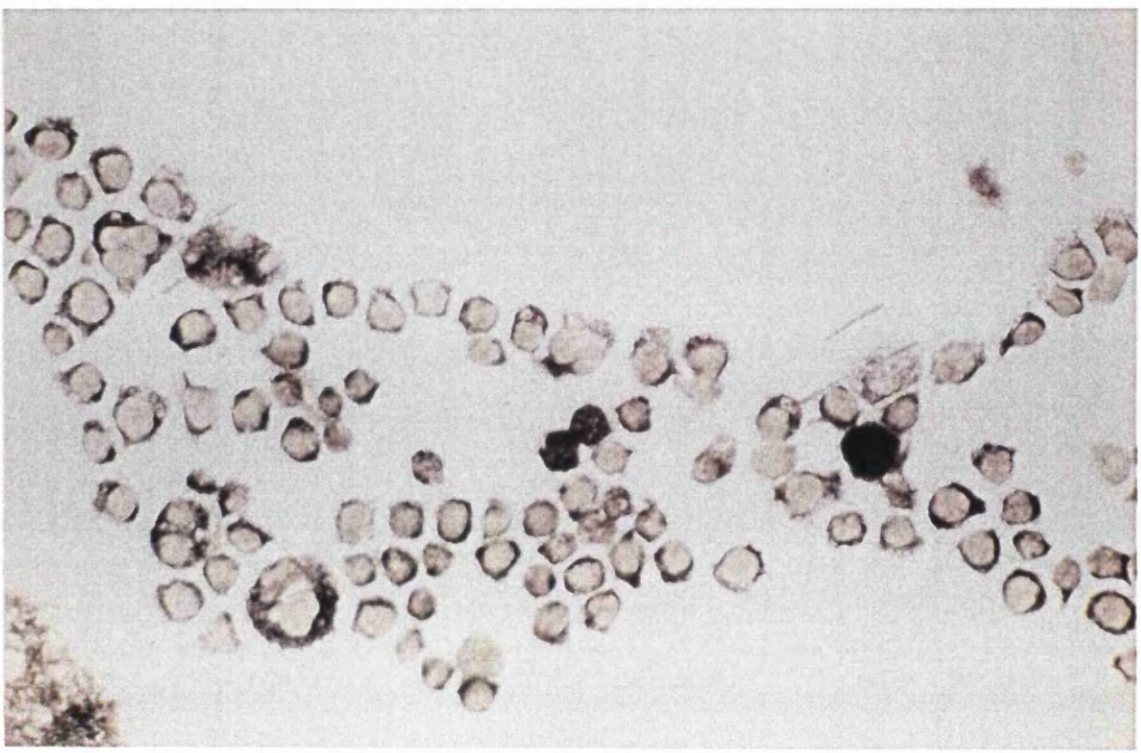
Figure 2.2.6b

In situ hybridisation showing *bcl-2* mRNA expression in the myeloma cell line JIM-1 using Digoxygenin-labeled sense (a) and antisense (b) riboprobes. Counterstaining was with Light Green. Cytospins were further labeled with anti-Dig-AP conjugate with colorimetric detection using NBT/BCIP. Slides were viewed under light microscopy (magnification x 10).

a)



b)



Both paraffin sections and cytospins were then treated in the same way:

Slides were incubated in proteinase K (PK) solution at 37°C (80µl/slide) for 30 minutes in a humidified atmosphere (the PK incubation serves to remove protein and unmask the mRNA). Slides were washed in 0.2% glycine/DEPC for 30 seconds (this is a PK inhibitor and stops digestion of proteins) and then in DEPC/H_2O, for 5 minutes before post-fixing at 4°C in 0.4% paraformaldehyde in DEPC/H_2O for 20 minutes (this acts as a weak fixative, fixing RNA to the tissue section but not masking it so that probe will still be accessible). Slides were rinsed in DEPC/H_2O, for 5 minutes, x2 before rinsing in Methylated spirits and allowed to air dry.

HYBRIDISATION

Probes (κ and λ) were diluted 1:1 in the negative control (hybridisation buffer) and 20µl per slide was added. Sections were covered with "Sigmacote" coated coverslips, and incubated at 37°C overnight in a humidifed atmosphere.

WASHING AND DETECTION

There was no need for DEPC-treated water from this stage as RNA was all bound. Cover slips were removed and slides washed in 2xSSC, for 5 minutes with shaking (this high stringency wash removes any unbound probe). Sections were then immersed in TBS (or PBS), 0.1% Triton X-100 for 10 minutes, with no shaking required. The slides were rinsed briefly in TBS x2 and were then incubated with 50µl of a 1:80 diluted FITC/AP antibody (in DEPC.PBS/1% Triton-X/3% BSA) at room temperature for 30 minutes before for 2x5 minutes in TBS. Slides were washed in substrate buffer, pH9.0 for 5 minutes before incubating with diluted enzyme substrate (BCIP/NBT) + endogenous alkaline phosphatase inhibitor (levamnisol) for 60 minutes at room temperature. Slides were washed briefly in ddH_2O x3 before counterstaining in Light Green for 3 minutes. Slides were washed in ddH_2O x3 before mounting in DAKO "Glycergel" and observing under light microscopy.

2.2.8 ISH for Digoxygenin labeled probes:

Pretreatments, PK incubation and post-fixation were as described in section (2.2.7).

Hybridisation buffer containing 30% formamide was used to dilute riboprobes. The probe dilution factor and PK concentration was determined by probe standardisation experiments.

For example, for the *bcl-2* sense and antisense riboprobes, a 1:500 probe dilution and PK20 was used and 20µl probe was added per slide. Hybridisation and washing were as described (2.2.7) except that detection was with a 1:500 dilution of anti-Digoxygenin-labelled alkaline phosphatase (Dig-AP), (Boehringer Mannheim) for 2 hours at room temperature in a humidified atmosphere. Slides were washed in TBS (or PBS) for 2x5 minutes and then in substrate buffer for 5 minutes before immersing in NBT/BCIP detection buffer for 1.0-1.5 hours at room temperature in the dark. Slides were observed for positivity (over-incubation with substrate solution causes it to precipitate), washed, stained and mounted in glycergel.

SOLUTIONS REQUIRED FOR ISH:

(See also section 2.1.11)

1. TBS,0.1% Triton X-100
2. TBS,0.1% Triton X-100,3% BSA Stored at 4°C, approx 1 week
3. Rabbit F(ab) anti-FITC/AP (alkaline phosphatase), (Dako code:K046): 1:80 dilution of antibody in TBS,0.1% Triton x-100,3% BSA.
4. Enzyme substrate (vial B - DAKO),NBT/BCIP - detects alkaline phosphatase activity, resulting in a blue-black precipitate. Diluted 1:50 with substrate buffer pH9.0
5. Substrate buffer containing: 0.1M Tris/HCl, 0.1M NaCl, 50mM $MgCl_2$, pH9.0
6. Proteinase K (PK) solution containing: Stock solution 500µg PK/ml in DEPC water aliquoted and stored at -20°C. Approriate dilutions of PK in digestion buffer (PK buffer made by varying PK concentrations for optimum detection of mRNA. Optimal [PK] for cytospins with most probes was found to be PK20 ie 20µg/ml.
7. PK diluting buffer containing: 0.1M Tris HCl, pH8.0, 50mM EDTA
8. DEPC.0.2N HCl
9. DEPC.0.2% Glycine
10. Levamnisol - endogenous alkaline phosphatase inhibitor
11. Hybridisation Buffer (30% formamide) containing: 0.1M Tris/HCl, pH 7.5, 12.5 x Denhardt's solution (2% BSA, 2% Ficoll, 2% PVP), 2 x SSC, 0.5% SDS, 30% formamide, 10% dextran sulphate, 2.5mg/ml salmon sperm DNA.

2.2.9 FLUORESCENT ANTIBODY LABELLING OF CYTOSPINS

Cytospins were fixed in 5% acetic acid in ethanol at 4°C for 15 minutes, washed in PBS for 5 minutes at 4°C and then stained with (for example) 20µl unconjugated mouse anti-human-*kappa* or -*lambda* antibody for 30 minutes at room temperature. Slides were washed in PBS for 3 x 5 minutes. Positive slides were detected by a second incubation with 20µl 1:10 Diluted (PBS) goat anti-mouse-FITC labeled Immunoglobulin ($F(ab')_2$) for 30 minutes at room temperature. Slides were washed for 15 minutes in PBS, mounted in glycergel and examined by fluorescence microscopy.

The myeloma cell lines used in this thesis were tested for monoclonal light chain expression using this method and the results were as described in section 2.1.1. A representative example is shown in Figure 2.2.9.

2.2.10 IMMUNOCYTOCHEMISTRY (ICC) OF MYELOMA CELL LINES - DETECTION OF CYTOPLASMIC BCL-2 PROTEIN

Cytospins of myeloma cell lines were immersed immediately in cytofix and left overnight (alternatively cytospins were air dried for at least I hour and then fixed in acetone for 10 minutes before air-drying).

ICC Protocol

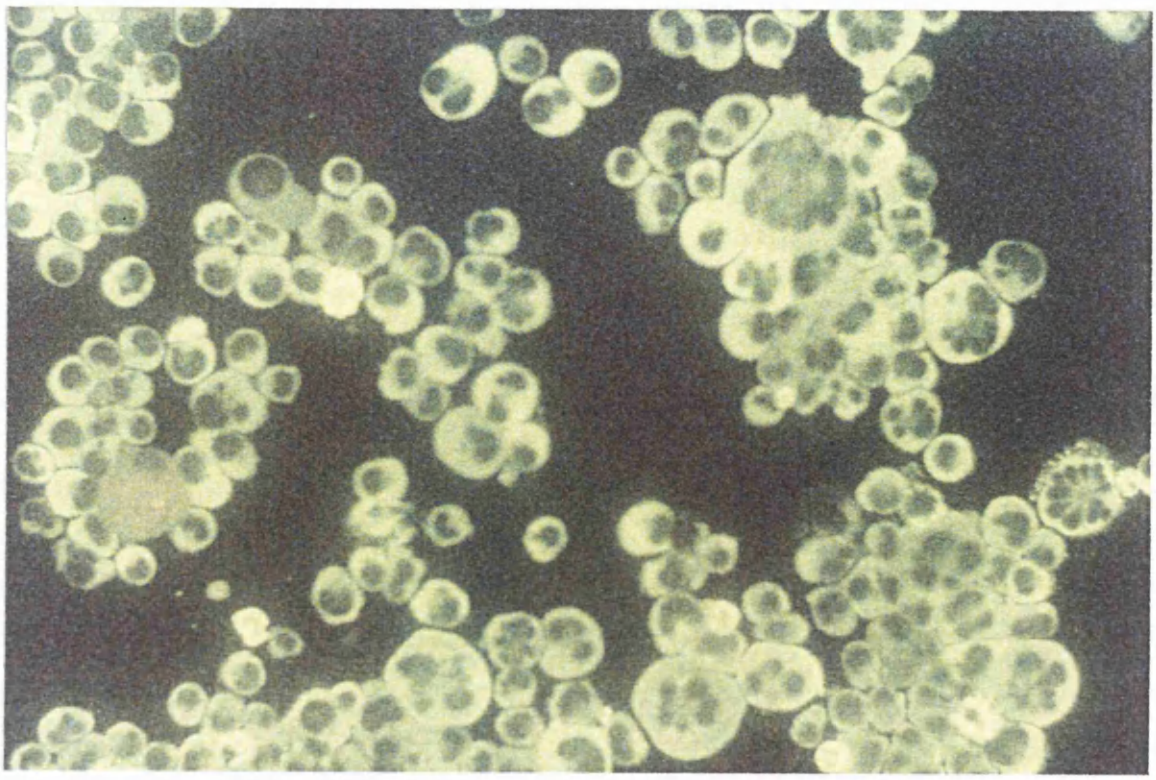
This is an adaptation of the method described by Malik DY and Damon ME (Ref. 260a) and describes a standard indirect alkaline phosphatase method of immunocytochemistry using New Fuschin Chromogen substrate for detection.

Cytospins were washed for 10 minutes in water and then in TBS at room temperature (RT). Non-specific binding was prevented by incubation with 50µl goat serum (1:5 dilution in TBS) in a humidified atmosphere for 15 minutes at RT. Cytospins were then incubated with 50µl mouse anti-human Bcl-2 protein, diluted 1:50 in TBS for 2 hours at RT. Slides were washed for 10 minutes in TBS, dried and incubated with 50µl AP-conjugated goat anti-mouse IgG (GAM-AP) diluted 1:20 in TBS with 5% NHS for 45 minutes at RT. Slides were washed in TBS for 10 minutes at RT then incubated with substrate solution (New

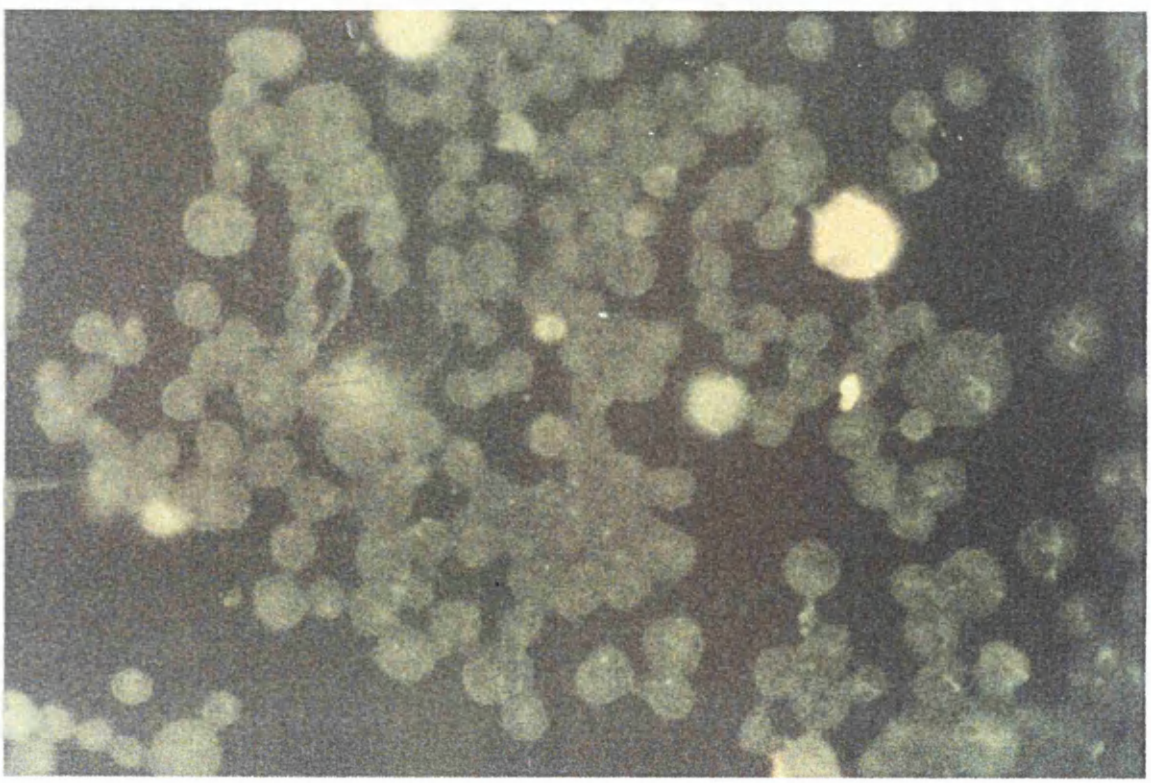
Figure 2.2.9

Detection of monoclonal, cytoplasmic immunoglobulin *kappa* (κ) light chains in the myeloma cell line RK following staining with a) anti-κ and b) anti-λ monoclonal mouse-anti-human antibodies. Positive staining was detected using an FITC-conjugated goat anti-mouse antiserum. Cytospins viewed under fluorescence microscopy (magnification x 40).

a)



b)



fuschin chromogen substrate), (Biogenix) for up to one hour until the colour was developed. Slides were then washed in water, counterstained in haematoxylin for 1 minute and rinsed in (STWS) until cytospins turned blue. Slides were dehydrated in histoclear and mounted in Harleco synthetic resin (HSR) dissolved in histoclear. Slides were observed under a light microscope, negative samples were blue with no red staining. Positive samples showed red staining mixed with blue counterstaining.

Note: an alternative method of ICC staining used for paraffin sections such as tonsil was a standard labeled-Streptavidin method. Slides were labeled with mouse-anti-human Bcl-2 as described above followed by goat anti-mouse antiserum conjugated to Biotin. The slides were then incubated with horse-radish peroxidase conjugated to Streptavidin and detection was with 0.04% Diaminobenzidine tetrahydrochloride (DAB). Positive samples were stained completely brown with blue nuclei and clear background staining of negative samples.

These two methods were used to test myeloma cell lines for Bcl-2 expression using tonsil sections as positive and negative controls with the non-Hodgkin's B cell line DoHH2 (2.1.1) as a further positive control. The protein was found to be expressed in all myeloma cell lines tested although their intensity of expression varied. Representative results for tonsil controls and the cell line JJN-3 are shown in Figures 2.2.10a and 2.2.10b respectively. Detailed studies of the B cells within the different compartments of tonsil germinal centres (136) have revealed that the expression of the protein product of the bcl-2 proto-oncogene is extremely localised. There is no Bcl-2 protein expressed in centroblasts of the dark zone or centrocytes of the light zone, where these cells are dying by apoptosis. There is a very low level of Bcl-2 protein expression in the B blasts of the apical light zone where B cells differentiate into plasma cells or memory cells but there is marked Bcl-2 expression in cells of the follicular mantle which comprises long-lived, recirculating $IgM^{+}IgD^{+}$ B cells (Figure 2.2.10a).

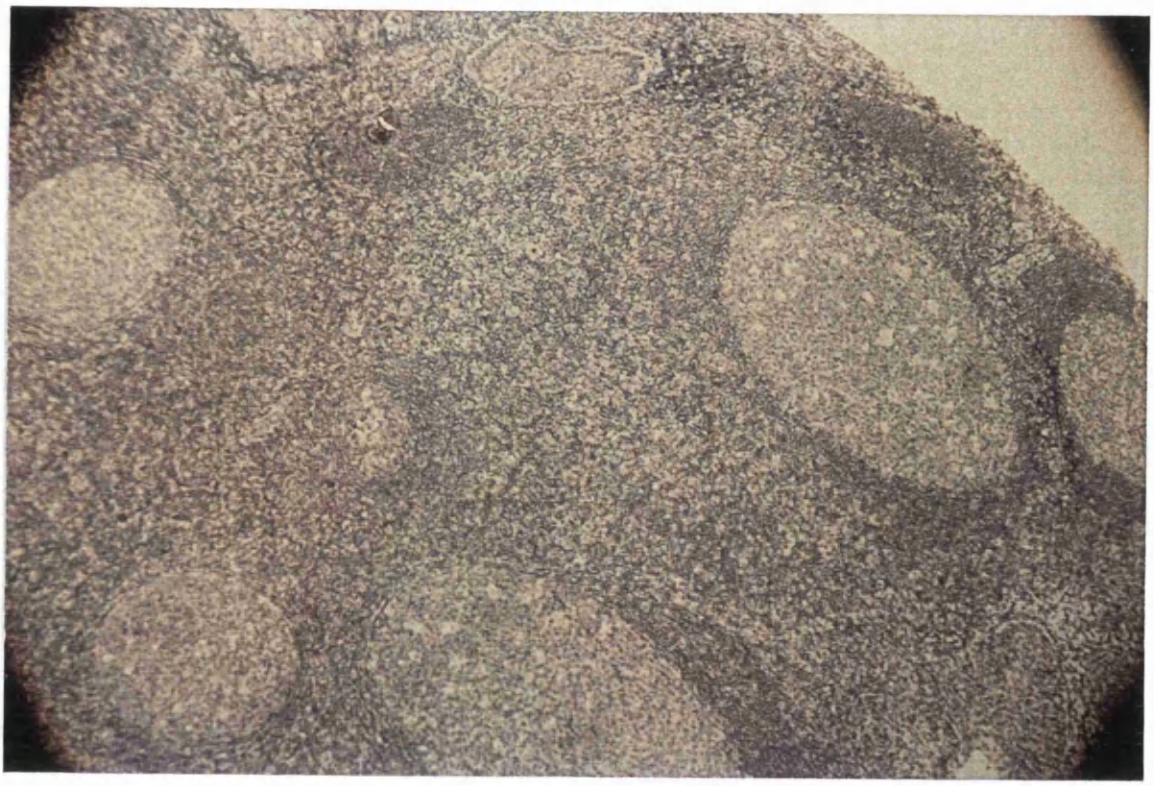
Solutions for ICC:

STWS; 0.35% $NaHCO_3$, 2% $MgSO_4$

Figure 2.2.10a

Immunocytochemistry showing Bcl-2 expression in human tonsil sections labeled with a) negative control mouse IgG antibody, b) mouse anti-human Bcl-2 followed by Biotin-conjugated goat anti-mouse antiserum. After then labeling with Horse-radish peroxidase conjugated to Streptavidin, detection was with DAB. Positive cells stained brown with negative cells having blue nuclei and a clear background. There is no positivity for Bcl-2 within the tonsil germinal centres but intense staining in the follicular mantle and areas around the germinal centres containing long-lived, recirculating B cells.

a)



b)

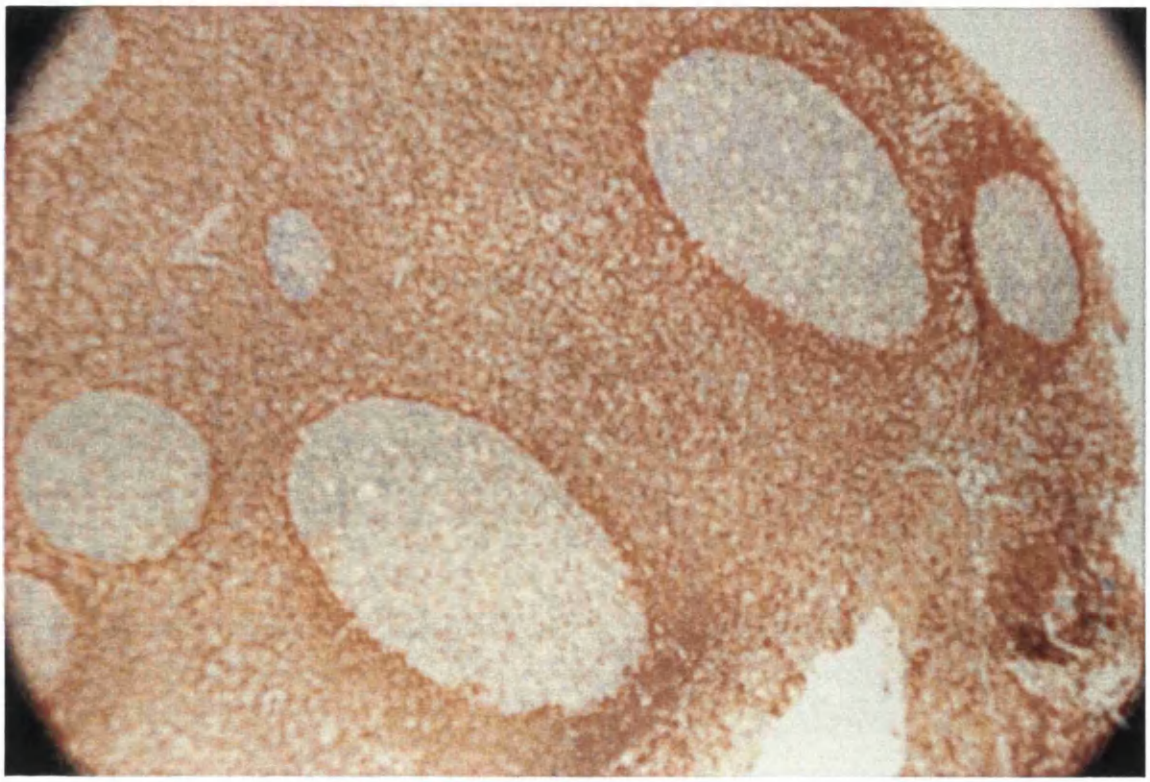
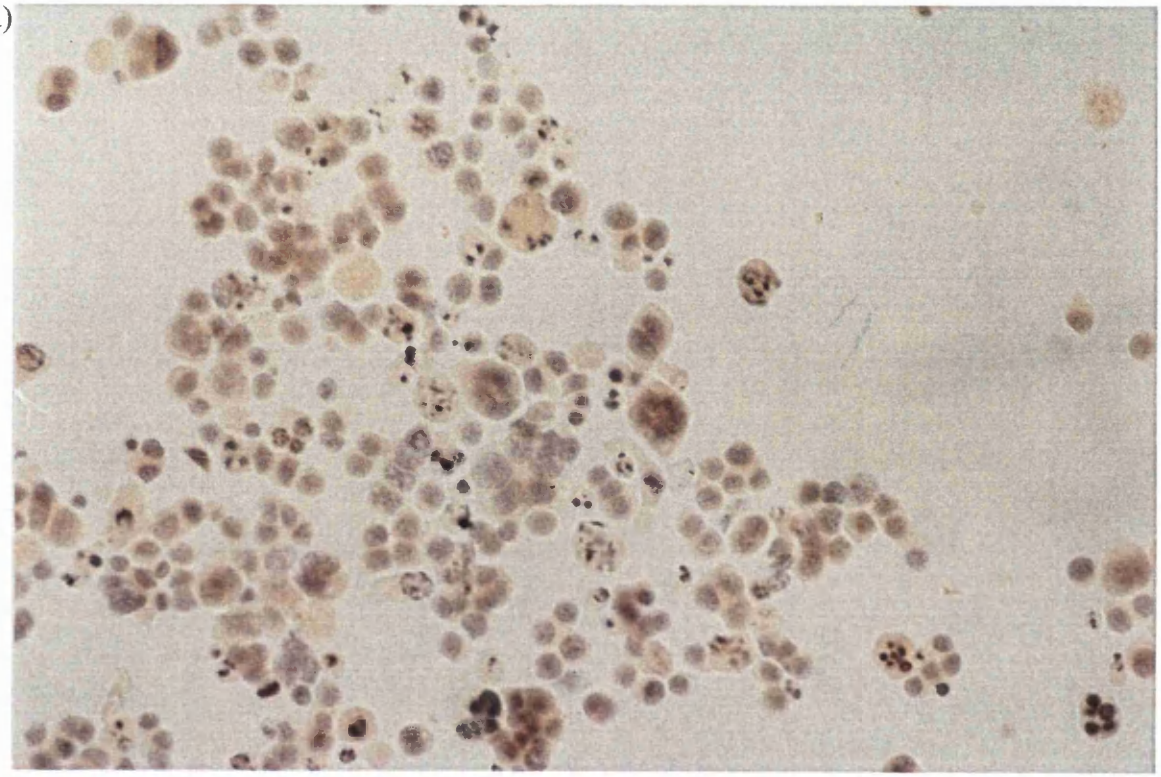


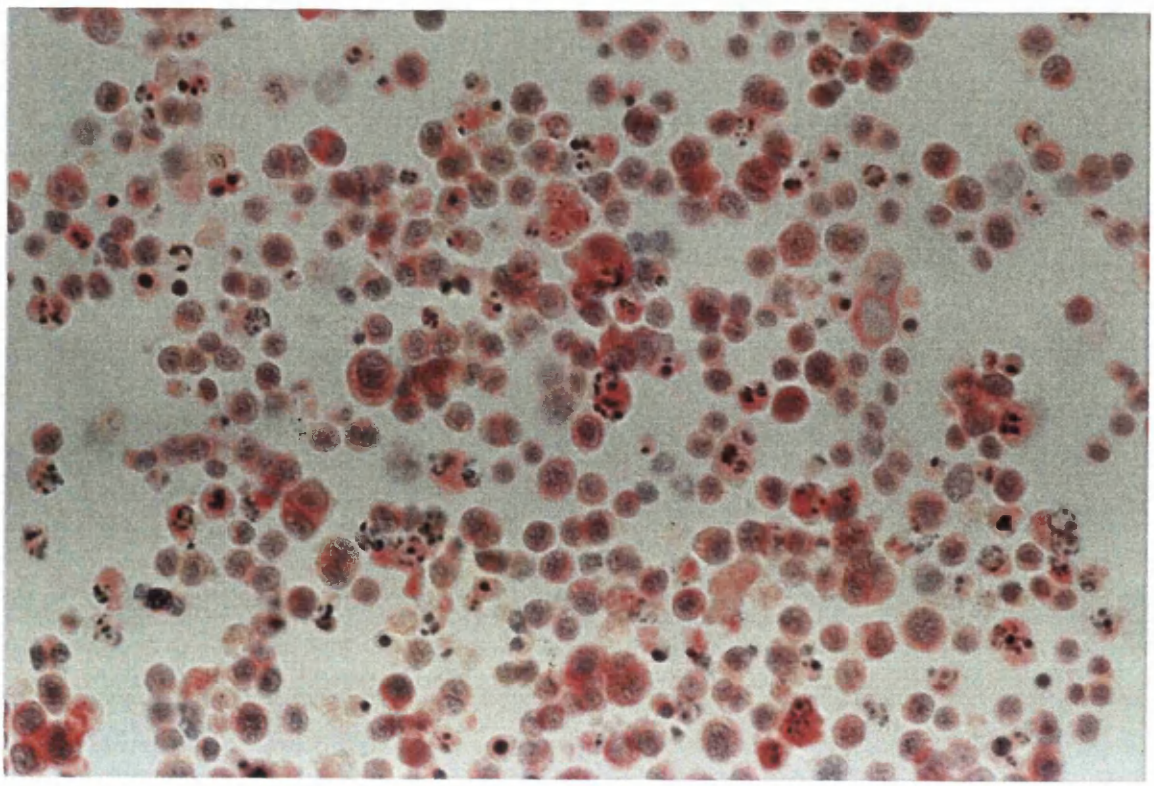
Figure 2.2.10b

Bcl-2 expression in the myeloma cell line JJN-3 detected by the standard indirect alkaline phosphatase method of immunocytochemistry using New Fuschin Chromogen substrate for detection. Cytospins were labeled with a) mouse IgG negative control or b) mouse anti-human Bcl-2. Cells were viewed under light microscopy (magnification x 10). Positive cells are stained red.

a)



b)



2.2.11 POLYMERASE CHAIN REACTION

This procedure was used to amplify cDNA encoding the genes of interest in this study in order to determine whether these genes were being transcribed in myeloma cell lines. Commercially synthesised sequence specific primers were used. In an initial step cDNA was produced from RNA by Reverse Transcription PCR (RT-PCR). The RNA was prepared as described in section 2.2.2.

The essential ingredients of a PCR reaction in order to amplify genomic DNA are:
DNA, a 3' (antisense) and a 5' (sense) primer, PCR buffer (containing Tris-HCl, KCl, $MgCl_2$, and gelatin), deoxyribonucleotides (dNTP's- dATP, dTTP, dGTP, dCTP), and DNA polymerase.

The method used was as described in the GeneAmp RNA PCR kit protocol and samples were amplifed using a Perkin Elmer Cetus 9600 PCR machine. Sense and antisense PCR primers 20 base pairs in length were chosen for the genes of interest in this study (*bcl-2*, *fas*, FasL, CD40, CD40L) from their cDNA sequences (synthesised by Cruachem Ltd.), (Table 2.2.11). Primers were selected with an average G+C content of 50% and a random base distribution. Primers were chosen which would result in a cDNA product which spanned introns to ensure that the product was not the result of DNA contamination of the RNA sample. IL-6 and IL-6R primers were purchased from Clontech Labs.Inc. RT-PCR reactions contained final concentrations of 5mM $MgCl_2$, 1x PCR buffer II, 1mM dNTP's, 1U/µl RNase inhibitor, 2.5U/µl reverse transcriptase, 2.5µM random hexamers, and ≤ 1µg total RNA. PCR reactions contained 2mM $MgCl_2$, 1XPCR buffer II, 2.5U/100ul Taq DNA polymerase and 0.15-0.2µM of each primer. The annealing temperature T_a was calculated as 5°C below the melting temperature (T_m).

The melting temperature (T_m) is the temperature at which 50% of the double-stranded hybridised species have dissociated and can be calculated using the following formula:

$$T_m = 16.6\log[M] + 0.41[P_{gc}] + 81.5 - P_m - B/L - 0.65[P_f]$$

Table 2.2.11

Sequences of synthetic primers used to perform RT-PCR reactions

	Primer 1	Primer 2			
cDNA	Sense	Antisense	Ta (deg.C)	Position in cDNA	Size
bcl-2	dGAGATGTCCAGGCAGCTGCA	dGCCGTACAGTTCCACAAAGG	65	371-640	269
CD40	dATGGTTCGTCTGCCTCTGCAGTGCGTCCT	dTAAAGACCAGCACCAAGAGG	64	48-690	642
CD40 (nested)	dGCGAATTCCTAGACACCTGG	dCCGATCCTGGGGACCACAGA	65	241-620	379
Fas	dAACCATGCTGGGCATCTGGA	dCATCTGCACTTGGTATTCTG	65	191-580	389
Fas (nested)	dACGTCTGTTGCTAGATTATC	dGGGCTTTGTCTGTGTACTCC	59	231-480	249
FasL	dAGATCTACTGGGTGGACAGC	dCATTCCAGAGGCATGGACCT	65	117-570	453
FasL (nested)	dCAGGCCTGTGTCTCCTTGTG	dTCTCCAAAGATGATGCTGTG	65	321-470	149

Probes synthesised in 5' to 3' direction

where:

M = the molar concentration of Na^+, to a maximum of 0.5 (1x SSC contains 0.165M Na^+)

P_{gc} = % of G or C bases in the oligonucleotide probe (30-70%)

P_m = % mismatched bases, if known - each 1% mismatch will alter the T_m by 1°C on average.

P_f = the percent of formamide in the buffer, B = 675 for synthetic probes up to 100 bases, L = probe length in bases

Standard reverse transcription PCR reaction: In the first step reverse transcriptase was used to transcribe RNA into cDNA using random primers (hexamers). In the second step cDNA encoding the gene of interest was amplified by Taq polymerase using sequence specific primers (Table 2.2.11).

1. Reverse transcription of RNA samples:

	1 tube	
$MgCl_2$ (25mM)	4µl	PCR reaction
10x PCR buffer II	2µl	
ddH_2O	1µl	10 minutes at 25°C
dNTP's (10mM)	2µl (of each)	30 minutes at 42°C
RNase inhibitor (20U/µl)	1µl	5 minutes at 99°C
Reverse transcriptase (50U/µl)	1µl	5 minutes at 4°C
Random Hexamers (50µM)	1µl	
RNA (1mg/ml)	2µl	Number of cycles = 1

2. cDNA amplifcation:

	1 tube	
$MgCl_2$ (25mM)	4µl	PCR reaction
10x PCR buffer I	8µl	1 minute at 94°C (denaturation)
ddH_2O	65.5µl	1 minute at T_a (55-70°C) (annealing)
DNA polymerase (2.5U)	0.5µl	1 minute at 72°C (extension)
cDNA (from above)	20µl	
primers (15-20µM)	1µl of each	Number of cycles = 25-30

The products of the second step PCR were visualised in a 1-2% agarose gel, containing ethidium bromide, depending on the size of the expected product. A molecular weight marker was run with the samples to determine the size of the products (2.1.6a).

A panel of myeloma cell lines were tested by RT-PCR using a series of sequence-specific primers to determine levels of transcription of the genes IL-6, IL-6R, *fas*, CD40 and *bcl-2*. Results are shown in Figures 2.2.11(a-d)

2.2.12 GENERATION OF cDNA PROBES

In order to synthesise riboprobes for use in *in situ* hybridisation or cDNA probes for Southern, Northern or dot blotting procedures, the cDNA encoding the gene of interest which had been previously cloned and inserted into a plasmid vector (eg pBluescript, pCRII or CDM8 plasmids,2.1.2), was transformed into competent E.Coli bacteria, grown to confluence and then harvested.. The plasmid containing the human cDNA insert was then extracted from the host bacteria (plasmid preparation). The cDNA was subsequently cut out of the plasmid by restriction enzyme digestion resulting in a cDNA probe. Alternatively, the plasmid containing insert was linearised, using an appropriate restriction enzyme, and the cDNA *in vitro* transcribed to manufacture an RNA probe (riboprobe) using the RNA polymerases T3 and T7 for pBluescript and T7 and SP6 for pCRII plasmids. Plasmid vectors containing the cloned and sequenced cDNAs of interest were generouly donated by the authors who cloned them for the *bcl-2*, *fas*, Fas ligand, CD40 and CD40 ligand genes. In order to generate a cDNA probe encoding the IL-6R gene, the β subunit of the interleukin-6 receptor (250 base pairs) was initially amplified by RT-PCR and the product cloned into the pCRII cloning vector. This was sequenced to verify authenticity of product, before subsequent amplification and restriction digestion (2.2.20).

cDNA inserts were transformed as follows; - those in the pBluescript vector were transformed into competent XL Blue (strain of E.Coli) - those in the CDM8 vector were transformed into competent MC1061/P3 (strain of E.Coli), (Ref. 261).

Probes were synthesised in this manner for the following human genes:

1) *bcl-2* - cDNA encoding the complete open reading frame (850 base pairs) inserted into the EcoRI and HindIII sites of the pBluescriptII SK+/- plasmid vector (2.96Kb) was generously donated by Dr Michael Cleary (Ref. 262).

Figure 2.2.11a

Reverse transcription polymerase chain reaction (RT-PCR) detection of mRNA in myeloma cell lines using IL-6 and IL-6R sequence-specific primers (Clonetech). Expected products are 628bp and 251bp respectively. Marker (M) is MspI cut pBR322 DNA.

Sample (RNA)	Products	
	IL-6	IL-6R
1. JIM-1	-	+
2. JIM-3	-	+
3. JJN-3	-	-
4. U266	+	+
5. RPMI-8226	-	+
6. IM-9	-	-
7. ANBL-6 (22/5)	-	+
8. ANBL-6 (27/2)	-	+
9. ANBL-6 (6/3)	-	+
10. ANBL-6 (17/2)	-	+ (weak)
11. IL-6 positive control RNA	+	-
12. IL-6R positive control RNA	-	+
13. no RNA	-	-
14. no reverse transcriptase+JIM-1 RNA	-	-

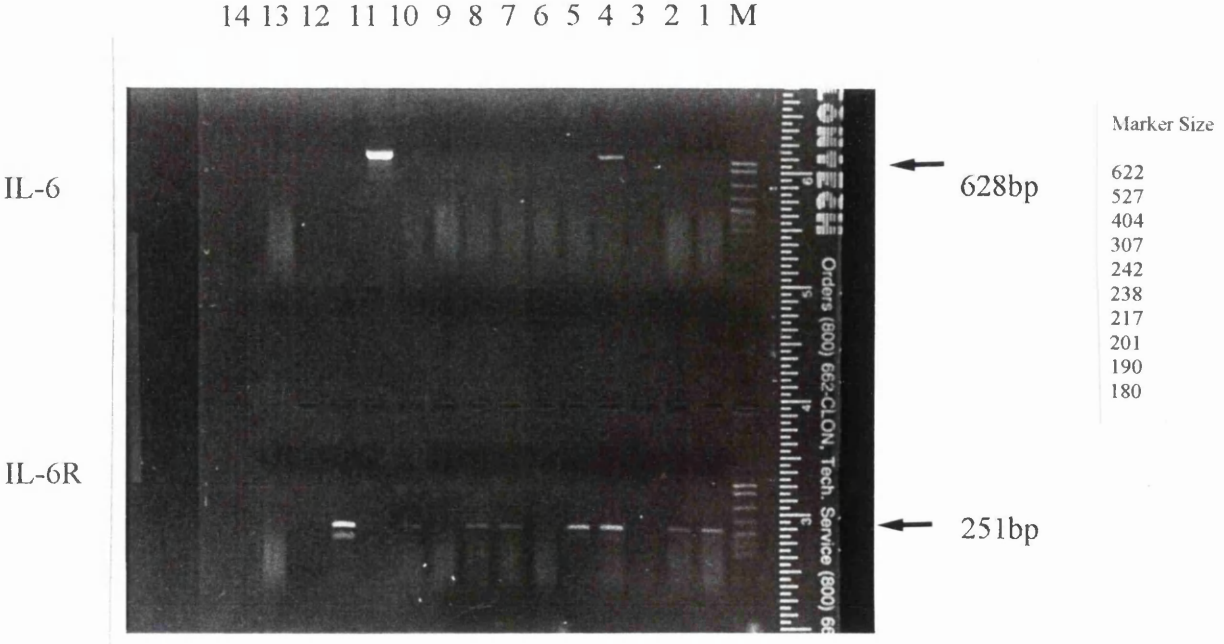


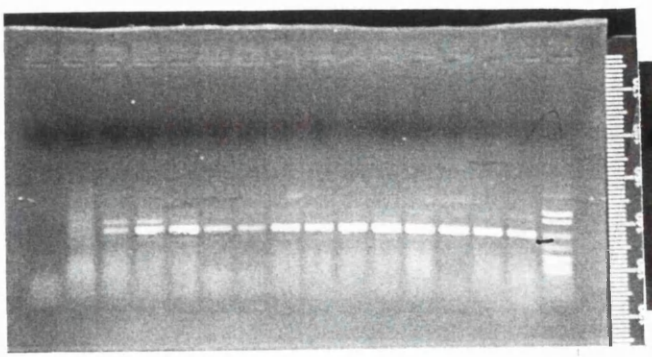
Figure 2.2.11b

Detection of Fas mRNA expression in myeloma cell lines using a) sequence-specific primers (product - 383bp) followed by nested PCR (product - 249bp). A 1:50 dilution of the products from (a) were amplified by primers chosen from within the original region of amplified DNA using Taq DNA polymerase. Marker (M) is MspI cut pBR322 DNA.

See page 70 for marker sizes

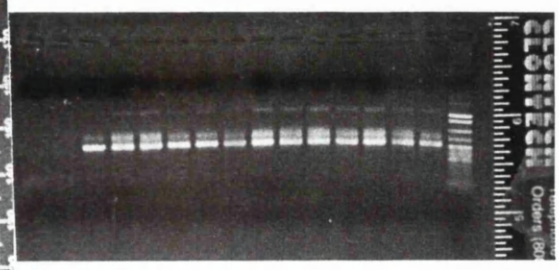
Sample (RNA)	Products		
	Fas	Fas (nested)	
1. JIM-1	+	+	
2. JIM-3	+	+	
3. JJN-3	+	+	
4. U266	+	+	
5. RPMI-8226	+	+	
6. IM-9	+	+	
7. ANBL-6 (22/5)	+	+	
8. ANBL-6 (27/2)	+	+	
9. ANBL-6 (6/3)	+	+	
10. ANBL-6 (17/2)	+	+	
11. SD-1	+	+	(ALL cell line)
12. MOLT-4	+	+	(T cell leukaemia cell line)
13. KYO-1	+	+	(CML cell line)
14. no RNA	-	-	
15. no reverse transcriptase+JIM-3 RNA	-	-	

15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 M



a) Fas RT-PCR

15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 M



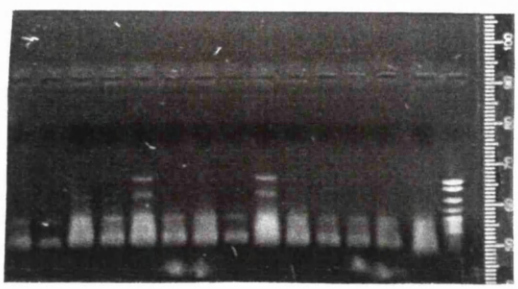
b) Fas nested PCR

Figure 2.2.11c

Detection of CD40 mRNA in myeloma cell lines by nested PCR. a) RT-PCR of RNA using CD40-specific primers spanning intron 1 (product - 642bp). No products detected. Samples from (a) were amplified b) by nested PCR (product - 379bp) to determine whether the copy number of CD40 gene expression was too low to be detected by the initial PCR. This was found to be the case since products were detected for all samples following nested PCR. Marker was MspI cut pBR322 DNA. See page 70 for marker sizes

Sample (RNA)	Products	
	CD40	CD40 (nested)
1. JIM-1	-	+
2. JIM-3	-	+
3. JJN-3	-	+
4. U266	-	+
5. RPMI-8226	-	+
6. IM-9	+	+
7. ANBL-6 (22/5)	-	+
8. ANBL-6 (27/2)	-	+
9. ANBL-6 (6/3)	-	+weak
10. SD-1	+	+ (ALL cell line)
11. MOLT-4	-	+ (T cell leukaemia cell line)
12. KYO-1	-	+ (CML cell line)
13. EJM	-	+
14. no reverse transcriptase+JIM-3 RNA	-	-

14 13 12 11 10 9 8 7 6 5 4 3 2 1 M

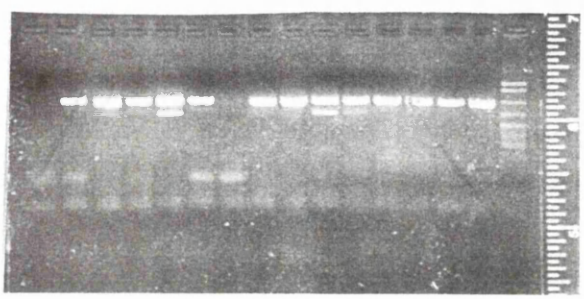


a) CD40 RT-PCR

379bp→

← 642bp

14 13 12 11 10 9 8 7 6 5 4 3 2 1 M

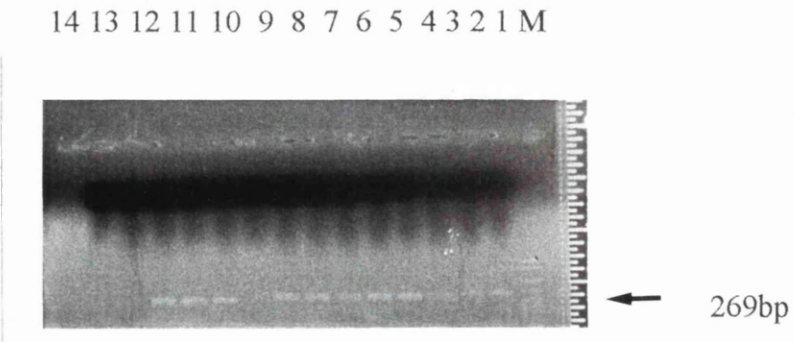


b) CD40 nested PCR

Figure 2.2.11d

Detection of *bcl-2* mRNA in myeloma cell lines by RT-PCR using *bcl-2* sequence-specific primers. Product - 269bp, marker - MspI cut pBR322 DNA. See page 70 for marker sizes

Sample (RNA)	**Products**
	bcl-2
1. JIM-1	+
2. JIM-3	+
3. EJM	+
4. JJN-3	+
5. U266	+
6. IM-9	+
7. RK	+
8. ANBL-6	+
9. no RNA	-
10. SD-1	+
11. MOLT-4	+
12. KYO-1	+
13. no reverse transcriptase+JIM-3 RNA	-
14. no RNA	-



bcl-2 RT-PCR

2) CD40 - cDNA encoding the complete open reading frame (900bp) inserted into the NotI and ScfI sites of the CDM8 plasmid vector (4.4Kb) was generously donated by Professor Ivan Stamenkovic (Ref. 98). This CD40 cDNA was subsequently subcloned into pBluescriptII SK+/- in order to provide promotor sites for *in vitro* transcription.

3) CD40L - cDNA encoding the complete open reading frame (1.1Kb) inserted into the EcoRI site of the pBluescriptII SK+/- plasmid was generously donated by Dr RJ Armitage (Ref. 94).

4) *fas* - cDNA encoding the full length (pBLF58-1,2.6Kb) gene inserted into the XhoI site of pBluescriptII SK+/- was generously donated by Dr Shigekazu Nagata (Ref. 101).

5) Fas ligand - full length cDNA (970bp) inserted into the XbaI site of pBluescriptII SK+/- was generously donated by Dr S. Nagata (Ref. 263).

6) IL6R - 250bp cDNA encoding IL6R beta subunit (complete open reading frame 1401bp) was amplified by polymerase chain reaction using IL6R specific primers and cloned into the EcoRI site of the pCRII cloning vector (Invitrogen, section 2.2.20), (Ref. 264).

2.2.13 PREPARATION AND TRANSFORMATION OF COMPETENT E.COLI

A. Preparation of competent E.Coli This procedure was performed in a Category 1 containment suite. All solutions were autoclaved or filter sterilised. A loopful of the XLBlue strain of E. coli was streaked onto a petri dish containing L-Broth (2.5%), agar (1.5%) with appropriate antibiotics and grown overnight at 37°C. A single colony was then transferred to a sterile universal container with 5ml L-Broth and expanded overnight at 37°C with shaking. This was then expanded further in 100ml L-Broth until the OD_{600} was between 0.2 - 0.4. Cells were spun at 3000rpm for 10 minutes at 4°C, the pellet resuspended in 50ml ice-cold 10mM $MgSO_4$. and left on ice for 20 minutes. Cells were spun again and the pellet resuspended in 20 ml ice-cold 50mM $CaCl_2$ and left on ice 30 minutes. After a further spin cells were resuspended in 1ml ice-cold 50mM $CaCl_2$. and left on ice for at least 30 minutes.

B. Transformation of competent E. Coli with pBluescriptII SK+/- containing cDNA inserts.

This describes the transformation of E.Coli with the cDNA insert encoding the open reading frame of the *bcl-2* gene inserted at the EcoRI and HindIII sites of pBluescript. The same procedure was used to transform XLBlue with pBluescript containing insert cDNAs encoding human CD40L, *fas* and Fas ligand genes. In summary, a plasmid DNA suspension was prepared at 1-10ng/μl in transformation buffer consisting of 10mM Tris HCl, pH8.0, 10mM $CaCl_2$, 10mM $MgCl_2$ (total volume 100μl). 100μl competent E.Coli was added and the mixed suspension incubated on ice for 20 minutes. Cells were heat shocked at 42°C for 2 minutes in order to render them susceptible to the uptake of plasmid DNA. Cells were left at room temperature for 10 minutes to ensure maximum uptake. After the addition of 2.5ml L-Broth to each tube, cells were incubated at 37°C for 90 minutes before plating out onto agar plates containing appropriate antibiotics. Plates were incubated at 37°C overnight. Routine transformation efficiencies of 1.5-1.8 x 10^5col/μg were obtained.

Method and calculation of transformation efficiency:

1. Dilutions of 0-10ng/μl plasmid DNA in 100μl Transformation buffer of plasmid DNA were prepared as follows:
   tube 1: zero DNA
   tube 2: 1ng/μl DNA
   tube 3: 5ng/μl DNA
   tube 4:10ng/μl DNA

2. 100μl competent E coli (from **A**) was added to each of tubes 1- 4, mixed by inversion and left to sit on ice for 20 minutes.

3. Cells were heat shocked at 42°C for 2 minutes to allow plasmid DNA to be taken up by E.Coli and then allowed to stand at room temperature for 10 minutes.

4. 2.5ml L-Broth was added to each tube and tubes were incubated at 37°C for 90 minutes before plating out on agar plates with appropriate antibiotic to detect plasmid (pBluescript plasmid is Ampicillin resistant). Three plates per dilution were prepared (100μl/plate) and plates were incubated upside-down overnight at 37°C in oven (to avoid condensation dripping onto colonies and spreading them).

5. The number of colonies on each plate was counted to determine the average number of colonies per plate per dilution.

The Transformation efficiency was calculated as follows:

Transformation Efficiency = number of colonies per microgram
ie: (colony forming units/μg) = CFU/μg

$$\text{ie} \quad \frac{\text{CFU in control plate}}{\text{ng insert used in transformation}} \times \frac{1 \times 10^3 \text{ng}}{\mu\text{g}} \times \text{dilution factor}$$

eg: plate 2 has 10ng insert cDNA in Bluescript
average number of colonies for three plates at this dilution = 66
volume in which 10ng added to produce these colonies = 2.7ml
(100μl competent XL Blue in 1ml $CaCl_2$ added to 100ul diluted pBluescript plasmid containing insert, added to 2.5ml L Broth)

CFU =66, dilution factor = 10 (10ng DNA in 100μl transformation buffer),
ng/ml insert used in transformation = 10/2.7 = 3.7

$$\text{so, TE} = \frac{66 \text{ CFU}}{3.7} \times \frac{1 \times 10^3 \text{ng}}{\text{ug}} \times 10 = 1.78 \times 10^2$$

ie: 10ng cDNA in 2.7ml = 3.7ng/ml total cDNA
100μl of this 3.7ng cDNA per ml = 66 colonies
(100μl streaked per plate = 66 colonies)
ie 0.37ng cDNA added per plate = 66 colonies
number of colonies per ng = $66/0.37 = 1.78 \times 10^2$
number of colonies per ug = 1.78×10^5
ie Competent cells have a Transformation efficiency of 1.78×10^5col/μg

C. Transformation of competent MC1061/P3 strain of E.Coli with CDM8 plasmid vector containing the CD40 cDNA insert, (Ref.265). This was performed according to the Invitrogen protocol supplied.

Briefly, the already ultracompetent E coli provided in the kit were transformed with an aliquot of the CDM8 plasmid vector containing the cloned insert of human CD40 cDNA as described in section 2.2.12 as follows:

1. One aliquot of ultracompetent MC1061/P3 cells (0.3ml) was removed from -80°C freezer and thawed on ice and mixed gently by hand.

2. An aliquot of 100μl of these cells was added to each of two pre-chilled 15ml Falcon tubes - 100μl for CDM8 containing insert (CD40 cDNA) and 100μl for the pUC18 positive control. The remaining cell suspension was stored at -80°C.

3. 5µl 0.5M beta mercaptoethanol was added to each 100µl aliquot and the tubes swirled gently. Incubation was continued on ice for 10 minutes, with swirling every 2 minutes.

4. 10ng (1µl) CD40 cDNA in CDM8 vector or 10ng of pUC18 control was then added and the tubes swirled evenly to mix DNA and cells. Cells were then incubated on ice for 30 minutes.

5. Cells were then heat shocked by placing the tube in a 42°C water bath for 75 seconds, returned to ice and chilled for 2 minutes.

6. 900µl SOC (2.2.20) medium was added and the cells were incubated at 37°C with moderate agitation (225rpm) for 60 minutes.

7. 100µl per plate was plated onto LBroth/agar plates with antibiotics as follows:

	Resultant growth expected (and found)
a) No antibiotics	lawn of growth
b) Kanamycin (Kan) @ 50µg/ml	lawn of growth
c) Kan, Tet (10µg/ml), amp (30µg/ml) containing MC1061 + CDM8 insert.	discrete colonies
d) Ampicillin only	discrete colonies in positive control plate.

The positive control pUC18 plasmid (1ng/µl) is ampicillin resistant and results in the formation of discrete colonies on plates containing only ampicillin.

E.Coli harbouring the plasmid P3 permit selection and maintainance of plasmids which encode the tRNA suppressor F gene (*sup*F). The P3 episomal plasmid in MC1061 E.Coli encodes the kanamycin resistance gene as well as amber mutants of the tetracycline and ampicillin resistance genes. Therefore, strains which harbour P3 alone are resistant to kanamycin but sensitive to both tetracycline and ampicillin. When E.Coli carrying the P3 plasmid are transformed with *sup*F plasmids (eg CDM8), they are rendered resistant to both tetracycline and ampicillin by suppression of the amber mutations.

2.2.14 PLASMID PREPARATIONS

Six individual plaques from **B**, plates containing ampicillin and tetracyclin (pBluescript containing insert in XLBlue) and **C**, plates containing kanamycin, tetracyclin and ampicillin (CDM8 containing insert in MC1061/P3), were picked and expanded in 5ml L Broth overnight at 37°C. The plasmid was then separated from EColi by plasmid preparation (Qiagen Ltd.) and digested with the appropriate restriction enzymes to verify that the correct cDNA insert had been selected (Figure 2.2.15d, see below). The best of the six plasmid minipreps was then selected for large scale expansion and plasmid preparation (Qiagen maxiprep DNA purification system) in order to either cut out cDNA insert and use directly as a probe or to linearise the plasmid and synthesise a riboprobe.

2.2.15 RESTRICTION DIGESTION OF PLASMID DNA

Restriction maps of all cDNA inserts were performed to verify authenticity of products following transformation and expansion in E.coli (Figures 2.2.15a, 2.2.15b and 2.2.15c). In all cases restriction products were as expected.

For example:

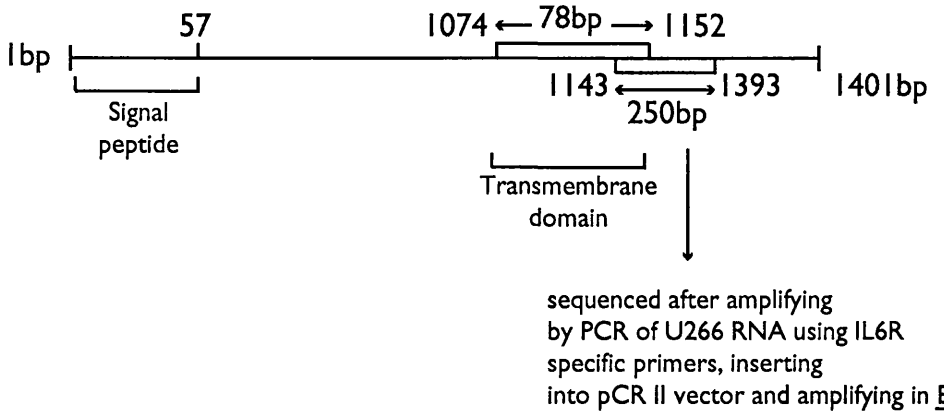
a) pBluescript plasmid (2.96 kilobases) contains a cDNA insert of human *bcl-2* gene, 850 base pairs in length. The cDNA insert was added by cutting the pBluescript II SK +/- at the EcoRI and HindIII restriction sites. A restriction digest was set up to cut out the insert at these sites. The products were then run in an agarose gel to verify the size and purity of the plasmid preparartion.

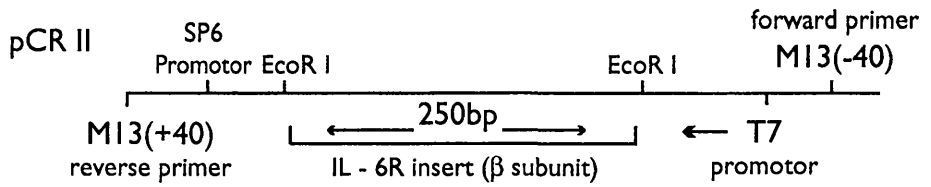
b) CDM8 plasmid vector (4.4Kb) contains CD40 cDNA insert (900bp) which is cloned into the MC1061 strain of E.Coli at the NotI and ScfI sites. The insert can also be excised by cutting the plasmid at the XbaI or XhoI restriction sites.

Restriction digest of *bcl-2* cDNA in pBluescript		
Master Mix: 1 reaction tube	10x reaction buffer	2µl
	ddH_2O	6µl
	EcoRI	1µl
	HindIII	1µl
	plasmid DNA (approx 1ug)	10µl

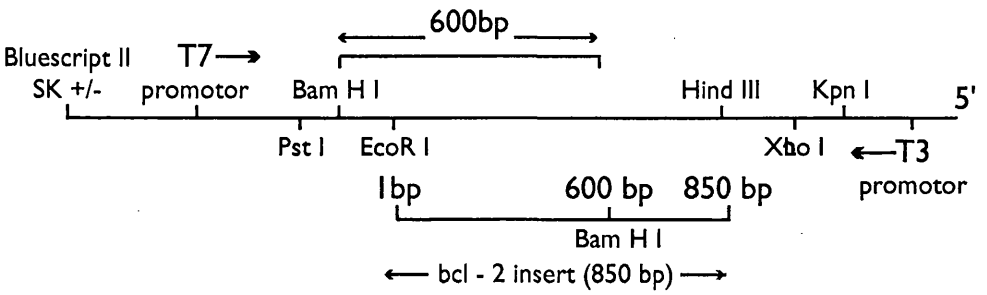
Figure 2.2.15a
RESTRICTION MAPS OF CLONED GENES FOR USE AS PROBES

1) **IL6R - open reading frame = 1401 bp (467 codons)**





2) **bcl - 2 restriction map**



3) **CD40L restriction map**

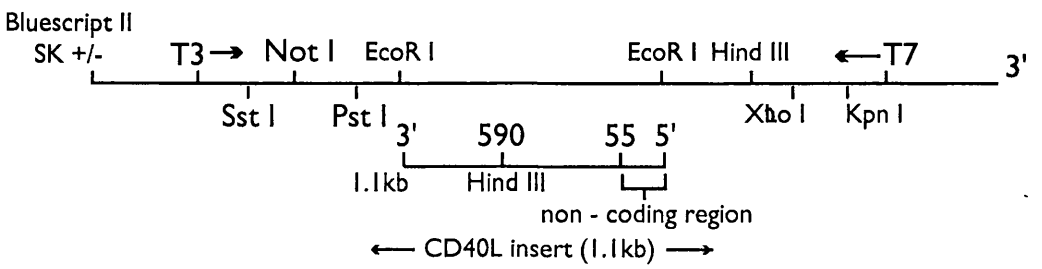
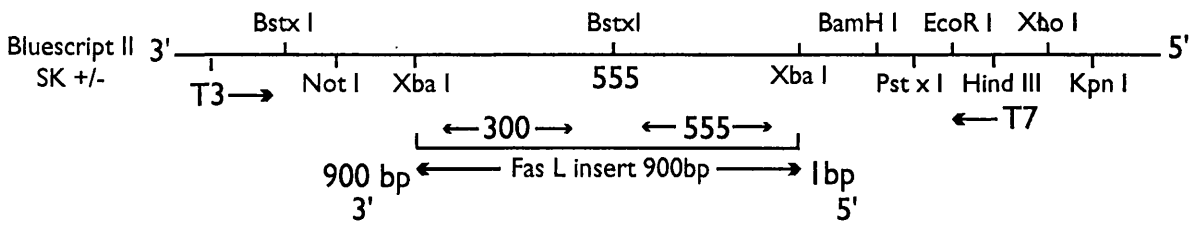
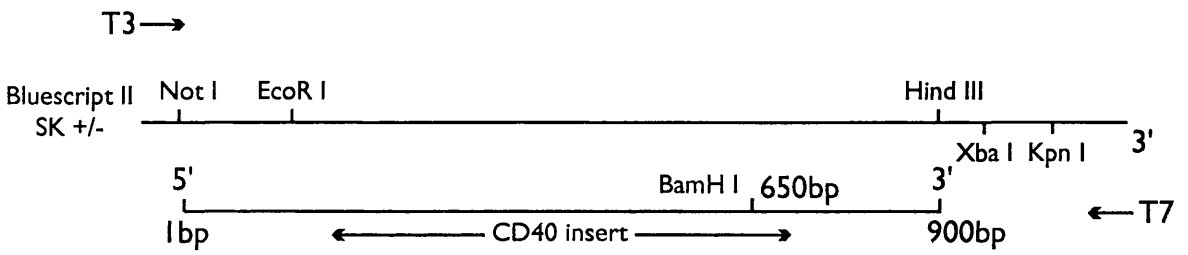


Figure 2.2.15b
RESTRICTION MAPS OF CLONED GENES FOR USE AS PROBES

4) Fas L restriction map



5) CD40 restriction map



6) Fas restriction map

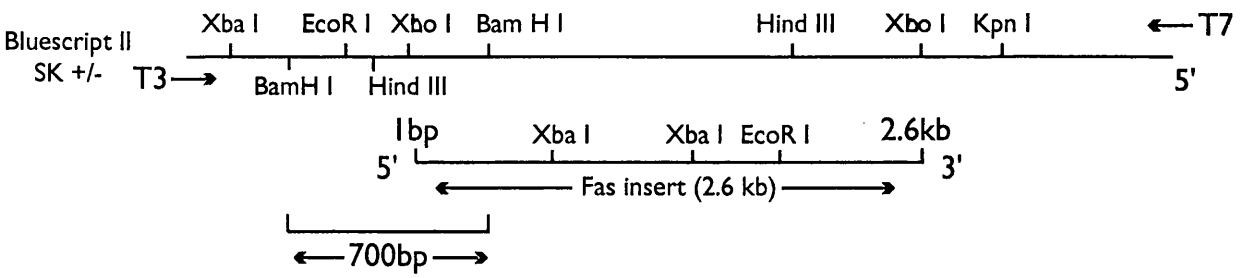


Figure 2.2.15c

Restriction map of *fas* in pBluescript cut with the following restriction enzymes:

	Products*
1) XbaI+XhoI	400bp, 700bp, 1.1kb, 3kb
2) XbaI+BamHI	400bp, 800bp, 4kb
3) XbaI	400bp, 1.1kb, 4kb
4) XhoI	2.5kb, 3kb
5) BamHI	700bp, 5kb
6) uncut	

Marker- EcoRI+HindIII λDNA.

*In all cases products of restriction digestion were as expected from restriction maps.

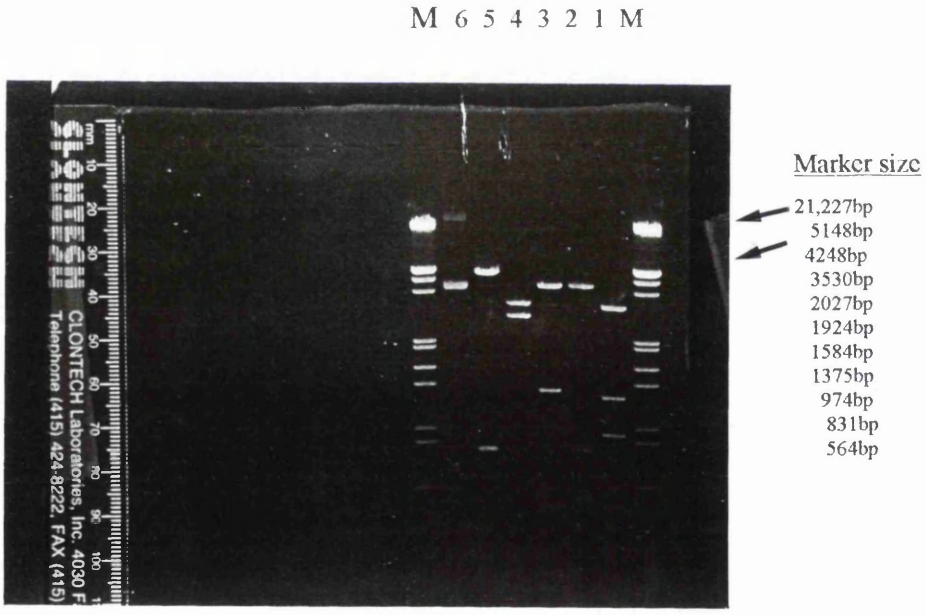
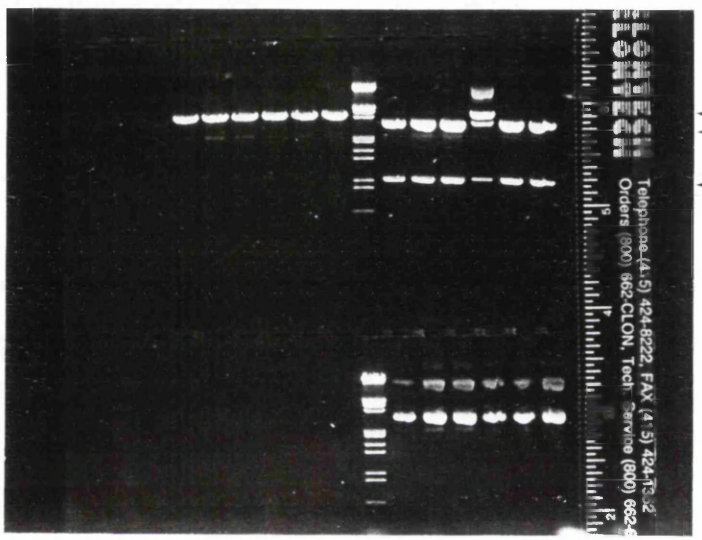


Figure 2.2.15d

1. Restriction digest of mini plasmid preparations (samples 1-6) of human Fas ligand cDNA inserted into pBluescript following transformation into XLBlue, expansion and harvesting (2.2.14). Samples were visualised on a 1% agarose gel containing ethidium bromide and were digested with a) no enzyme, b) NotI (product is linearised plasmid containing insert, 3.9kb) or c) XbaI (products are plasmid, 3kb and insert, 843bp). The marker was EcoRI+HindIII λ cut DNA. See page 81 for marker sizes

6 5 4 3 2 1

b)

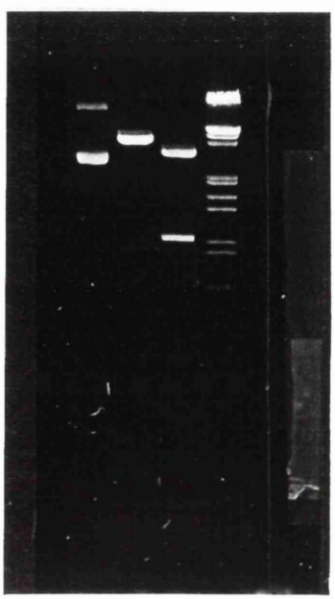


c)

a)

2. Miniprep sample 2 (above) chosen to expand further in XLBlue for subsequent maxi plasmid preparation. Restriction digest of maxiprep cut with a) XbaI, b) NotI or c) no enzyme. Marker (M) was EcoRI+HindIII λDNA.

c b a M



Digests were performed for each of 6 tubes (plasmid preparations) and 6 control tubes containing no restriction enzymes. These were incubated in a water bath at 37°C for 1-2 hours, then run in 1% agarose gel containing 0.25μg/ml ethidium bromide at 125 volts for 30 minutes. The gel was observed under ultraviolet light and photographed. This procedure was used to isolate cDNA encoding all the genes of interest. Figure 2.2.15d shows the agarose gel visualising restriction digests of mini plasmid preparations and subsequent maxi plasmid preparation of the Fas ligand cDNA in pBluescript as an example.

Once product size and purity was confirmed :

A maxi plasmid prep of insert DNA was performed. The $OD_{260/280}$ was calculated to assess the yield of purified CD40 cDNA. This was between 1.8-2.0 verifying the purity of the DNA preparation and enabled calculation of the DNA concentration.

In order to separate cDNA insert from plasmid to use as a cDNA probe a 200μl preparative digest using restriction enzymes which excised the insert was performed. Products were run in 1.2% low melting temperature agarose gel (containing EtBr), 100v for approximately 2 hours. The cDNA insert was excised under UV light stored at 4°C and used for subsequent Northern and Dot blotting experiments.

eg, For the *bcl-2* insert:

200μl preparative restriction digest:

10X reaction buffer		20μl
ddH_2O		108.45μl
EcoRI		10μl
HindIII		10μl
50ug (plasmid+*bcl-2*) DNA		51.55μl
	Total	200μl

CALCULATION OF cDNA INSERT CONCENTRATION

Maxiprep DNA purification results in DNA preparation of:

plasmid + insert

The following formula was used to calculate the overall DNA concentration:

OD_{260} x dilution of sample x 50 = [DNA] of plasmid + insert in μg/ml

eg: *bcl-2* plasmid preparation:
OD_{260} = 1.94 (1:10 dilution)
ie pBluescript + *bcl-2* insert [DNA] = 1.94 x 10 x 50 μg/ml
= 970μg/ml

200μl restriction digest to separate plasmid from insert contains 50μg DNA (plasmid + insert) ie 50μg DNA is equivalent to :

plasmid (pBluescript)	3000bp in length
insert (*bcl-2*)	850bp in length
total length	3850bp

Insert = (850/3850)% ie 22% of total

ie [insert DNA] = 22% of 50μg =11μg

This is contained in the agarose cut out from the 200ul restriction digest = approx 2.5ml (the tube is weighed before and after adding DNA/agarose, 1 gram = approx 1ml, or alternatively, DNA/agarose could be melted at 65°C pipetted and the volume quantitated).
ie 11μg = 2.5ml, so 4.45μg = 1ml, ie 4.45ng/ul.

The *bcl-2* cDNA probe is at a concentration of 4.45ng/ul.

2.2.16 SUBCLONING OF CD40 cDNA FROM CDM8 PLASMID VECTOR INTO pBLUESCRIPT

In order to provide two promotor sites for in vitro transcription CD40 cDNA was excised from CDM8 and subcloned into pBluescript by ligation with DNA ligase. The insert was cut out using the restriction enzymes NotI and HindIII and ligated into the same restriction sites of pBluescript (the original insert was inserted at the NotI and ScfI sites of CDM8 but ScfI requires a temperature of 50°C with bovine serum albumin added to the restriction digest so HindIII was used instead).

Preparative digests of CD40 cDNA in CDM8 plasmid vector and of pBluescript containing no insert were carried out. The pBluescript digest was phenol extracted (2.2.3) to remove any residual restriction enzyme activity which would interfere with the ligation reaction. The entire CDM8 digest was run in a 1% low melting agarose gel to separate CD40 insert from plasmid (no ethidium bromide was added to the gel since this, in combination with light,

causes nicks in the DNA which reduces the transformation efficiency). The CD40 insert was cut out of the gel under UV light after staining for 10 minutes in ethidium bromide to visualise the product.

Preparative digest of CD40 cDNA from CDM8 plasmid vector

1. CD40 cDNA was cut out of CDM8 using the restriction enzymes NotI and HindIII.
2. pBluescript plasmid DNA (vector DNA, no insert) was also cut at the NotI and HindIII restriction sites of the multiple cloning site (MCS).
3. Competent XLBlue cells were prepared and kept on ice for transformation with ligation reaction products.

Restriction digests	CD40 in CDM8	pBluescript (No insert)
10x reaction buffer	40µl	20µl
ddH_2O	46µl	160µl
NotI	10µl	5µl
HindIII	10µl	5µl
DNA (CD40 in CDM8,20µg) @68µg/ml	294µl	(pBluescript,10µg) @ 1mg/ml 10µl
TOTAL	400µl	200µl

Samples were incubated at 37°C for one hour. Then a further 2µl of each restriction enzyme was added and samples incubated for a further hour (or overnight) to ensure complete digestion. Following electrophoresis, the excised CD40 cDNA was extracted from the low melting temperature agarose by electroelution using the dialysis tubing method as follows:

Dialysis tubing (Sigma) was boiled in 0.06% EDTA for 10 minutes. The gel containing the CD40 insert was inserted into this tubing containing 1ml TE buffer with a knot tied at both ends. The tubing was placed onto the platform of a gel electrophoresis tank containing 1x TBE buffer and ethidium bromide and electrophoresed at 125 volts until all of the DNA had been eluted from the agarose into the TE buffer. This could be visualised under UV light. The polarity was reversed and electrophoresis continued for 10 minutes to collect any residual DNA which may have stuck to the walls of the dialysis tubing.. The extracted DNA in TE buffer was phenol extracted and the $OD_{260/280}$ was calculated to assess the yield of purified CD40 cDNA. This was between 1.8-2.0 verifying the purity of the DNA preparation and enabled calculation of the DNA concentration.

LIGATION OF CD40 cDNA INTO pBLUESCRIPT

Ligations containing 10-50ng CD40 cDNA and 10-50ng vector pBluescript DNA were set up with appropriate controls (uncut pBluescript, pBluescript cut with NotI only (+/- ligase), pBluescript cut with HindIII only (+/- ligase)). Ligation was performed using T_4 DNA ligase and ligation buffer containing 50mM Tris.HCl, pH7.8, 10mM $MgCl_2$, 10mM DTT, 1mM ATP, (New England Biolabs.). Reaction mixes were incubated at 20°C overnight.

Procedure:

1) Ligation of CD40 cDNA (1.14µg in 400µl TE) into pBluescript cut with NotI and HindIII (5µg in 100µl TE). Ligations were set up containing 50ng, 20ng and 10ng CD40 cDNA + ligase (reactions 10+11+12).

Controls:

2) Uncut pBluescript with no insert DNA (reaction 1)
3) pBluescript cut with HindIII only (+/- ligase) with no insert DNA (reactions 2+3)
4) pBluescript cut with NotI only (+/- ligase) with no insert DNA (reactions 4+5)
5) pBluescript cut with NotI+HindIII (+/- ligase) with no insert DNA (reactions 6+7)
6) No pBluescript + insert DNA (+/- ligase), (reactions 8+9)

LIGATION

	1	2	3	4	5	6	7	8	9	10	11	12
10x R.buffer	2. 5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
DNA 1 (50ng/ul)	1µl[1]	1µl[2]	1µl[2]	1µl[3]	1µl[3]	1µl[4]	1µl[4]	-	-	1µl[4]	1µl[4]	1µl[4]
DNA 2 (2.85ng/ul)	-	-	-	-	-	-	-	17.5µl (50ng)	17.5µl	17.5µl	7µl (20ng)	3.5µl (10ng)
ddH_2O	21.5µl	20.5	21.5	20.5	21.5	20.5	21.5	4	5	3	13.5	17
T_4DNA ligase(4U/ul)	-	1µl	-	1µl	-	1µl	-	1µl	-	1µl	1µl	1µl
TOTAL	25µl	25µl	25µl	25µl	25µl	25µl	25µl	25µl	25µl	25µl	25µl	25µl

DNA 1 - pBluescript @ 50ng/µl

DNA 2 - CD40 cDNA @ 2.85ng/µl

superscripts (DNA 1): [1] Uncut pBluescript
[2] pBluescript cut with HindIII only
[3] pBluescript cut with NotI only
[4] pBluescript cut with NotI and HindIII

Reaction mixes incubated at 20°C overnight (RT).

Transformation of ligation reactions into XL Blue strain of E.Coli was carried out as described in section 2.2.12. Positive colonies were selected from L-Broth/agar plates containing approriate antibiotics and Xgal (Gibco BRL) as follows:

1. The contents of each ligation reaction (25µl) were added to 75µl transformation buffer, ie 100µl total.
2. This 100µl was added to 100µl of competent XL Blue as previously described (2.2.12) and 100µl plated out onto LBroth/agar plates (2 plates per ligation reaction, ie 24 plates in total) as follows:

a) L Broth/agar containing ampicillin @ 100µg/ml
tetracycline @ 7.5µg/ml
XGal* @ 40mg/ml, 25µl/plate

* Xgal results in the formation of white and blue colonies (2.1.4). White colonies contain insert and are therefore selected to perform plasmid preparations and assess whether insert is present following restriction analysis.

b) Control plates - LBroth/agar+tetracycline alone - show a lawn of growth as XLBlue is tetracycline resistant.

Results:

1. Uncut pBluescript, no ligase - colonies all blue (βGal gene not interrupted)
2. pBluescript cut with HindIII + ligase - all blue colonies (pBluescript ligated back together)
3. pBluescript cut with HindIII - no ligase - very few colonies, all blue
4. pBluescript cut with NotI + ligase - several colonies, all blue
5. pBluescript cut with NotI - no ligase - very few colonies, all blue
6. pBluescript cut with NotI and HindIII + ligase - colonies all blue, not many as the MCS has been cut out
7. pBluescript cut with NotI and HindIII - no ligase - no colonies
8. CD40 insert alone cut out of CDM8 with NotI and HindIII + ligase - no colonies
9. CD40 insert cut out of CDM8 with NotI and HindIII - no ligase -no colonies
10. pBluescript (double cut, 50ng) + CD40 (insert cut out,50ng) + ligase - blue and white colonies - white colonies contain pBluescript+CD40 insert
11. pBluescript (double cut,50ng) + CD40 (20ng) + ligase - blue and white colonies. White colonies contain insert
12. pBluescript (double cut,50ng) + CD40 (10ng) + ligase - blue and white colonies.

Fourteen white colonies were picked from plates 10,11,12 and 14 mini plasmid preparations were performed. The resultant DNA was digested with the NotI and HindIII restriction enzymes and the products run on a 1% agarose gel. This resulted in the visualisation of two bands - one band at 900bp (CD40 cDNA), one band at 3Kb (pBluescript). Control digests were performed by picking 3 blue plaques, performing a plasmid prep, and digesting the DNA with NotI and HindIII. A resultant band at 3kb was seen following electrophoresis with an equivalent size to pBluescript without insert.

The best miniprep sample was chosen and the remainder of that expanded plaque grown up and used to perform a preparative (maxi) restriction digest.This was subsequently phenol extracted and used as a template for *in vitro* transcription.

2.2.17 RNA DOT BLOT HYBRIDSATION

The cDNA cut out of its plasmid vector using appropriate restriction enzymes was run in a low melting point agarose gel (2.2.16) until plasmid and insert were completely separated. The insert DNA cut out of the gel was used to synthesise a cDNA probe. cDNA probes were generated with ^{32}P-dCTP using Random Hexamers by labeling isolated plasmid insert DNA with ^{32}P-labelled dCTP using random hexamers as primers (Ref. 266).

Method:

cDNA probe preparation:

Excised cDNA (20ng) was heat denatured at 99°C for 10 minutes and added to a 50µl reaction mix containing 1x oligo labeling buffer (OLB), 30µCi ^{32}P-CTP and 5U Klenow Fragment. The mixture was incubated in a 37°C water bath for at least 1 hour or for several hours at room temperature. The product is smaller than the original 850bp cDNA in the case of *bcl-2* as the Klenow fragment does not synthesise the entire strand but is of the order of 300-400 base pairs long. A microtitre grid template was prepared on a PALL Biodyne B Nylon Transfer Membrane which was wet in DEPC.H_2O, then in 20 x SSC before blotting with 3MM paper and air dried.

Doubling Dilution Assay: A stock solution of RNA in 20µl DEPC.H_2O (500µg/ml for total RNA) was prepared, heat denatured at 65°C for 15 minutes and snap cooled on ice. 20ul of each RNA sample was put into the first well of a microtitre plate and 10ul ddH_2O in wells 2-12. 10µl from well 1 was mixed with the contents of well 2, 10µl transfered to well 3 with doubling dilutions continued up to well 12. 4µl of each sample was spotted onto the hybridisation filter and it was air dried. The filter was baked for 30 minutes at 80°C then the RNA was immobilised by cross-linking under UV light for 2 minutes.

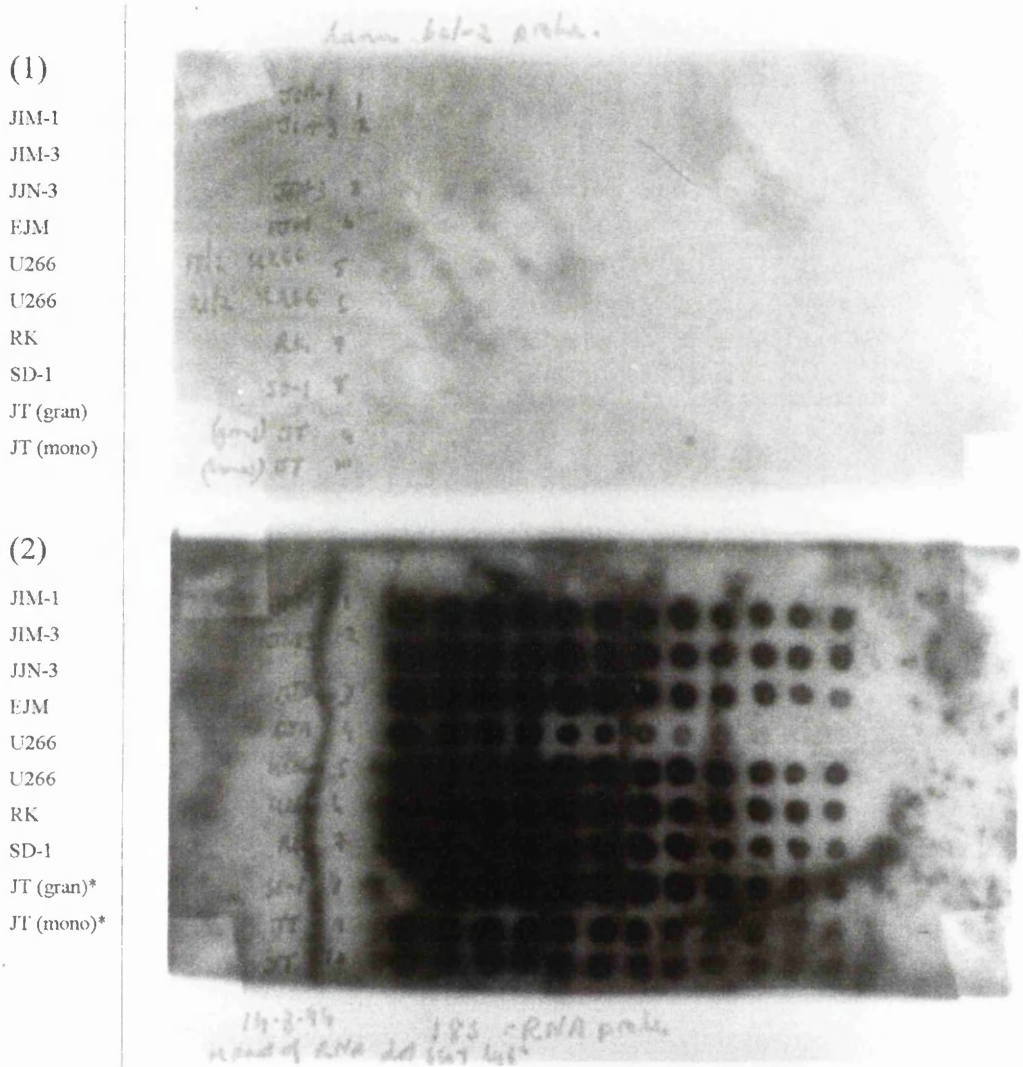
Hybridisation with cDNA ^{32}P-labelled probe: Filters were prehybridised at 65°C in 10ml Aqueous Dextran Buffer. Heat denatured salmon sperm DNA (1mg\ml) was added just prior to pre-hybridisation to block non-specific binding of the labeled probe. The labeled probe was passed through a NICK Sephadex column (Pharmacia Biotech) to separate nick-translated DNA from unincorporated ^{32}P-labeled nucleotides. It was heated to 99°C for a couple of minutes to dissolve the agarose prior to adding to the hybridisation tube. Hybridisation was at 65°C overnight. Filters were subsequently washed to a final SSC concentration of 0.1x SSC and then autoradiographed. Figure 2.2.17 shows hybridisation of a human *bcl-2* cDNA probe, prepared as described above, to dot-blotted RNA prepared from the myeloma cell lines; JIM-1, JIM-3, JJN-3, EJM, U266 and RK. RNA prepared from SD-1 (an acute lymphoblastic leukaemia cell line) and both granulocyte and monocyte RNA preparations of a normal control (JT) which were used as negative controls. A ^{32}P-labeled 18S rRNA was used as a positive control probe. The cell lines JIM-1, JIM-3, JJN-3, EJM, U266, RK were all positive for *bcl-2* mRNA expression with SD-1 and JT (granulocyte and monocyte preparations) all negative.

2.2.18 cDNA dot-blot hybridisation with Digoxygenin-labeled riboprobes (to test specificity of probes following IVT). See Figure 2.2.18.

The cDNA products of RT-PCR reactions were dot-blotted onto Hybond-N^+ filters (Amersham Life Science) and hybridised with digoxygenin-labeled riboprobes (2.2.19) using "DIG Easy Hyb" (Boehringer Mannheim) hybridisation solution for nucleic acid blots using Dig-labeled probes according to the supplied protocol as follows:

Figure 2.2.17

Dot-Blot hybridisation of RNA from myeloma cell lines with a ^{32}P-labeled human *bcl-2* cDNA probe (1). A ^{32}P-labeled 18S rRNA probe was used as a positive control (2).



All myeloma samples showed positive hybridisation to the 18S control probe. The cell lines; JIM-1, JIM-3, JJN-3, EJM (weak), U266 and RK were positive for *bcl-2* RNA expression with SD-1 (an ALL control cell line) and normal controls JT (granulocyte preparation) and JT (monocyte preparation) all negative. *JT is not the same patient as the myeloma cell line JT used in the rest of this thesis. Two different RNA preparations of U266 were compared. The second one consisted of older RNA and was subsequently shown to be degraded (no hybridisation was observed in this dot-blot).

RK is a myeloma cell line developed by the author (2.1.1).

Figure 2.2.18

Specificity of riboprobes tested using dot-blots of RT-PCR products.

A. In an initial step myeloma cell line RNA was reverse transcribed using sequence-specific primers and the products run on a 1% agarose gel. The primers used were specific for the following cDNAs; a) *bcl-2* (product 269bp), b) CD40 (product 642bp), c) FasL (product 453bp), d) *fas* (product 389bp). See photographs of gel electrophoresis. Markers- MspI cut pBR322 DNA and HindIII cut λDNA.

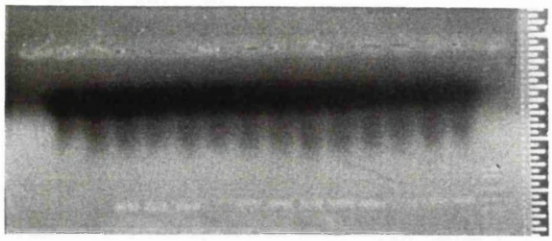
Summary of Results

Sample (RNA)	**RT-PCR Products**			
	bcl-2	**CD40**	***fas***	**FasL**
1. JIM-1	+	-	+	-
2. JIM-3	+	-	+	-
3. EJM	+	-	+	-
4. JJN-3	+	-	+	-
5. U266	+	-	+	-
6. IM-9	+	+	+	-
7. RK	+	-	+	-
8. ANBL-6	+	-	+	-
9. RPMI-8226	-	-	-	-
10. SD-1	+	+	+	-
11. MOLT-4	+	-	+	-
12. KYO-1	+	-	+	-
13. no reverse transcriptase+JIM-3 RNA	-	-	-	-
14. no RNA	-	-	-	-

Figure 2.2.18 (cont.)

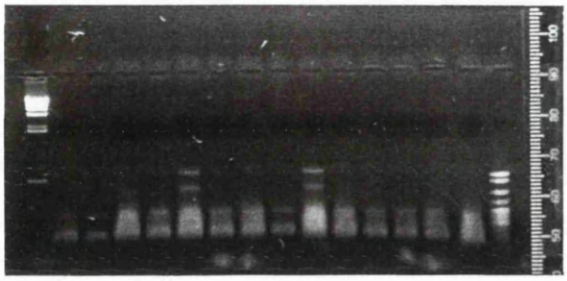
a) *bcl*-2 RT-PCR

14 13 12 11 10 9 8 7 6 5 4 3 2 1 M



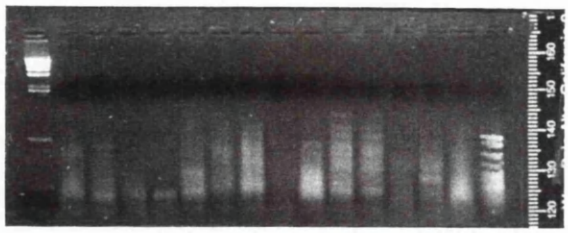
-265bp

b) CD40 RT-PCR



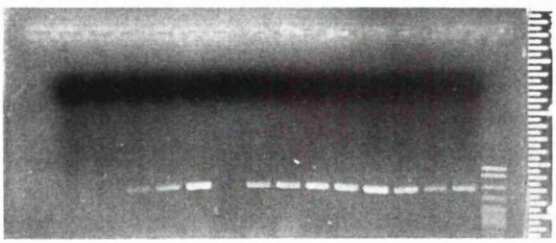
-642bp

c) FasL RT-PCR



-453bp

d) Fas RT-PCR



-389bp

Figure 2.2.18 (cont.)

B) RT-PCR products were then dot-blotted onto nitrocellulose filters and hybridised with Digoxygenin-labeled riboprobes to test for specificity of hybridisation (2.2.18) using: a) *bcl-2* sense, b) *bcl-2* antisense, c) CD40 sense, d) CD40 antisense, e) FasL sense, f) FasL antisense, g) *fas* antisense and h) *fas* sense riboprobes. All probes (both sense and antisense) were found to bind specifically to positive dot-blots of cDNA encoding the same genes. Sense probes also bound since the dot blot consisted of denatured, double-stranded DNA.

Dot-blots of CD40 (1) and *bcl-2* (2) RT-PCR products (from A) hybridised with:

a) Digoxygenin-labeled *bcl-2* sense riboprobe.

b) Digoxygenin-labeled *bcl-2* antisense riboprobe

c) Digoxygenin-labeled CD40 sense riboprobe

d) Digoxygenin-labeled CD40 antisense riboprobe

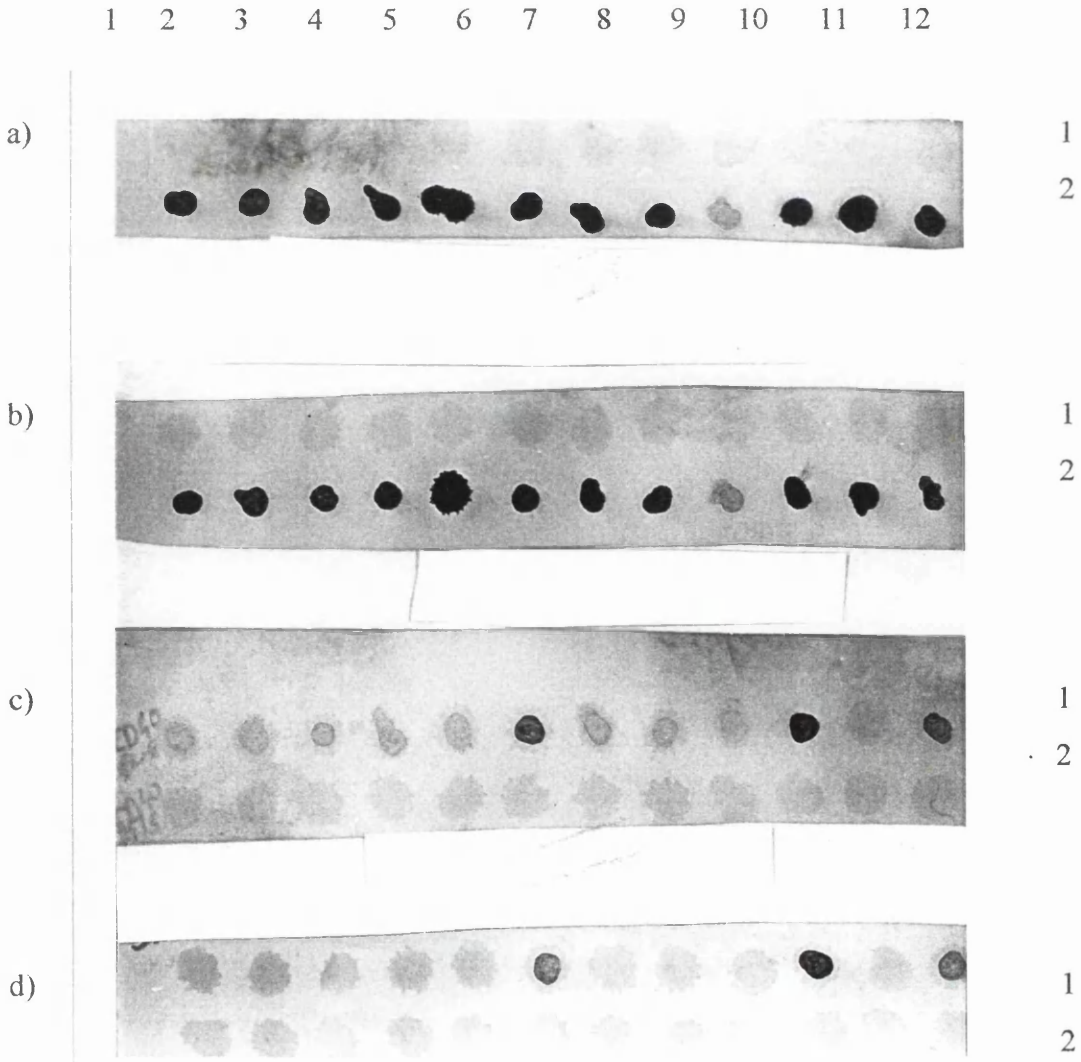


Figure 2.2.18 (cont.)

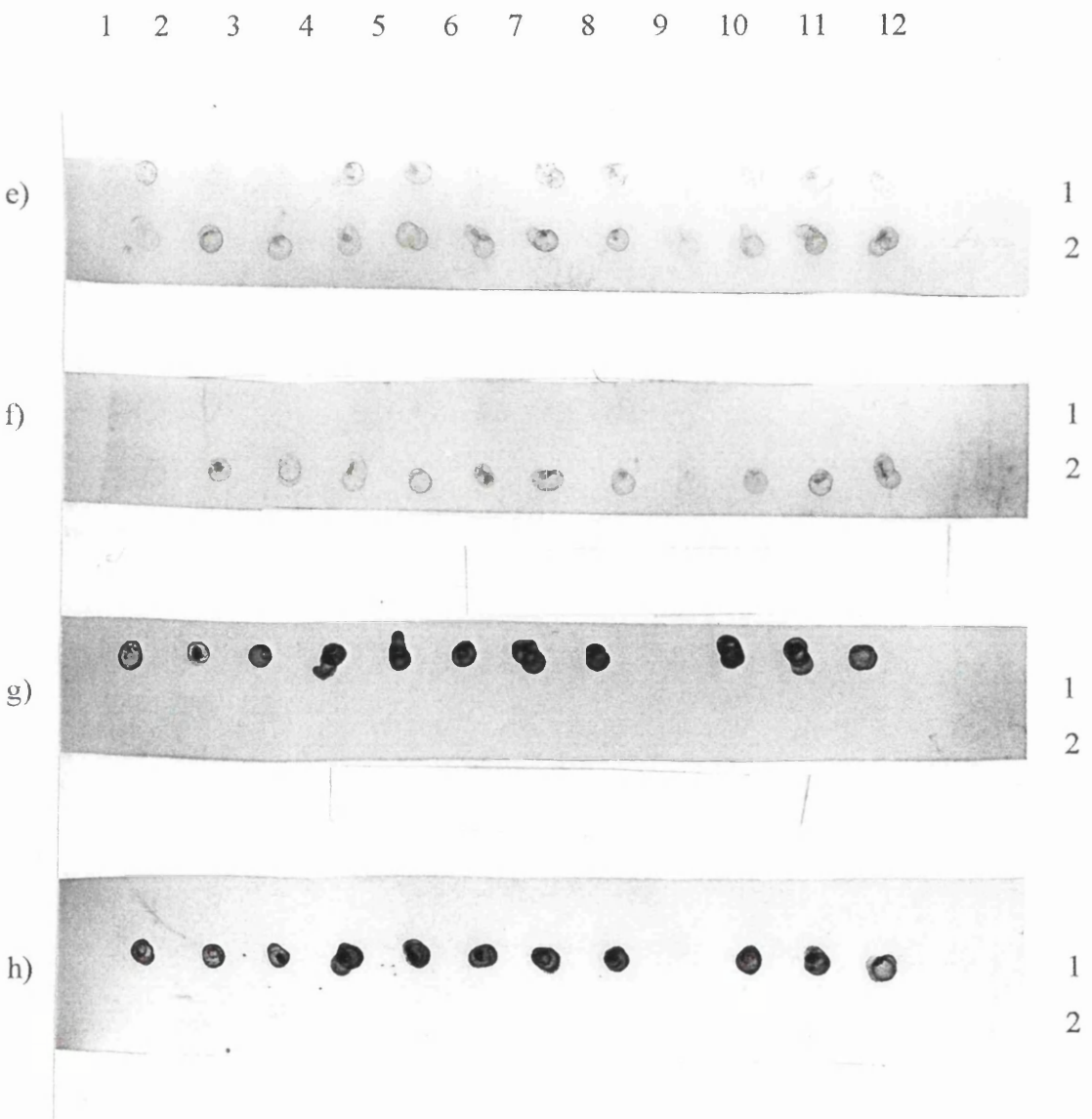
Dot-blots of *fas* (1) and FasL (2) RT-PCR products from (A) hybridised with:

e) Digoxygenin-labeled FasL sense riboprobe

f) Digoxygenin-labeled FasL antisense riboprobe

g) Digoxygenin-labeled *fas* antisense riboprobe

h) Digoxygenin-labeled *fas* sense riboprobe



Preparation of dot-blots

RT-PCR products (5µl of each sample) were alkaline denatured with 5µl of 1M sodium hydroxide. 2µl was spotted onto identical Hybond N^+ filters, pre-wetted with distilled water and then 20xSSC before air drying. Filters were baked at 80°C for 2 hours and then UV irradiated for 2 minutes to cross-link the denatured DNA.

Hybridisation and detection

Filters were pre-hybridised at 50°C with 20ml/100cm^2 of DIG Easy Hyb for 30 minutes with constant rotation. This was discarded and 1ml of each Digoxygenin-labeled riboprobe (@100ng/ml) in DIG Easy Hyb previously heated to 50°C was added. Hybridisation was allowed to continue overnight at 50°C. Filters were washed for 2 x10 minutes with 2x SSC, 0.1% SDS and then with 0.1x SSC, 0.1% SDS for 2x 15 minutes before detection with anti-Dig-AP conjugate and NBT/BCIP as described in 2.2.19.

2.2.19 PREPARATION OF RIBOPROBES

Riboprobes are complementary strands of RNA prepared by an RNA polymerase-catalysed transcription of mRNA in the 3' to 5' direction. Riboprobes were generated from *bcl-2*, CD40, CD40L, *fas*, and FasL cDNAs in pBluescript by *in vitro* transcription (IVT) of linearised plasmid using Digoxygenin-labeled-UTP (Boehringer Mannheim) incorporated into the RNA *in vitro* transcription kit (Stratagene) in place of a radioactively labelled-UTP. The protocol was followed as described by Stratagene using T_3 and T_7 RNA polymerases. Plasmids containing insert were linearised using the appropriate restriction enzymes and were then phenol extracted to purify the product and destroy any RNase activity prior to *in vitro* transcription.

This describes the method of preparation of the *bcl-2* riboprobe. The same procedure was used to generate riboprobes from CD40, CD40L, *fas*, and FasL cDNAs in pBluescript as follows:

1. CD40 in pBluescript was linearised using KpnI (and subsequently *in vitro* transcribed (IVT) to produce a sense riboprobe using the RNA polymerase T3) and with SstI (*in vitro* transcribed with T7 to generate an antisense probe).

2. CD40L in pBluescript linearised with KpnI (IVT using T3 to generate a sense riboprobe) and with PstI (IVT using T7 to generate an antisense probe).

3. *fas* in pBluescript linearised with PstI (IVT using T7 to generate a sense probe) and with KpnI (IVT using T3 to generate an antisense probe).

4. Fas ligand in pBluescript linearised with HindIII (IVT using T3 to generate a sense probe) and with NotI (IVT using T7 to generate an antisense probe).

A *bcl-2* riboprobe (RNA probe) labeled at both ends with digoxygenin was prepared by taking 10μg of pBluescript plasmid containing the human *bcl-2* cDNA insert, linearising it using the restriction enzymes EcoRI and HindIII in two separate restriction digests, and performing *in vitro* transcription of these digests using the RNA polymerases T_7 and T_3 to produce sense and antisense RNA probes respectively.

1. Two restriction digests of 10μg pBluescript *bcl-2* plasmid preparation (@ 0.97mg/ml) were performed using the EcoRI and HindIII restriction enzymes (RE).

2. The linearised plasmid containing the *bcl-2* insert was then phenol extracted (2.2.3) to purify the product and destroy any RNase activity prior to *in vitro* transcription. The final pellet was resuspended in 20μl fresh, ribonuclease free DEPC treated sterile distilled water.

3. *In vitro* transcription of linearised plasmid containing the *bcl-2* insert was performed using digoxygenin-labeled-UTP as follows:

a) Antisense riboprobe synthesis:

5x transcription buffer	50μl
DNA template (phenol extracted, HindIII digest,10ug)	20μl
10mM Dig-UTP 10x labeling mix*	25μl
0.75M DTT	10μl
T_3 RNA polymerase (20U,@10U/μl)	2μl
DEPC.H_2O (up to 250μl)	143μl
Total	250μl

b) Sense riboprobe synthesis:

5x transcription buffer	50µl
DNA template (EcoRI digest, 10µg)	20µl
10mM Dig-UTP,10X conc.	25µl
0.75M DTT	10µl
T_7 RNA polymerase (20U,@10U/µl)	2µl
DEPC.H_2O (up to 250µl)	143µl
Total	250µl

* The labeling mix also contained unlabeled ATP, GTP and CTP

Samples were incubated at 37°C for 30 minutes, then 10U additional RNA polymerase was added and samples incubated for a further 30 minutes at 37°C (to ensure that all of the substrate was used).

Purification of riboprobes:

Riboprobes were fractionated on a G25 sephadex column and eluted with $T_{10}E_1$ buffer containing 0.1% SDS (destroying any ribonuclease activity in the column). Eluates (12x150µl) were dot blotted and labeled with anti-Digoxygenin antibody to assess which eluates contained labeled probe. Eluates were also run in a 1% agarose gel to detect which of them contained RNA and to check that these were the same samples which were positive by dot blotting. Samples were stored at -20°C until ready to perform dot-blots as follows:

Probe detection on nitrocellulose:

A nitrocellulose membrane was rinsed in distilled water and then washed in 20x SSC buffer in DEPC.H_2O for 10 minutes before blotting and air drying. 1µl of each eluate fraction was spotted onto the membrane which was then air dried and baked at 80°C for 2 hours. The membrane was rehydrated in detection buffer 1 (2.1.11) for 1 minute and then put in a heat seal bag with 20ml detection buffer 2 and incubated for 30 minutes at 37°C. A 1:1000 dilution (20ml) of anti-Dig/AP/conjugate was added after removal of buffer 2 and the membrane labeled for 1 hour, with shaking, at room temperature. The membrane was washed x2 for 20 minutes in detection buffer 1 and then in detection buffer 3 for several

minutes before detection using NBT/BCIP (2.1.11). Positive dots were observed after about 30 minutes after which the reaction was stopped by wetting the filter in dH_2O and storing it in the dark after blotting dry. Those fractions with intense reaction (2-3 dots) were pooled and freeze dried. Samples could be stored indefinately at -20°C in this state but were reconstituted in 50-200µl DEPC.H_2O according to the intensity of the dot blots as required.

Figure 2.2.19 shows the dot-blots performed following *in vitro* transcription resulting in the generation of Fas ligand sense and antisense riboprobes as an example. Eluates 4,5 and 6 contained Digoxygenin-labeled RNA by dot-blot analysis and this was confirmed by gel electrophoresis. Similar results were obtained following IVT of CD40, CD40L, *fas, bcl-2* and IL-6R sense and antisense riboprobes (results not shown).

2.2.20 PREPARATION OF INTERLEUKIN-6 RECEPTOR RIBOPROBE

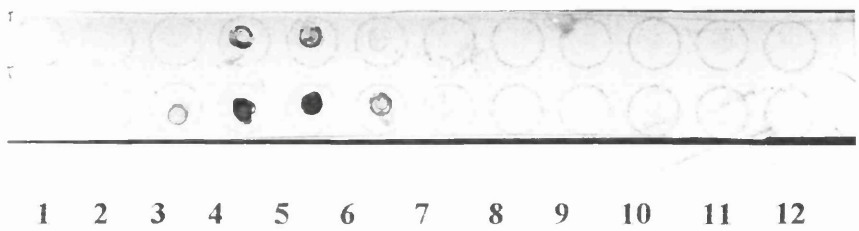
The U266 myeloma cell line (Ref. 249) is known to express a large amount of interleukin-6 receptor (IL-6R) on its cell surface (10^4 sites/cell), (Ref. 267).

RNA was prepared from the U266 cell line, and reverse transcribed using IL-6R-specific primers to provide enough cDNA to insert into a cloning vector. The IL-6R DNA was then cloned into the pCR™ vector using the TA cloning system (Invitrogen Corp.), sequenced and used to generate a Digoxygenin labeled riboprobe as previously described (2.2.18). The manufacturers protocol was used to perform the cloning procedure. Ligation of the IL-6R PCR product with the PCR vector was set up as a 1:1 molar ratio of vector:PCR insert. Following ligation the pCRII plasmid containing insert was transformed into competent INVαF' cells. The pUC19 plasmid vector was used as a positive control (258). The SOC transformation recovery medium consisted of 2% Bacto tryptone, 0.5% Bacto yeast extract, 10mM NaCl, 2.5mM Kcl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose. Positive colonies were selected from L-Broth/agar plates containing appropriate antibiotics and Xgal. Following sequencing the IL6R cDNA in pCRII was linearised and *in vitro* transcribed using NotI (+SP6 RNA polymerase to generate a sense probe) and HindIII (+T7 to generate an antisense probe).

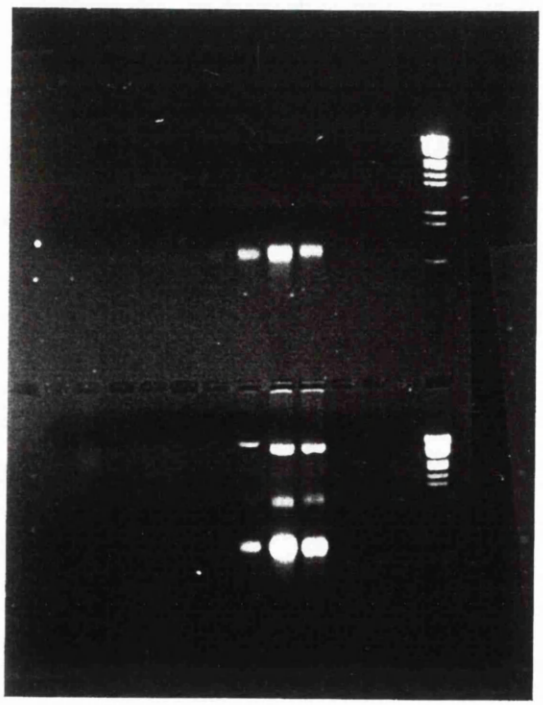
Figure 2.2.19

Riboprobes were fractionated on a G25 sephadex column and eluted with $T_{10}E_1$ buffer containing 0.1% SDS. Eluates (12x150µl) were dot blotted and labelled with anti-Digoxygenin antibody to assess which eluates contained labelled probe (a). Eluates were also run in a 1% agarose gel to detect which of them contained RNA (b).

a) Dot-blot of 12 column eluates as in b)



b) G25 column eluates of FasL IVT products of 1) FasL sense and 2) FasL antisense digoxygenin-labeled riboprobes run on a 1% agarose gel.



See page 81 for marker sizes

RT-PCR

IL-6R cDNA was prepared from U266 RNA by RT-PCR as described in section 2.2.11 using IL-6R specific primers (Clonetech). $\beta_2$microglobulin primers were used as a positive control and a sample containing no RNA polymerase was used as a negative control. Amplification of the cDNA in a reaction volume of 50µl was as follows:

45 seconds	94°C	
45 seconds	60°C	
2 minutes	72°C	
Hold, 7 minutes	72°C	
Hold	4°C	Cycle number = 35

NB: It would normally be necessary to run a control RT-PCR reaction containing no RT to ensure there is no DNA contamination of the RNA preparation but, since these IL-6 primers span the intron/exon boundaries, any amplified DNA would be larger than the amplified RNA so it was not necessary to run this control.

Correct product size was verified by running 5µl of the product in a 2% agarose gel.The positive control IL-6R DNA and the U266 cDNA product results in a band of 250 base pairs (See figure 2.2.11a). The size of the $\beta_2$microglobulin product was 300bp.

The remaining 45µl (stored at 4°C) of amplified cDNA could then be used as an IL-6R cDNA probe or subsequently cloned into a suitable vector (pCRII, see below) and further amplified in order to generate a riboprobe.

CLONING OF IL-6R cDNA using the TA Cloning system

(Invitrogen version 1.3).

The TA cloning system provides a quick, one-step strategy for direct insertion of a polymerase chain product into a plasmid vector. The procedure eliminates any enzymatic modifications of the PCR product such as Klenow or T_4 Polymerase treatment to create blunt ends and it does not require the use of specifically designed primers containing restriction sites.

This cloning system takes advantage of the non-template dependant activity of thermostable polymerases used in PCR that add single deoxyadenosines to the 3' end of all duplex

molecules. These A-overhangs are used to insert the PCR product into a specifically designed vector providing single 3'T-overhangs at the insertion site. The vector is supplied with the kit as a linear molecule with the 3'dT-overhangs ready for insertion of PCR product.

1. **Cloning of PCR product into pCR™ vector.**

a) One vial of lyophilised TA Cloning vector - pCR™ vector was resuspended in 8.8µl TE buffer resulting in a final concentration of 25ng/µl

b) The transformation efficiency of the TA cloning "One Shot" cells is at least 5 x 10^7 transformants per microgram supercoiled plasmid (positive control). The cells were tested by transformation of the supercoiled (SC) plasmid (pUC18 @ 10ng/µl). The lyophilised SC plasmid was reconstituted in 100ul sterile water to prepare a 1ng/µl stock (Stored at -20°C). This was diluted 1:100 in TE buffer to a final concentration of 10pg/ul and 1ul was used instead of ligated PCR product by adding directly to competent E Coli and spread onto L-Broth plates containing Ampicillin.

.

c) Ligation of PCR product (IL-6R-cDNA, 10ng/µl) with the pCR™ vector was set up as a 1:3 molar ratio of vector:PCR insert. 50ng (2µl) of the resuspended vector was used per ligation.

Size of pCR™ plasmid = 3.9kb MW of 1 base pair = 660
MW " " " = 3900 x 660
= $\underline{2.574 \times 10^6}$

Size of IL-6R cDNA = 250bp
MW " " " = 250 x 660
= $\underline{1.65 \times 10^5}$

ie vector is 15.6x larger than the PCR product.

A 1:1 molar ratio of vector:product
= 50ng:3.2ng = 2µl:0.3µl
A 1:3 molar ratio of vector:product
= 50ng:10ng
= 2µl:1µl

A TA cloning ligation reaction with varying molar ratios of vector:product was set up as follows:

Ligation reaction:

	1	2	3	4[1]
Sterile dH_2O	6	4.5	2	7µl
10x ligation buffer	1	1	1	1µl
pCR™ vector (25ng/ul)	2	2	2	2µl
PCR product (~10ng/ul)	1	2.5	5	-µl
T_4 DNA Ligase	1	1	1	1µl
Total	11	11	11	11µl

[1] Reaction 4 is self ligation control, plated onto L-Broth+Amp+X-Gal

Reaction mixes were incubated at 12°C for 4 hours or overnight.

2. **Transformation with competent E.Coli:**

The pCR™ plasmid is ampicillin resistant (amp_R). Competent E.Coli containing plasmid + insert were selected for by spreading the amp.Lbroth/agar plates with XGal (as described in section 2.2.16). The transformation reaction was performed according to the manufacturers protocol and was essentially as described in section 2.2.13.

Briefly, L-Broth/agar plates containing 100µg/ml ampicillin and overlayed with 25µl X-Gal (40mg/ml) were prepared (1 plate for each TA cloning ligation reaction). The tubes containing the ligation reactions were spun briefly and placed on ice. 2µl of the 0.5M β-ME was added to a 50µl vial of competent INVαF' E coli cells, one for each reaction, and mixed by tapping gently. 1µl of each TA cloning ligation reaction (and 1µl of diluted, supercoiled plasmid as a test of transformation efficiency) was pipetted directly into the competent cells and mixed by tapping gently. The remaining ligation mixture was stored at -20°C. The vials were incubated on ice for 30 minutes followed by 60 seconds at 42°C. Vials were removed from the water bath and replaced on ice for 2 minutes. 450µl pre-warmed SOC medium was added to each vial which were then shaken at 225rpm at 37°C for exactly one hour before replacing on ice. Aliquots of 25µl and 100µl from each transformation vial were spread on separate, labeled L-Broth plates containing amplicillin and X-Gal. After overnight incubation white colonies were picked for plasmid isolation and restriction analysis, PCR or sequencing.

Colonies formed after overnight incubation at 37°C:

Plate	1		2		3		4		5		6	
Spread	**25µl**	**100µl**	**25µl**	**100µl**	**25µl**	**100µl**	**25µl**	**100µl**	**25µl**	**100µl**	**25µl**	**100µl**
Cols.												
Blue	**3**	**25**	**39**	**14**	**6**	**8**	**1**	**49**	**lawn of growth**		**103**	**>300**
White	**1**	**20**	**47**	**27**	**0**	**9**	**0**	**0**	-	-	-	-
Total	4	45	86	41	6	17	1	49			103	>300

REACTIONS:

1-3. Competent E.Coli transformed with ligations 1-3

4. Self ligation control (no PCR product added to ligation reaction). The percentage of white colonies on this plate should be less than 5% of the total number of colonies. Any blue colonies result from a T:T mismatch self-ligation of the vector.

5. SC plasmid control transformation plated onto L-Broth without Ampicillin.

6. SC plasmid control transformation plated onto L-Broth with ampicillin.

Calculation of Transformation efficiency:

Volume in transformation: Competent E Coli (INV alpha F')	50µl
β-ME	2µl
Vector + or - insert	1µl
SOC buffer	450µl
Total	503µl

1. Plate 6, SC plasmid (pUC18)

10pg SC plasmid added in 503µl to plate 6.
25µl added to plate, 103 colonies on plate.

503µl = 10pg SC plasmid
1000µl = 10 x (1000/503) = 19.88pg/ml
ie, 25µl = 0.025ml contained 19.88 x 0.025 pg SC plasmid
= 0.497pg SC plasmid/plate
ie, 0.497pg SC plasmid resulted in 103 colonies, = 207.24 colonies/pg
= 2×10^8 cols/µg SC plasmid

(Expect at least 5×10^7 transformants per microgram supercoiled plasmid.)

2. Plate 1 - plasmid (pCR™) + ~10ng insert (IL-6R)

25µl plate:
503µl = ~10ng insert DNA (1ul of IL-6R PCR product)
ie 19.88ng/ml insert x 0.025 = 0.497ng insert DNA/plate
0.497ng resulted in 4 colonies,
ie 8 x 10^3 colonies per microgram insert DNA

100µl plate:
503µl = ~10ng insert DNA
ie 19.88ng/ml insert x 0.100 = 1.988ng/plate
1.988ng resulted in 45 colonies,
ie 2.26 x 10^4 colonies per microgram insert DNA

3. Plate 2 - plasmid + ~25ng insert (2.5µl)

25µl plate: Total of 86 colonies
ie 1.24ng insert DNA per plate produced 86 colonies.
ie 6.9 x 10^4 colonies per microgram insert DNA

100µl plate: 4.97ng insert DNA produced 41 colonies.
ie 8.2 x 10^3 colonies per microgram DNA

4. Plate 3: - plasmid + ~50ng insert DNA (5µl)

25µl plate: Total 6 colonies, 2.4ng DNA produced 6 colonies
ie 2.4 x 10^3 colonies per microgram insert DNA

100µl plate: Total 17 colonies, produced by 9.94ng insert DNA
ie 1.7 x 10^3 colonies per microgram insert DNA

Expansion of white colonies - positive for insert cDNA

Twelve individual white colonies and two control blue colonies from plates 1 and 2 were expanded in 2.5ml of L-Broth containing ampicillin overnight. Plasmid minipreps were performed and the products verified by restriction analysis. Samples with 250 base pair insert and 3.9 kilobase plasmid bands on agarose gel were selected to perform a maxi DNA plasmid prep. If the bands were faint the DNA was phenol extracted and the digest repeated.

The samples selected with positive bands on agarose gel electrophoresis after phenol extraction and repeat EcoRI digest were 1,2,3,5,9,10,11,12 (Lanes 13 and 14 only

generated bands of 3.9kb corresponding to plasmid without insert ie blue colonies on L-Broth plates). The remaining 40µl of the chosen miniprep sample (3) was phenol extracted prior to sequencing. Glycerol stocks of selected samples were stored at -80°C (200µl glycerol added to a 1ml sample). Sample number 3 from plasmid minipreps was sequenced and was proven to have the correct sequence for the 250 base pair segment of the IL-6R gene which had been amplified by PCR.

Sequencing was performed as described (2.2.21) using one reaction with the M13 -40mer and one reaction using the M13 +40mer in order to sequence the IL-6R cDNA from both ends. Following sequencing the IL6R cDNA in pCRII was linearised using NotI (+SP6 RNA polymerase to generate a sense probe) and HindII (+T7 to generate an antisense probe).

2.2.21 DNA SEQUENCING (DOUBLE STRANDED TEMPLATE)

(Ref. 268)

Sequencing apparatus	- Hoeffer "Poker Face II" nucleic acid sequencer.
Sequencing kit	- United States Biochemical "Sequenase" kit, version 2.0

The Chain-Termination sequencing method was used. This involves the synthesis of a DNA strand by a DNA polymerase *in vitro* using a single-stranded DNA template. Synthesis is initiated at only one site where an oligonucleotide primer anneals to the template. The synthesis reaction was terminated by the incorporation of a nucleotide analogue which will not support continued DNA elongation. The chain-terminating nucleotide analogues are the 2',3' -dideoxynucleoside 5' triphosphates (ddNTP's). These lack the 3'-OH group necessary for DNA chain elongation. When proper mixtures of dNTPs and one of the four ddNTPs are used, enzyme-catalysed polymerisation is terminated in a fraction of the population of chains at each site where the ddNTP can be incorporated. Four separate reactions, each with a different ddNTP provide complete sequence information. A radioactively labeled nucleotide was also included in the synthesis, so that labeled chains of various length could be visualised by autoradiography after separation by high resolution electrophoresis.

The pCR™ plasmid used to clone the IL-6R cDNA contains an M13 replication origin so that the IL-6R insert could be amplified using the M13 primer once it had been rendered single-stranded. The DNA synthesis was carried out in two steps. The first was the labeling step (using ^{35}S-dATP) performed after annealing of the primer. The second being the chain termination step using dideoxynucleotides.

In the first step, the primer was extended using limiting concentrations of the deoxynucleoside triphosphates, including radioactively labeled dATP. This step continues to complete incorporation of labeled nucleotide into DNA chains which are distributed randomly in length from several nucleotides to hundreds of nucleotides. In the second step, the concentration of all the deoxynucleoside triphosphates is increased and a dideoxynucleoside triphosphate is added. Processive DNA synthesis occurs until all growing chains are terminated by a dideoxynucleotide. During this step, the chains are extended, on average, only several dozen nucleotides. The reactions were terminated by the addition of EDTA and formamide. They were then heat denatured and run on a polyacrylamide electrophoresis gel (PAGE).

Samples (double-stranded plasmid preparation, containing IL-6R insert) were alkaline-denatured, ethanol precipitated and redissolved in distilled water with "sequenase" reaction buffer and M13 sequencing primer prior to commencing the labeling reaction.

Preparation of 8% Polyacrylamide sequencing gel (19:1):

A 19:1, acrylamide:bis-acrylamide gel mix (Boehringer Mannheim) was used

1. Calculation of volumes for 500ml of an 8% gel mix:

$Va = A.Vt/40$

where:
Va = Volume of PAGE 1™ Sequencing Gel Mix
Vt = Total volume of gel-casting solution (ml)
A = Percentage acrylamide desired

Therefore, for 500ml of an 8% polyacrylamide gel:

$Va = (8x500)ml/40 = 100ml$

2. Gel mix (Ref. 269):

100ml sequencing gel mix
50ml 10x TBE buffer (10%)
180g urea (6M)
This was made up to 500ml in distilled water and filter steriled using a vacuum pump.

1ml fresh 10% ammonium persulphate and 40µl TEMED were added for each 100ml of gel mix. which was poured immediately, ensuring no air bubbles or leakage. A "Shark Tooth" comb (62 wells) was inserted, flat side in towards gel and the gel left flat to set.

Sequencing reaction:

1) Alkaline denaturation of double-stranded template:

An equal volume 0.4M NaOH, 0.4mM EDTA was added to a 100 µl sample of pCRII-IL-6R (1µg) to give a final volume of O.2M NaOH, 0.2mM EDTA and this was incubated at 37°C for 30 minutes. The mixture was neutralised by adding 0.1 volumes (10µl) of 3M sodium acetate (pH4.5-5.5) and the DNA precipitated with 2-4 volumes (300µl) ethanol at 70°C for 15 minutes. After spinning in a microfuge for 5 minutes samples were washed with 70% ethanol and air dried before resuspending in 7µl distilled water.

2) Annealing template and primer:

a) For each set of 4 sequencing lanes (A,C,T,G), a single annealing (and subsequent labeling) reaction was used:

Primer (M13 -40mer or +40mer	1µl
"Sequenase" reaction buffer	2µl
ssDNA (approx. 1-5ug*)	7µl
Total	10µl

* 1. For single stranded control M13 (M13mp18 @ 0.2µg/µl) DNA 1µg was used, ie 5µl control DNA + 2µl water.

2. For plasmid DNA, 1-5µg was used, ie 7µl above.

b) Reaction tubes were warmed for 2 minutes at 65°C, then allowed to cool slowly to room temperature over a period of about 30 minutes. Once the sample temperature reached 30°C samples were put on ice since annealing was complete. Samples were used within 4 hours.

3. Labeling Reaction:

To the annealed template-primer (above) the following was added on ice:

Template-primer (above)	10.0µl
DTT (0.1M)	1.0µl
Diluted labeling mix (dNTP's)	2.0µl
[alpha-^{35}S]dATP (10µCi/µl)	0.5µl
Diluted sequenase	2.0µl
Total	15.5µl

This was mixed thoroughly, avoiding air bubbles and incubated for 2-5 minutes at room temperature or cooler (incubation for longer or warmer leads to sequence artifacts within 100 bases of the primer).

4. Termination Reactions:

1. Four PCR tubes were labeled for each sample. 2.5µl ddGTP termination mix was added to a tube marked "G" and repeated for A,T and C. The tubes were capped and put in PCR machine at 37°C for at least one minute.

2. When the labeling reaction was completed, 3.5µl was added to each of the tubes labeled "G", "A", "C" and "T" and this was repeated for each sample. The tubes were mixed, centrifuged and the incubation continued at 37°C for a total of 3-5 minutes (to a maximum of 30 minutes).

3. 4µl of "Stop Solution" was added to each of the termination reactions, tubes were mixed thoroughly and stored on ice until ready to load the sequencing gel.

4. Immediately prior to loading the gel the samples were heated to 75-80°C for 2 minutes and then 2-3µl was loaded into each lane.

5. PAGE (Polyacrylamide gel electrophoresis).

1. An 8% (19:1) gel was prepared and assembled in electrophoresis apparatus. 5µl tracking dye was added to two wells and the gel was pre-run at 1500-2000 volts for about 30 minutes until it heated up to at least 55°C. This was to ensure that the samples remained denatured when running the gel.

2. Samples were then added (stop solution contained marking dye) and the gel was run for 1.5 hours at about 1800 volts. The electrophoresis was stopped at this point and a further 3µl of each sample (G,A,T,C) was added. Electrophoresis was continued for a further 90 minutes. This means that a longer sequence could be read since the initial run allows resolution of sequences further up the DNA strand, whilst the second run of sample resolves the early sequence clearly.

3. The sequencing gel was removed and soaked in fixative for 15 minutes at room temperature without agitation (5% acetic acid, 15% methanol). The gel was dried onto 3MM paper at 80°C for 1 hour, put in X-Ray cassette with fast film overnight at room temperature and the autoradiograph developed the next day in order to read the sequence. A comparison of the 250bp sequence obtained between base pairs 1143-1393 (complete open reading frame 1401bp) was found to be identical to the published sequence for IL-6R (264).

2.2.22 FLUORESCENT ANTIBODY CELL SORTING BY FLOW CYTOMETRY.

Cells from fresh blood or marrow samples or cultured cells were incubated with fluorescent monoclonal anti-human antibodies which were then analysed by flow cytometry. Fluorescent labels used to conjugate monoclonal antibodies in this study were: Fluorescein Isothyocyanate (FITC) and Phycoerythrin (PE).

Direct Immunofluorescent staining:

Cells ($5x10^5$-10^6) were washed twice in 3-4ml Phosphate buffered saline (PBS) containing 0.1 % azide, resuspended in 200µl PBS and incubated with 10-20µl of fluorochrome-conjugated mouse-anti-human monoclonal antibody for 20 minutes in the dark at room temperature. For dual labeling, antibodies conjugated to different fluorochromes were mixed in the same reaction tube. Negative controls consisted of either FITC- or PE-labelled mouse IgG_1 or IgG_2. Labeled cells were washed x2 in PBS and resuspended in 400µl of PBS prior to FACS analysis.

Indirect Immunofluorescent staining:

For unconjugated monoclonal antibodies a sandwich labeling technique was adopted. Cells were incubated first with the unconjugated mouse anti-human antibody for 20 minutes with unconjugated mouse IgG as negative control. After washing, these cells were incubated for a further 20 minutes with a fluorochrome-labeled anti-mouse IgG before washing and analysing as above.

IL-6R expression on the surface of myeloma cells was detected by this sandwich labeling technique. Cells were initially labeled with mouse anti-human IL-6R MoAb and then, after washing, the cells were labeled with an FITC-conjugated monoclonal goat anti-mouse immunoglobulin. This procedure was necessary since a fluorescein-conjugated anti-IL-6R antibody was not commercially available. Results are shown in Figure 2.2.22a and are sumarised in Table 2.2.22.

Detection of cytoplasmic and nuclear antigens:

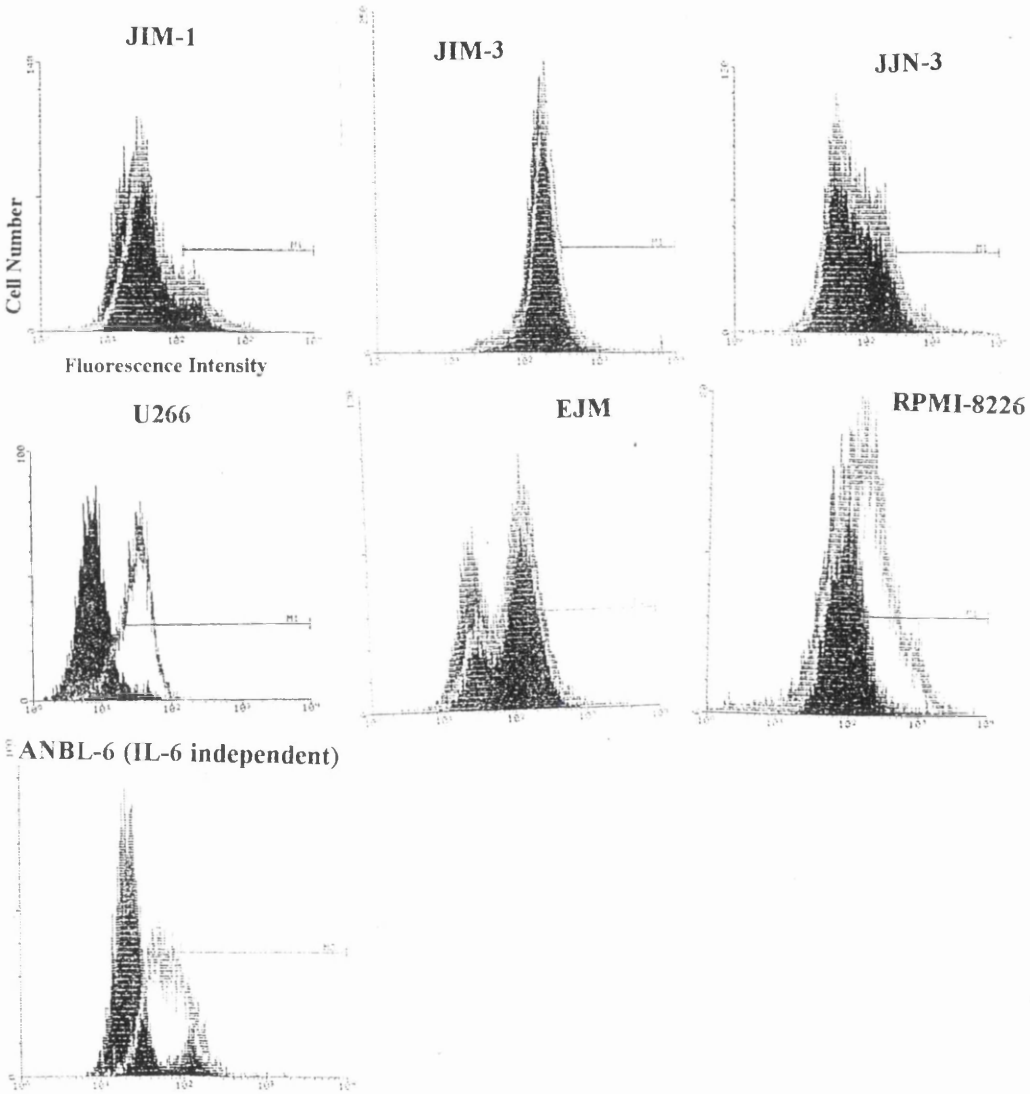
Cells were permeabilised prior to labeling by incubating them in 2ml of Ortho Permeafix (Ortho Diagnostic Systems Ltd.) for 40 minutes at room temperature. After centrifugation at 1000rpm for 5 minutes cells were resuspended in PBS, incubated at room temperature for 5 minutes, spun and labeled with an FITC-conjugated antibody for 40 minutes at room temperature, washed again and analysed by flow cytometry.

Cytoplasmic Bcl-2 protein was detected using this method by permeabilisation of myeloma cells and labeling them with an FITC-conjugated, monoclonal anti-human Bcl-2 antibody. Results are shown in Figure 2.2.22b and are sumarised in Table 2.2.22.

Note: PE is too large to enter permeabilised cells therefore PE-labeled antibodies were not employed for this procedure.

Figure 2.2.22a

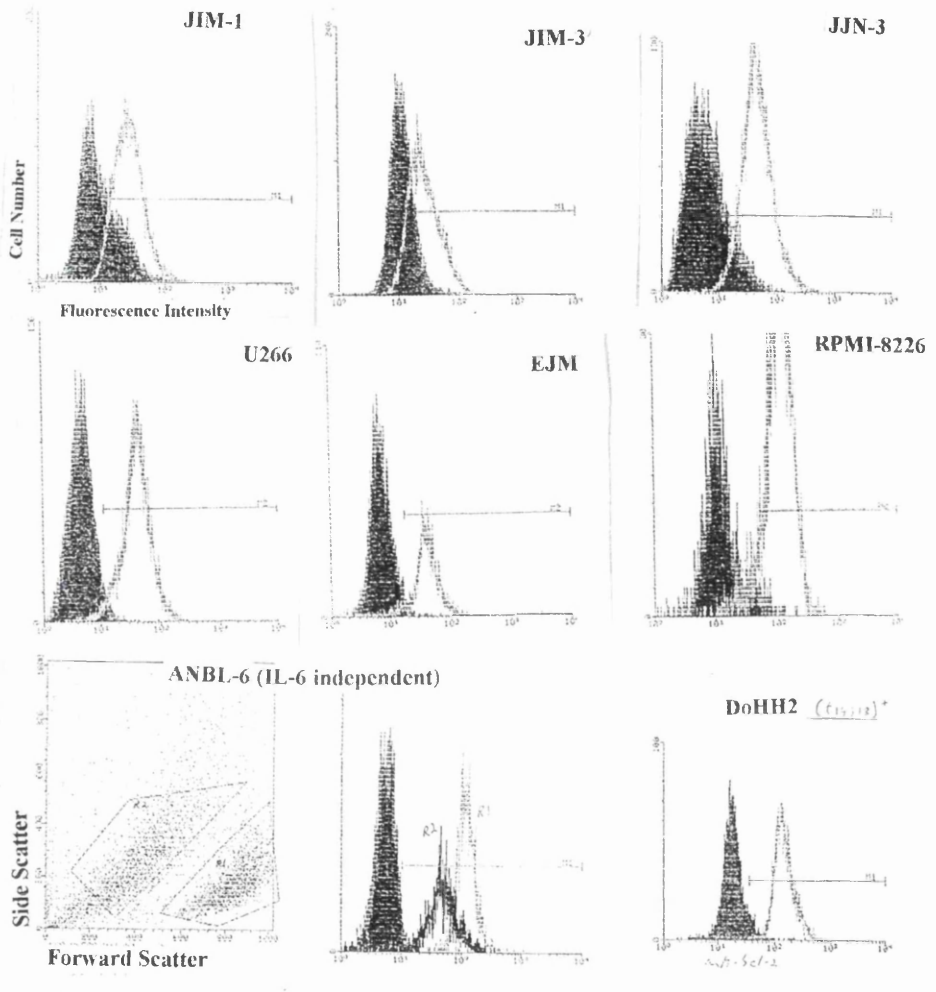
IL-6R expression in Myeloma cell lines by flow cytometric analysis



A panel of myeloma cell lines was assessed for IL-6 receptor expression by labeling 10^6 cells with mouse-anti-human IL-6R MoAb, using mouse IgG as negative control (black background), followed by an FITC-conjugated goat anti-mouse antiserum. 10,000 cells per experiment were analysed. JIM-1, JIM-3, JJN-3 and EJM were negative for IL-6R expression with U266, RPMI-8226 and IL-6 independent passages of ANBL-6 proving positive.

Figure 2.2.22b

Bcl-2 expression in Myeloma cell lines by flow cytometric analysis



A panel of myeloma cell lines was assessed for Bcl-2 expression by labeling 10^6 cells with FITC conjugated mouse-anti-human Bcl-2 MoAb, using mouse IgG-FITC as negative control (black background). 10,000 cells per experiment were analysed. The cell line DoHH2 was used as positive control. All the myeloma lines tested proved to be Bcl-2 positive. Two populations of ANBL-6 cells were assessed (R1 and R2). Both regions were found to be positive for Bcl-2 expression with R2 showing the apoptotic and R1 showing the viable cell populations respectively. The apoptotic cells manifested a downregulated Bcl-2 expression compared to the viable population.

Table 2.2.22

Cell line	Bcl-2 expression	IL-6R expression
JIM-1	+	-
JIM-3	+	-
JJN-3	+	-
U266	+	+
EJM	+	-
RPMI-8226	+	+
ANBL-6 (IL-6 independent)	+	+

2.2.23 MYCOPLASMA TESTING OF CELL LINES:

Cell lines were mycoplasma tested twice a year using the method described by Chen TR, (Ref 269a). All lines were consistently found to test negative for mycoplasma. Cell-free medium from known positive cell lines (local to our laboratory) were used as positive controls. This method of detection was used by three other laboratories known to the author as the most reliable method of mycoplasma detection.

Hoechst 33258 staining for mycoplasma

Principle

Hoechst 33258 is a fluorescent stain for DNA. The method relies on the discrimination of extranuclear DNA staining as indicative of mycoplasma infection.

Outline

Fixed cells were stained in aqueous solution, wet mounted in buffer and examined by immunofluorescence. An indicator cell line such as NRK, Vero or 3T6 is used to incubate the medium from the cells under test. This line should, of course, be mycoplasma free.

Protocol

NRK rat fibroblasts grown in Modified Dulbecco's medium containing 10% FCS were set up in 6cm petri dishes @ 10^4 cells per dish in 4ml fresh medium. One dish being the negative control. Cell free medium(2ml) was added to each dish from cells to be tested which had been in contact with the cells for at least 2 days. Dishes were incubated for 3-4 days at 37°C. After this period medium was removed and cells washed twice with PBS prior to fixation for 10 minutes. Fixative was removed and cells stained for 10 minutes with 5ml Hoechst 33258 in PBS (0.05μg/ml). Cells were rinsed twice in distilled water mounted in McIlvaine's buffer (pH5.5) and examined by fluorescence microscopy. Negative cells showed fluorescent nuclei with no cytoplasmic staining. Small foci of cytoplasmic staining was observed in positive controls.

Reagents consisted of: Hoechst 33258 stain (2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-perpazyl)-benzimidazol-trihydrochloride),stock solution @ 1mg/ml in PBS, stored at -20°C was diluted 1:20,000 (1μl in 20ml PBS) for use; Fixative: 3 parts methanol, 1 part glacial acetic acid; McIlvaine's Buffer, pH5.5:0.2M Na_2HPO_4,0.1M Citric Acid

2.2.24 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (MNC)

4ml of heparinised peripheral blood was layered carefully onto 8ml of lymphocyte separation medium (Flow laboratories) in a 15ml centrifuge tube and was spun at 400xg for 30 minutes (approximately 1800rpm). The top layer containing plasma was discarded and the band at the interface between the serum and the separation medium containing the MNC was collected. The cells were washed in at least 7 volumes of PBS (sterile) by spinning for 10 minutes at 1000rpm, with a further wash in 10ml ice-cold PBS. Cells were resuspended in 5ml ice-cold PBS and counted using an automated counter or haemocytometer.

CHAPTER 3

MYELOMA CELL ADHESION ASSAYS

Table 3

Immunophenotyping of myeloma cell lines by flow cytometric analysis. Summary of at least 3 different experiments expressed as percentage positivity. 10,000 cells counted per experiment.

PHENOTYPE	Bcl-2	Fas	CD40	CD38	B-B4	HLA-DR	CD58	CD56	CD54	CD45	CD44	CD11a	CD49d	CD49e	IL-6R
					Percentage positivity (range)										
CELL LINE															
JIM-1	50 (40-60)	15 (5-23)	15 (4-25)	86 (82-90)	100	0	100	90 (75-97)	35 (10-66)	50 (45-68)	0	6 (4-11)	100	38 (30-40)	90
JIM-3	60 (15-70)	66 (30-83)	13 (10-16)	83 (80-86)	92 (89-95)	22 (7-36)	100	52 (22-68)	0	39 (28-43)	0	0	100	22 (13-40)	0
EJM	50 (50-90	80 (64-95)	12 (6-22)	98	96	0	100	27	100		50 (36-63)	0	87 (85-90)	28 (17-36)	0
JJN-3	75 (50-94)	82 (70-94)	29 (21-34)	75 (60-90)	85 (80-90)	94 (87-100)	100	60 (42-81)	93 (92-94)	89 (78-92)	84 (81-86)	24 (10-37)	98	30 (31-62)	0
U266	(80-100)	91 (88-93)	17 (7-28)	85 (80-90)	98	89 (76-100)	100	50 (28-66)	100	48 (42-53)	53 (42-67)	22 (6-33)	50 (44-56)	18 (13-23)	95
RPMI-8226	100	79 (56-88)	94 (93-94)	65 (60-70)	85	96 (92-100)	95 (92-100)	65 (33-91)	97 (94-100)	16 (12-18)	14 (11-20)	12 (5-25)	50 (33-71)	16 (8-23)	60
IM-9	100	100	100	60 (43-70)	90	98	98	100	100	100	85 (73-92)	99	99	0	0
JT	100 (70-100)	66 (40-100)	51 (10-94)	90	90	20 (5-32)	97	49 (27-96)	18 (7-29)	63 (35-96)	34 (28-44)	20 (10-36)	98	43 (7-84)	
ANBL-6 (IL-6 dependent)	50 (21-45)	40 (21-58)	55 (20-81)	98	89 (79-99)	15 (13-17)	95	17 (5-30)	73 (36-99)	59	90 (71-100)	22 (15-29)	84 (74-94)	52 (25-84)	70
ANBL-6 (IL-6 independent)	50 (39-84)	66 (43-97)	14 (9-43)	99	95 (86-99)	27 (6-96)	96	48 (22-94)	10 (4-19)	80 (53-91)	0	16 (12-22)	99	38 (16-97)	0

MYELOMA CELL ADHESION ASSAYS

3.1 Introduction

Multiple adhesive interactions are undoubtedly occuring as a dynamic process controlling the localisation and adhesion of myeloma plasma cells in the bone marrow These, in turn, appear to regulate cytokine production from either the stroma or the myeloma cells to support tumour expansion. The proteins of the extracellular matrix (ECM), fibronectin, collagen, laminin are components of the bone marrow microenvironment and tumour cells may adhere to these proteins via various adhesion molecules. A summary of the phenotype of all the myeloma cell lines used in this thesis is shown in Table 3 and includes cytoplasmic Bcl-2 expression. Fibronectin is a multifunctional adhesive glycoprotein found as an insoluble form in extracellular matrices and basement membranes and as a soluble form in plasma. Expression of surface fibronectin and fibronectin mRNA (detected by Northern blot analysis) has also been shown on myeloma cell lines (66) which suggestes that homotypic FN-FN adhesion may also occur in the bone marrow stroma. Collagens constitute a highly specialised family of glycoproteins which are integral components of the extracellular matrix.

A striking feature of myeloma plasma cells is their tendency to remain in the bone marrow until the end stages of the disease with the onset of plasma cell leukaemia which results in circulating plasma cells. This indicates that the bone marrow stroma is of prime importance in the pathogenisis of the disease.

Beta1/alpha4 integrins are strongly expressed on myeloma-derived cell lines, indicating that VLA-4 is the principal integrin on these cell lines (65,66 and Table 3). These myeloma cell lines adhere to fibronectin through VLA-4 as well as through arginine-glycine-aspartic acid (RGD)-dependent mechanisms. Both VLA-4 and VLA-5 bind to fibronectin. VLA-5 binds to an RGD peptide on the fibronectin molecule (66,67) although this integrin is not universally expressed on myeloma cell lines and the intensity of expression varies with

repeated passage (66 and personal findings). Mature B and T cells do not express much fibronectin on their surface, so it is possible that adundant fibronectin expression on myeloma cells is a specific and important phenomenon in the pathogenesis of the disease.

The ability of myeloma cells to bind to fibronectin through VLA-4 and RGD-dependent mechanisms may contribute to their localisation in the bone marrow and, conversely, the loss of fibronectin receptors may lead to extravasation into the circulation. Recent migration studies of leukaemic cells suggest that VLA-4 may regulate adhesion whilst VLA-5 controls the motility of these cells (74).

It was decided therefore to investigate the adhesion of a panel of myeloma cell lines to various extracellular matrix molecules. It was hoped to determine which myeloma cell surface adhesion molecules were important in binding to individual stroma and whether or not this binding could be blocked by incubation with monoclonal antibodies against these molecules.

Pilot experiments using myeloma cell lines incubated in flasks were initially carried out (3.1.2). The adherent cells were removed by washing and then by vortexing and their phenotype before and after incubation on fibronectin, collagen or fibronectin-like engineered polymer (FN-RGD) was examined. Adhesion assays were subsequently set up with myeloma cell lines adhering to microtitre plates coated with the same ECM proteins (3.1.4) with or without previous incubation with monclonal antibodies against the cell surface receptor molecules (VLA-4, VLA-5) in an attempt at adhesion blockade.

3.1.1 Preparation of stromal layers in flasks

Flasks (F25 Falcon flasks) were coated with either fibronectin, collagen or the synthetic RGD peptide (FN-RGD).

Preparation of stromal layers

i) Collagen: (Sigma C-8919, type I, obtained from calf skin)
Stock solution at 0.1% was diluted 1:10 in sterile dd$H_2$0. Two millilitres were added per F25 flask and allowed to air dry overnight. The following day excess fluid was removed and the flask rinsed with sterile PBS before use.

ii) Fibronectin: (Sigma F2006 from human plasma)
A stock solution at 1mg/ml in was made up in sterile ddH_2O, diluted 1:50 in sterile PBS and 1ml added per F25 flask. Flasks were air dried for at least 45 minutes at room temperature before use.

iii) FN-RGD: (Sigma F5022, synthetic polymer)
A stock solution at 1mg/ml (in Sigma diluent) was diluted 1:10 in sterile PBS and 2.5ml added per flask. The polymer was allowed to adhere for 1.5-2 hours at room temperature before removing excess fluid and immediately rinsing x2 with PBS.

Immediately prior to performing an adhesion assay flasks were incubated for 1 hour at 37^{0}C with 2ml 1% BSA in PBS (which had been heat denatured @ 56^{0}C for 30 minutes) to prevent non-specific binding. This solution was removed before addition of the cells.

3.1.2 Adhesion of myeloma cell lines to flasks coated with extracellular matrix proteins

i) The antigenic profile of cell lines was assessed prior to adhesion by immunophenotyping as described in materials and methods (2.2.22).

ii) Myeloma cells (2x10^6) in 5ml medium were added per flask and the cells were incubated for 30 minutes at 37°C (fibronectin) or for 24 hours (collagen and FN-RGD).

iii) After incubation the contents of the flasks were removed by gentle pipetting and spun down (rinsed cells). A further 2ml of PBS was added to each flask before passing it over a vortex for 30 seconds. The contents were removed and the cells spun down (vortexed cells). These cells (rinsed and vortexed) were then immunophenotyped by flow cytometry.

Results **Table 3.1.2**

Results are expressed as percentage positivity of each antigen tested by flow cytometric analysis before (pre) and after (post) incubation in flasks coated with fibronectin, collagen or FN-RGD. Cells removed by rinsing flasks were compared with those harvested after vortexing flasks.

SURFACE MARKER / CELL LINE	CD38 PRE	CD38 POST R	CD38 POST V	B-B4 PRE	B-B4 POST R	B-B4 POST V	CD56 PRE	CD56 POST R	CD56 POST V	VLA-5 PRE	VLA-5 POST R	VLA-5 POST V
JIM-1												
FIBRONECTIN	95	98	98	100	96	96	75	84	72	30	40	81
COLLAGEN		98	NC		99	NC		95	NC		45	NC
FN-RGD		99	NC		99	NC		-	NC		-	NC
JIM-3												
FIBRONECTIN	100	95	100	100	97	97	60	59	79	10	21	60
COLLAGEN		98	NC		93	NC		42	NC		31	NC
FN-RGD		100	NC		94	NC		-	NC		-	NC
U266												
FIBRONECTIN	97	97	95	98	98	99	66	40	41	13	16	18
COLLAGEN		97	NC		98	NC		-	NC		-	NC
FN-RGD		98	NC		98	NC		-	NC		-	NC
JJN-3												
FIBRONECTIN	96	98	97	99	99	97	42	32	48	31	17	50
COLLAGEN		98	NC		99	NC		-	NC		-	NC
FN-RGD		98	NC		97	NC		-	NC		-	NC

ABBREVIATIONS:

R = rinsed, V = vortexed, NC = no cells, - = not tested

Conclusion

In the fibronectin coated flasks the intensity of VLA-5 expression of JIM-1, JIM-3 and to a lesser extent JJN-3 increased significantly in those cells removed after vortexing the flask compared to those pre-adhesion and those removed by rinsing the flask. This effect was not observed with U266. CD56, B-B4 and CD38 antigen expression remained unchanged following incubation with these extracellular matrix molecules. There was almost a 100% recovery of the myeloma cells added after rinsing the flasks coated with collagen and FN-RGD following the incubation period. This indicates that VLA-5 on the surface of the myeloma cell lines JIM-1, JIM-3 and JJN-3 adheres to fibronectin but not to collagen or FN-RGD and CD56, B-B4 and CD38 do not contribute to the adhesion of myeloma cells to fibronectin..

3.1.3 Preparation of stromal layers in microtitre plates (Ref. 270)

In order to attempt to standardise adhesion assays, the method describes by Van Riet et al. (270) was adopted. Assays were performed by initially coating the surface of microtitre plate wells with commercially available extracellular matrix molecules fibronectin, collagen and FN-RGD (3.1.1). Myeloma cell lines were then allowed to adhere with or without previous incubation with monclonal antibodies against VLA-4 and VLA-5 to ascertain if these surface antigens were potentially responsible for myeloma cell adhesion to the extracellular matrix.

Preparation of stromal layers

Flat-bottomed 96 well microtitre plates (Nunc Immunosorb) were coated overnight with 100μl of a solution of fibronectin (used @ 10-50μg/ml in PBS), collagen (used at a final concentration of 0.1μg/ml or FN-RGD (used at 10μg/ml final concentration).

3.1.4 Adhesion assay and adhesion blockade.

This describes the optimised method which was used following several experiments (detailed below) to obtain the best conditions for adhesion. Variations in this protocol are described for each experiment.

i) Before use, pre-coated plates and a negative control uncoated plate were washed with RPMI-1640 (no additives) and non-specific binding was blocked by incubation for 2 hours with 100µl 1% BSA in RPMI-1640 (37°C). Myeloma cells, grown in a humidified atmosphere of 5% carbon dioxide in air, were washed x3 in RPMI-1640 medium without additives, resuspended at $2x10^6$ cells/ml and seeded at 100µl/well into microtitre plates.

ii) To determine the functional involvement of the integrin receptors VLA-4 and VLA-5 in cellular attachment, cells were, prior to plate binding, incubated for 30 minutes at 37°C with monoclonal antibodies to the alpha-4 chain (Immunotech, The Binding Site, Birmingham, clone HP2/1) and the alpha-5 chain (Immunotech, clone SAM-1) using anti-CD51 (Immunotech, clone AMF7), monoclonal antibody to the vitronectin receptor as control antibody. These antibodies (stock @ 0.2mg/ml) were added at a final concentration of 20µg/ml.

iii) After incubation 100µl of cell suspension per well was introduced to pre-coated microtitre plates and incubated overnight at 37°C. Thereafter wells were gently washed x5 in RPMI-1640 to remove non-adherent cells.

iv) Adherent cells were fixed for at least 6 hours in 100µl 3.5% formaldehyde in PBS at 4°C and then stained overnight at room temperature in 100µl 1% toluidine blue in 3% formaldehyde/PBS. Toluidine blue stains mucopolysaccharides in human blood cells.

v) Excess stain was removed by 4 washing cycles in PBS (microplate washer (Organon Teknika Microwell system, washer 400), wells were blotted dry and adherent cells were

permeabilised by addition of 100µl 1N hydrochloric acid (HCl). Before reading the optical density, the contents of each well was homogenised by intensive pippeting.

vi) The absorbance at 620nm was measured at intervals, using an Organon Teknika Microwell system plate reader, commencing with 15 minutes post HCl addition in order to establish the optimum conditions for assessing the optical density. One to two hours proved to be the optimum time to read the OD_{620}.

3.1.5 Comparison of cell adhesion to fibronectin between previously coated and freshly coated plates Adhesion blockade of myeloma cells adhering to fibronectin-coated plates.

A pilot experiment was performed to see whether plates coated more than one month before the adhesion assay affected results compared with freshly coated plates (1-3 days prior to assay).

Method

i) Wells of two microtitre plates coated with fibronectin @ 10µg/ml in PBS were prepared. One plate coated 6 weeks prior to the assay and the second plate was coated one day prior to the assay. A plate with no extracellular matrix (ECM) coating was used as a control.

ii) Myeloma cells at $2x10^5$/well, in 100µl serum free medium were added in triplicate to plates after incubation with a 1:10 dilution of blocking or control antibodies for 30 minutes at 37°C.

iii) Plates were incubated for 30 minutes at 37°C, the medium removed and wells were washed x3 with serum free medium..

iv) Adherent cells were fixed overnight at 4°C with 100µl/well of 3.5% formaldehyde in PBS. Wells were stained with toluidine blue for 4 hours at 37°C.

vi) Plates were washed in a microplate washer x4 in PBS prior to addition of 100µl 1N HCl.

vii) The optical density at 620nm was read at intervals from 30 minutes to 20 hours following the addition of hydrochloric acid.

Results

Plate 1: no ECM

OD_{620} (time)	30 mins	60 mins	90 mins	120 mins	150 mins	20 hours
negative control, no cells (subtracted)	0.061	0.057	0.103	0.179	0.188	0.112
ANBL-6	0.099	0.170	0.165	0.099	0.116	0.006
JJN-3	0.014	0.148	0.138	0.096	0.112	0.015

Plate 2: Fibronectin coated (fresh)

OD_{620} (time)	30 mins	60 mins	90 mins	120 mins	150 mins	20 hours
negative control, no cells (subtracted)	0.177	0.200	0.203	0.229	0.232	0.088
ANBL-6						
no Ab	0.099	0.234	0.145	0.144	0.254	0.184
neg (mouse IgG)	0.153	0.280	0.324	0.238	0.314	0.288
anti-VLA4	0.092	0.172	0.242	0.174	0.224	0.203
anti-VLA5	0.239	0.262	0.283	0.272	0.283	0.325
anti-VLA4+5	0.196	0.234	0.271	0.269	0.274	0.223
JJN-3						
no Ab	0.278	0.312	0.345	0.340	0.351	0.392
neg IgG	0.404	0.456	0.484	0.473	0.474	0.440
anti-VLA4	0.287	0.310	0.324	0.322	0.335	0.295
anti-VLA5	0.372	0.424	0.456	0.448	0.457	0.508
anti-VLA4+5	0.321	0.382	0.412	0.406	0.425	0.380

Plate 3: Fibronectin coated (6 weeks previously)

OD_{620} (time)	30 mins	60 mins	90 mins	120 mins	150 mins	20 hours
negative control, no cells (subtracted)	0.069	0.071	0.072	0.077	0.080	0.088
ANBL-6						
no Ab	0.111	0.130	0.133	0.132	0.131	0.131
neg (mouse IgG)	0.128	0.144	0.150	0.149	0.146	0.142
anti-VLA4	0.070	0.078	0.080	0.079	0.075	0.068
anti-VLA5	0.290	0.353	0.352	0.352	0.350	0.364
anti-VLA4+5	0.138	0.155	0.154	0.152	0.150	0.154
JJN-3						
no Ab	0.294	0.321	0.339	0.342	0.342	0.358
neg IgG	0.237	0.260	0.275	0.277	0.276	0.280
anti-VLA4	0.187	0.206	0.215	0.215	0.213	0.219
anti-VLA5	0.223	0.225	0.234	0.236	0.235	0.241
anti-VLA4+5	0.031	0.031	0.032	0.030	0.027	0.036

Summary of results at 120 minutes post-addition of hydrochloric acid

OD_{620} (time)		120 minutes
ANBL-6 (no ECM)		0.099
(FN coated, fresh)	no Ab	0.144
	neg (mouse IgG)	0.238
	anti-VLA4	0.174
	anti-VLA5	0.272
	anti-VLA 4+5	0.269
(FN coated,6 weeks)	no Ab	0.132
	neg (mouse IgG)	0.149
	anti-VLA4	0.079
	anti-VLA5	0.352
	anti-VLA 4+5	0.152
JJN-3 (no ECM)		0.096
(FN coated, fresh)	no Ab	0.340
	neg (mouse IgG)	0.473
	anti-VLA4	0.322
	anti-VLA5	0.448
	anti-VLA 4+5	0.406
(FN coated, 6 weeks)	no Ab	0.342
	neg (mouse IgG)	0.277
	anti-VLA4	0.215
	anti-VLA5	0.236
	anti-VLA 4+5	0.030

Conclusions:

1) The optimum time to read the OD_{620} is 2 hours after the addition of hydrochloric acid.

2) Myeloma cells still adhere to the plate previously coated with fibronectin and stored for one month at 4°C, ie adhesion is not adversely affected by coating plates up to one month prior to assay being performed.

3) In the previously coated plate only, for the cell line ANBL-6, anti-VLA-4 provides best adhesion blockade (50%) but a combination of anti-VLA-4+anti VLA-5 or anti-VLA-5 alone do not block adhesion. For the cell line JJN-3, a combination of anti VLA-4 + anti-VLA-5 provides a 90% block in adhesion with either anti-VLA-4 or anti-VLA-5 alone

resulting in only a 22% and 15% adhesion blockade respectively. These effects were not observed in the freshly fibronectin-coated plate.

3.1.6 Optimisation of adhesion assays.

A pilot experiment was performed to determine

1. The optimum fibronectin concentration for adhesion assays.
2. Whether toluidine blue staining overnight instead of for four hours results in higher optical density readings
3. Whether intensive pippeting of the contents of wells immediately prior to reading OD_{620} results in higher readings.

Note: Vortexing of microtitre plates was also tried but no difference in optical density readings between vortexed and intensively pippeted cells was observed.

Method

a) Preparation of stromal layers

Three plates were prepared as follows:

1) No ECM
2) Fibronectin coated @ 10μg/ml
3) Fibronectin coated @ 50μg/ml

Plates were washed in serum free medium and incubated for 3 hours with 1% BSA in serum free medium (heat inactivated).

b) Adhesion assay

i) Myeloma cells at $2x10^6$/ml were washed x3 in serum free medium before incubating with a 1:10 dilution of blocking or control antibodies for 1 hour at 37°C.

ii) Cells were then plated (100μl/well) into triplicate wells of microtitre plates and incubated overnight at 37°C.

iii) Wells were washed x5 with serum free medium, fixed for 6 hours at 37°C in 3.5% formaldehyde/PBS and stained with 100µl/well toluidine blue overnight at room temperature.

iv) After washing the plates x4 with PBS, wells were thoroughly dried by hitting the plate hard upside down onto a paper towel. The bottom of the plate was cleaned and dried before reading the OD_{620}.

v) 100µl of 1N HCl was added and the contents of the wells pipetted vigourously prior to reading the optical density. Readings were taken at intervals from 30 minutes to 28 hours.

Results **Plate 1: No ECM**

OD_{620} (time)	**30 mins**	**90 mins**	**6 hours**	**28 hours**
negative control, no cells (subtracted)	0.056	0.142	0.115	0.116
IM-9				
no Ab	0.008	0	0.016	0
neg (mouse IgG)	0.006	0	0.004	0
anti-VLA-4	0.010	0	0	0
anti-VLA-5	0.009	0	0.002	0
ANBL-6 (IL-6 independent)				
no Ab	0.004	0	0.022	0
neg IgG	0.003	0	0	0
anti-VLA-4	0.009	0	0.015	0.019
anti-VLA-5	-	-	-	-
JIM-1				
no Ab	0.004	0.048	0.017	0
neg IgG	0	0.104	0.019	0.020
anti-VLA-4	0.004	0.101	0.013	0.019
anti-VLA-5	-	-	-	-
JIM-3				
no Ab	0.013	0.023	0.025	0
neg IgG	0	0.111	0.002	0
anti-VLA-4	0	0.094	0.010	0.013
anti-VLA-5	-	-	-	-

- = not tested

Conclusion: These myeloma cell lines do not adhere to uncoated plates and blocking antibodies obviously have no effect on this.

Plate 2: Fibronectin @ 10μg/ml

OD_{620} (time)	30 mins	90 mins	6 hours	28 hours
negative control, no cells (subtracted)	0.083	0.086	0.089	0.088
IM-9				
no Ab	0.262	0.306	0.323	0.328
neg (mouse IgG)	0.398	0.467	0.507	0.530
anti-VLA-4	0.319	0.362	0.392	0.407
anti-VLA-5	0.266	0.303	0.326	0.324
ANBL-6 (IL-6 independent)				
no Ab	0.309	0.383	0.428	0.462
neg IgG	0.269	0.326	0.372	0.396
anti-VLA-4	0.276	0.339	0.389	0.413
anti-VLA-5	-	-	-	-
JIM-1				
no Ab	0.442	0.534	0.593	0.624
neg IgG	0.278	0.351	0.399	0.433
anti-VLA-4	0.359	0.446	0.505	0.544
anti-VLA-5	-	-	-	-
JIM-3				
no Ab	0.446	0.503	0.551	0.574
neg IgG	0.379	0.438	0.482	0.513
anti-VLA-4	0.353	0.414	0.460	0.488
anti-VLA-5	-	-	-	-

- = not tested

Conclusion

No significant adhesion blockade was achieved for ANBL-6, JIM-1 or JIM-3 with the blocking antibodies anti-VLA-4 or anti-VLA-5. A 23% blockade of IM-9 adhesion however, was observed with anti-VLA-4 and a 36% blockade of this cell line was achieved anti-VLA-5.

Plate 3: Fibronectin @ 50μg/ml

OD_{620} (time)	30 mins	90 mins	6 hours	28 hours
negative control, no cells (subtracted)	0.089	0.096	0.097	0.095
IM-9				
no Ab	0.356	0.441	0.473	0.492
neg (mouse IgG)	0.296	0.356	0.378	0.400
anti-VLA-4	0.231	0.287	0.304	0.317
anti-VLA-5	0.219	0.265	0.288	0.295
ANBL-6 (IL-6 independent)				
no Ab	0.538	0.671	0.728	0.689
neg IgG	0.407	0.519	0.567	0.617
anti-VLA-4	0.445	0.562	0.617	0.674
anti-VLA-5	-	-	-	-
JIM-1				
no Ab	0.420	0.538	0.586	0.633
neg IgG	0.352	0.467	0.502	0.556
anti-VLA-4	0.351	0.446	0.494	0.560
anti-VLA-5	-	-	-	-
JIM-3				
no Ab	0.453	0.543	0.586	0.616
neg IgG	0.382	0.475	0.516	0.558
anti-VLA-4	0.372	0.458	0.495	0.537
anti-VLA-5	-	-	-	-

- = not tested

Conclusion

In the cell line IM-9, 20% adhesion blockade (above negative) was achieved with anti-VLA-4 and 26% blockade achieved with anti-VLA-5. No blockade was achieved with either anti-VLA-4 or anti-VLA-5 with ANBL-6, JIM-1 or JIM-3.

Summary: Comparison of OD_{620} at 6 hours for 3 plates

CELL LINE	IM-9	ANBL-6	JIM-1	JIM-3	NO CELLS
No Ab					
no ECM	0.016	0.022	0.017	0.025	0.115
FN (10μg/ml)	0.323	0.428	0.593	0.551	0.089
FN (50μg/ml)	0.473	0.728	0.586	0.586	0.097
IgG neg					
no ECM	0.004	0	0.019	0.002	0.115
FN (10μg/ml)	0.507	0.372	0.399	0.482	0.089
FN (50μg/ml)	0.378	0.567	0.502	0.516	0.097
anti-VLA4					
no ECM	0	0.015	0.013	0.010	0.115
FN (10μg/ml)	0.392	0.389	0.505	0.460	0.089
FN (50μg/ml)	0.304	0.617	0.494	0.495	0.097
anti-VLA5					
no ECM	0.002				
FN (10μg/ml)	0.326				
FN (50μg/ml)	0.288				

Results expressed as OD_{620}, blanks (no cells) are already subtracted.

Conclusion:

Increasing the concentration of fibronectin to 50μg/ml, combined with increased staining time in toluidine blue and intensive pipetting of microtitre wells immediately prior to plate reading improves adhesion. No significant adhesion blockade of the myeloma cell lines ANBL-6, JIM-1 and JIM-3 was observed using the monoclonal anti-VLA-4 and -VLA-5 antibodies. IM-9 was partially blocked (24-36%) by anti-VLA-5 but only 20-23% blocked by VLA-4 blockade.

3.1.7 Comparison of different clones of antibodies used in adhesion blockade

The adhesion-blocking antibodies used in these assays were tested by flow cytometry for their ability to bind the cell surface adhesion molecules VLA-4, VLA-5 or CD51 (vitronectin receptor control) on a myeloma cell line in order to establish whether different clones of monoclonal antibody directed against the same antigen but perhaps recognising a different epitope would result in differences in their ability to cause adhesion blockade.

Method

a) "Camfolio" antibodies directed against VLA-4 (clone L25.3) and VLA-5 (clone MAb.16) used in adhesion blocking in the previous experiments were compared with "Immunotech" anti-VLA-4 (clone HP2/1) and anti-VLA-5 (clone SAM-1) used in adhesion blocking by van Riet et al. (270) and "Serotec" FITC-labelled anti-VLA-4 (clone 44H6) and VLA-5 (clone SAM-1). All of these antibodies were mouse anti-human MoAbs.

b) The myeloma cell line JJN-3 was chosen to compare detection of VLA-4 and VLA-5 expression using these antibodies since it had been shown by flow cytometric analysis to express virtually 100% positivity for VLA-4 (65,66 and Table 3) and 30% positivity for VLA-5 (Table 3). $5x10^5$ JJN-3 cells were washed x2 in PBS before staining for 15 minutes with 10-20μl of either a) FITC-labeled antibody (direct labeling) or b) unlabeled antibody followed by 2 washes in PBS and a further 15 minutes incubation with 4μl of FITC-labeled goat anti-mouse (GAM) MoAb (indirect labeling).

Results

	Cell line	FACS	Result
	JJN-3		(intensity of expression by FACS)
1)		neg, mouse IgG-FITC	
2)		anti-VLA4-FITC (Serotec, clone 44H6)	++
3)		anti-VLA4-FITC (Immunotech,HP2/1)	+++
4)		**anti-VLA5-FITC (Serotec, SAM-1)	-
5)		neg, mouse IgG+GAM-FITC	
6)		anti-VLA4+GAM-FITC (Immunotech, HP2/1)	+++
7)		anti-VLA4+GAM-FITC (Camfolio, L25.3)	+(weak)
8)		anti-VLA5+GAM-FITC (Immunotech, SAM-1)	-
9)		*anti-CD51+GAM-FITC (Immunotech, AMF7)	++

* Anti-CD51 is used as negative control antibody in adhesion assays.
** VLA-5 expression in JJN-3 had been shown to range from 31-62% using the Serotec antibody (Table 3) but the molecule was obviously not expressed above background (0-15%) on this occasion.

Conclusion

These results indicate that perhaps the Camfolio anti-VLA-4 blocking antibody was not binding to cell surface VLA-4 on myeloma cells as well as the Immunotech anti-VLA-4 MoAb. Therfeore, the Immunotech anti-VLA-4 and -5 blocking antibodies were used in future adhesion assays (although the Camfolio anti-VLA-5 antibody was not tested in this experiment). It was decided that it would be more economical to purchase all the antibodies from the same source and the Immunotech anti-VLA-5 antibody had already been shown to have some effect on blocking adhesion in some, but not all, myeloma cell lines (Ref. 270).

3.1.8 Effect of prolonged incubation with myeloma cell lines on fibronectin coated plates

In order to try and improve the optical densities in these adhesion assays an assay was performed without any adhesion blockade. Myeloma cell lines were incubated for either 2 hours or overnight in wells of microtitre plates coated with fibronectin @ 10μg/ml.

Method

a) Two plates each of the following were prepared:

1. no ECM
2. FN @ 10μg/ml poured 3 months previously (3m)
3. FN @ 10μg/ml poured 1 day previously (1d)

b) After blocking with BSA/RPMI, 100μl/well of myeloma cells @ $2x10^6$/ml were added in triplicate to duplicate plates 1-6.

c) Plates 1-3

Cells were allowed to adhere for 2 hours before washing wells x5 in serum-free RPMI-1640, fixing overnight in formaldehyde/PBS and staining for 4 hours in toluidine blue at 37°C.

Plates 4-6

Cells were allowed to adhere overnight before washing as above, fixing for 6 hours and staining overnight in toluidine blue at room temperature.

d) Plates were washed as described previously, adherent cells permeabilised and the optical density measured.

Results expressed as OD_{620} with blanks subtracted.

Cell Line / Plate	Time (OD_{620})	JIM-1	JIM-3	IM-9	JJN-3	ANBL-6 (IL-6 indep.)	Neg no cells
1 no ECM,	15 min	0.039	0.177	0.068	0.007	0.058	.052
	1 hr	0.038	0.190	0.040	0.005	0.066	.054
	2 hr	0.036	0.198	0.038	0.009	0.070	.060
2 FN, 3m,	15 min	0.196	0.468	0.309	0.439	0.294	.138
	1 hr	-	-	-	-	-	.082
	2 hr	0.185	0.448	0.368	0.452	0.363	.145
3 FN, 1d	15 min	0.050	0.259	0.207	0.392	0.160	.053
	1 hr	0.055	0.273	0.235	0.438	0.135	.054
	2 hr	0.066	0.291	0.236	0.460	0.136	.050
4 no ECM,	15 min	0.014	0.170	0.058	0.014	0.047	.072
	1 hr	0.016	0.095	0.058	0.014	0.031	.079
	2 hr	0	0.107	0.020	0.002	0.088	.181
5 FN, 3m,	15 min	0.514	0.863	0.111	0.672	0.451	.091
	1 hr	0.584	1.095	0.123	0.817	0.615	.091
	2 hr	0.579	1.044	0.149	0.823	0.689	.251
6 FN, 1d	15 min	0.475	0.620	0.238	0.621	0.166	.060
	1 hr	0.562	0.791	0.282	0.725	0.440	.091
	2 hr	0.592	0.811	0.295	0.756	0.212	.063

FN = Fibronectin, 3m = 3 months, 1d = 1 day

Conclusion

1. The best adherence was observed in plates 5 & 6 in which the cells were allowed to adhere overnight on fibronectin and then stained overnight with toluidine blue. There was a marked increase in adherence to fibronectin (coated either 3 months or one day previously) with JIM-1, JIM-3 and JJN-3 in these plates compared with those in which cells were only allowed to adhere for two hours. Adherence of ANBL-6 (IL-6 independent) in the fibronectin-coated plate coated 3 months previously was marked but not with the freshly-coated plate. IM-9 myeloma cells adhered less strongly to fibronectin with no significant increase in adhesion if cells were left to adhere overnight. Plates coated 3 months previously provided better adhesion than plates coated one day prior to the assay. This could be due to the freshly prepared fibronectin not being completely dissolved prior to coating the plates or the fibronectin was no longer functional due to repeated thawing. Therefore a new batch of 10x concentrated fibronectin was prepared, aliquoted into 100ul aliquots and stored at -20°C for all future assays.

3.1.9 Adhesion blockade by anti-VLA-4 of myeloma cell lines adhering to fibronectin-coated plates

Freshly prepared fibronectin (@ 50μg/ml) was used to coat plates one day prior to this assay and compared with a control plate with no ECM. Adhesion blockade was attempted using Immunotech anti-VLA-4 with anti-CD51 as negative control at a final concentration of 20μg/ml. Myeloma cells were allowed to adhere overnight at 37°C, washed, fixed for 6 hours then stained overnight at room temperature with toluidine blue.

Results are expressed as OD_{620}, read after permeabilisation of cells with HCl, blanks have already been subtracted.

Time after permeabilisation (OD_{620}) Cell line		15 min	1 hour	2 hours	4 hours
JIM-1	no ECM	no Ab	0.055	0.110	0.145	0.170
		anti-VLA4	0.049	0.069	0.079	0.089
		anti-CD51	0.025	0.042	0.040	0.045
JIM-1	FN	no Ab	0.128	0.133	0.135	0.134
		anti-VLA4	0.051	0.047	0.050	0.054
		anti-CD51	0.097	0.086	0.078	0.079
JIM-3	no ECM	no Ab	0.069	0.096	0.102	0.106
		anti-VLA4	0.060	0.089	0.095	0.100
		anti-CD51	0.145	0.182	0.191	0.156
JIM-3	FN	no Ab	0.303	0.379	0.427	0.461
		anti-VLA4	0.071	0.095	0.102	0.106
		anti-CD51	0.138	0.185	0.174	0.196
U266	no ECM	no Ab	0.129	0.073	0.088	0.085
		anti-VLA4	0.074	0.089	0.100	0.099
		anti-CD51	0.025	0.024	0.020	0.022
U266	FN	no Ab	0.776	0.984	1.041	1.158
		anti-VLA4	0.062	0.058	0.051	0.053
		anti-CD51	0.400	0.435	0.444	0.485
IM-9	no ECM	no Ab	0.217	0.145	0.154	0.301
		anti-VLA4	0.067	0.089	0.096	0.093
		anti-CD51	0.167	0.251	0.176	0.269
IM-9	FN	no Ab	0.412	0.366	0.380	0.400
		anti-VLA4	0.078	0.079	0.085	0.084
		anti-CD51	0.208	0.208	0.213	0.223
Blank	no ECM	(subtracted)	0.079	0.081	0.087	0.087
	FN		0.105	0.131	0.139	0.149

Conclusion

1. U266 adheres strongly to fibronectin with IM-9 adhering less strongly but adhesion of both these cell lines can be effectively completely blocked by prior incubation of the cells with anti-VLA-4.
2. JIM-1 and JIM-3 show only background adherence to fibronectin similar to that of the anti-Vitronectin control. Incubation with anti-VLA-4 has no significant effect on this.

Overall conclusion - Myeloma cell line adhesion assays

The myeloma cell lines tested, once the assay had been optimised IM-9, U266, JIM-1, JIM-3, JJN-3 and ANBL-6 (IL-6 independent), were all shown to adhere to fibronectin but not to either collagen or FN-RGD. Effective adhesion blockade was achieved with anti-VLA-4 in the myeloma cell lines IM-9, U266, JJN-3 and IL-6 independent passages of ANBL-6, but not with JIM-1 or JIM-3. JJN-3 was most effectively blocked (90%) by a combination of anti-VLA-4 and -5, with only 15-20% blockade achieved with either antibody alone (3.1.5). Anti-VLA-5 did not induce adhesion blockade in any of the other myeloma lines tested which is consistent with their low VLA-5 expression (Table 3), with the exception of the IL-6 independent passages of ANBL-6. VLA-5 expression in these lines were shown to range from 16-97% positive indicating that expression of this antigen varies during continued passage. These passages had only become recently IL-6 independent, the IL-6 dependent passages having a similar VLA-5 phenotype (25-84% positivity). It could be that expression of this antigen was low during these blockade experiments. Further experiments testing both IL-6-dependent as well as -independent passages of this cell line are warranted. The results in these assays were disappointingly inconsistent. The conclusions described here are only true for the individual experiments performed and should therefore not be extended to a general conclusion due to the variability in reproducibility in subsequent experiments.

CHAPTER 4

APOPTOSIS

4.1 Introduction

Apoptosis is the description of the process of programmed cell death (PCD) or activation induced cell death (AICD) in which cells are deleted, during their normal course of development by a programmed sequence of events as described in the introduction. In order to standardise the technique of recognising apoptotic cell death, the method described by Gregory et al. was adopted (271). An EBV negative, acute lymphoblastic leukaemia cell line L3055 (courtesy of Dr Chris Gregory, Dept. of Immunology, University of Birmingham Medical School (272) was used. This cell line has been shown to be extremely sensitive to calcium ionophore-induced programmed cell death. The degree of apoptosis was assessed by a comparison of flow cytometric analysis and fluorescence (or light) microscopy.

4.2 Standardisation of method using the L3055 cell line - induction of apoptosis with Calcium ionophore

Cultures of L3055 cells were set up in RPMI-1640 medium with 10% FCS and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were harvested when growth was logarithmic and were resuspended in medium at 10^6/ml. An initial sample was taken to measure base line apoptosis before adding calcium ionophore (Calbiochem, final concentration 1μg/ml, stock solution @ 1mg/ml in DMSO). Samples were taken at hourly intervals from 1-6 hours and finally at 24 hours after the addition of calcium ionophore and assessed for the extent of apoptotic cell death.

The two methods employed were: Morphological quantitative analysis by Acridine orange fluorescence microscopy (4.2.1 below) and semi-quantitative flow cytometric 90° light scatter analysis (4.2.2). The latter method of detection of apoptosis (4.2.2) does not distinguish between necrotic (background of 0-5%) and apoptotic cells since both lie within region R1 of the flow diagram. The extent of necrotic cell death in these experiments was always confirmed by morphological analysis however, and was found never to be above background level.

Results:

The percentage of apoptotic L3055 cells was found by both methods to increase to >50% 6 hours after addition of calcium ionophore with 100% apoptosis observed after 24 hours in culture (results not shown). These two methods were subsequently used concurrently to assess the degree of apoptosis of myeloma cell lines before and after treatment with various additives (anti-CD40, anti-Fas monoclonal antibodies, IL-4 or IL-6).

4.2.1 Acridine orange fluorescence microscopy:

Cells were fixed and stained with acridine orange and the percentage of apoptosing cells was scored upon examination under fluorescence microscopy (or by light microscopy after staining with Wright's stain, see note). Apoptotic cells were recognised by their characteristic features of membrane blebbing, cell shrinkage, condensation and fragmentation of chromatin and retention of cytoplasmic organelle structure. Necrotic cells were characterised by plasma membrane rupture, disruption of cytoplasmic organelles and absence of condensed chromatin. 2x 100 cells were examined for each slide if possible, and the percentage apoptosis was calculated.

Staining procedure:

(Acridine orange stock solution at 10mg/ml in PBS was used @ 10μg/ml).

1) 100μl cell suspension (approximately 10^6 cells/ml) was placed into a microfuge tube and spun briefly. The supernatant was removed and the cells were fixed in 100μl of fixative (25% acetic acid, 75% methanol), for 10 minutes at 4°C.
2) The cells were dropped onto glass slides and allowed to air dry.
3) The slides were stained in acridine orange solution in a coplin jar for 10 minutes at room temperature.
4) Slides were then rinsed in distilled water x2 and mounted in water.
5) The number of viable cells were assessed as those with rounded cell membranes and normal nuclei, non-shrunken cytoplasm with no membrane blebbing compared to those which were dying by apoptosis which had fragmented DNA in their nuclei and cytoplasm

with membrane blebbing. The percentage apoptosis was calculated by subtracting the background percentage of apoptotic cells in untreated control samples (which did not exceed 15%) from that obtained after induction.

Note: Cells (myeloma lines) in subsequent assays were also examined for signs of apoptosis by performing cytospins and staining with "Wright's" stain for examination by light microscopy. The percentage of apoptotic cells was found to be equivalent by both staining methods.

4.2.2. Flow cytometric light-scatter analysis:

Samples (0.5ml) taken concurrently with those for acridine orange staining were placed into sterile Falcon tubes and washed x2 in 5ml of PBS. Cells were then fixed in 1% formaldehyde (1/10 dilution of Becton Dickinson "Cell Fix" which contains 10% formaldehyde, 1% sodium azide in 10x buffered fixative). When all the samples had been collected, cells were washed in 5ml of PBS to wash off fixative and were resuspended in 0.5ml of PBS. Assessment of apoptosis was performed by monitoring the forward and 90° light scatter (forward versus side scatter) characteristics of the cells by flow cytometry. For each sample, 4000 cells were analysed using the Becton Dickinson FACScan analyser. An arbritary axis was drawn to distinguish between live and apoptotic cells. Apoptotic cells have an increased 90° light scatter due to increased granularity and a decreased forward scatter due to a decrease in size (Figure 4.2.2).

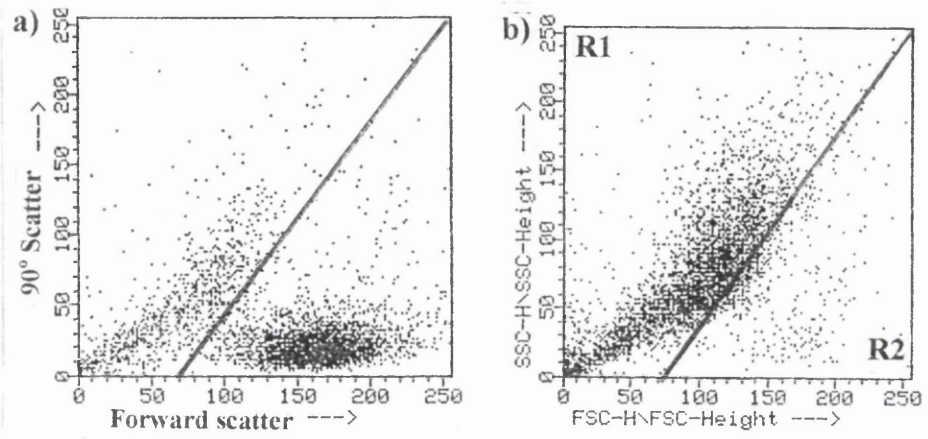
4.3 Induction of apoptosis in myeloma cell lines incubated with calcium ionophore.

Method

A panel of 6 myeloma cell lines was incubated for 24 hours in the presence of calcium ionophore @ 1μg/ml (final concentration). Samples of each cell line were taken at intervals from 0-24 hours and assessed by flow cytometry and acridine orange staining for signs of apoptosis.

Figure 4.2.2

Demonstration of the difference in light scatter amongst live and apoptotic cells: Flow cytometric light scatter analysis of the myeloma cell line IM-9 incubated for 72 hours with either no additive (a) or with anti-Fas (clone CH-11) at a final concentration of 100ng/ml (b). Regions containing dead and viable cells are shown as R1 and R2 respectively (see experiment 4.4.3).



The method of scoring apoptosis by flow cytometric analysis was as follows: - = no apoptosis, + = 50-70% apoptosis, ++ = 70-90% apoptosis, +++ = 100% apoptosis

Results Table 4.3, Figure 4.3

Table 4.3 Summary of results:

The degree of apoptosis, if any, assessed by flow cytometry and acridine orange staining:

Time (hours) Cell line	0	1	2	3	4	5	6	24
JIM-1	-	-	-	-	-	-	-	+
JIM-3	-	-	-	-	-	-	-	+
IM-9	-	-	-	-	-	-	-	+
JJN-3	-	-	-	-	-	-	-	+
RPMI-8226	-	-	-	-	-	-	-	+
***ANBL-6 (17.2.95)**	-	-	-	-	-	-	-	+
***ANBL-6 (22.5.95)**	-	-	-	-	-	-	-	+

* These two are IL-6-independent passages of the originally IL-6-dependent ANBL-6.

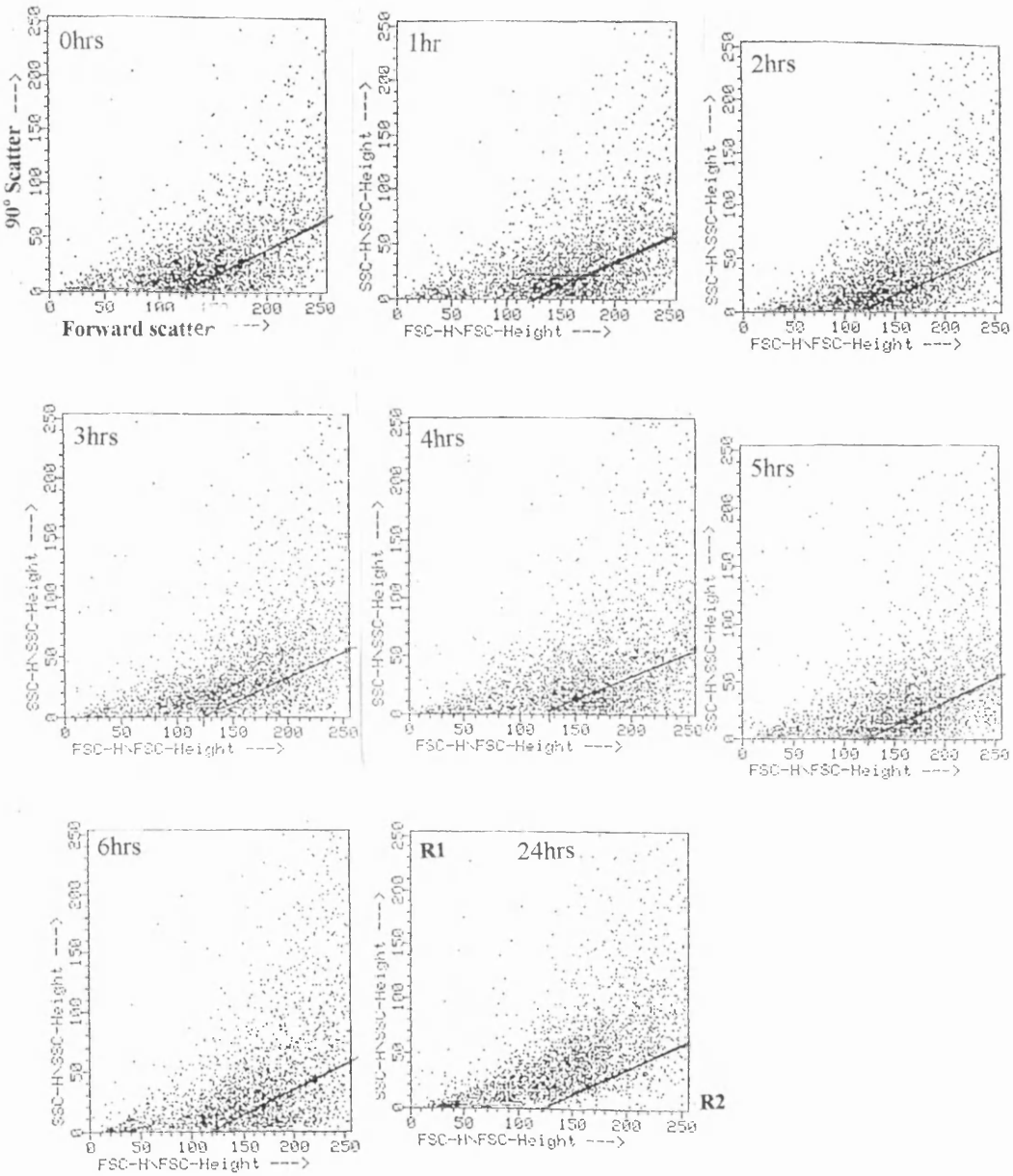
Figure 4.3 - As a representative experiment of all lines tested: flow cytometric light scatter analysis of the myeloma cell line RPMI-8226 is shown after a 24 hour incubation with calcium ionophore (1μg/ml). Samples (0.5ml) were harvested after 0, 1, 2, 3, 4, 5, 6 and 24 hours incubation and were analysed as described in 4.2.2. Apoptotic cells (region R1) were distinguished from live cells (region R2) by their decrease in size and increase in granularity. No apotosis was observed until the cells had been incubated with ionophore for at least 24 hours.

Conclusion

Only a percentage (20-30%) of all the myeloma cell lines tested were killed 24 hours after the addition of calcium ionophore. No apoptotic cell death was observed in samples incubated with calcium ionophore for less than 24 hours.

Figure 4.3

Flow cytometric light-scatter analysis of the myeloma cell line RPMI-8226 following a 24 hour incubation in the presence of Calcium Ionophore at a final concentration of 1μg/ml. Cells were harvested after 1,2,3,4,5,6 or 24 hours and 4000 cells per sample were analysed by flow cytometry. Live and apoptotic cells are found in regions R2 and R1 respectively.



4.4 Expression of Fas antigen on myeloma cell lines and the effect (over a period of 20 hours) of anti-Fas monoclonal antibody on the induction of apoptosis amongst these lines.

The expression of Fas antigen, activation of which has been demonstrated to induce apoptotic cell death in mature lymphocytes (170), was examined in a panel of myeloma cell lines (JIM-1, JIM-3, U266, IM-9 and IL-6 independent clones of ANBL-6) by flow cytometric analysis as described in 2.2.22. Cells were labeled with an FITC-conjugated mouse anti-human Fas monoclonal antibody and all the cell lines tested were found to express Fas antigen although JIM-1 showed a very weak expression (Figure 4.4a). These cell lines were then incubated over a 20 hour period in the presence or absence of anti-Fas agonistic antibody (Clone CH-11, Immunotech) in an effort to determine whether the cells could be induced to die by programmed cell death following activation of the Fas antigen on their cell surface. Samples were taken over this 20 hour incubation period and the degree of apoptosis, if any, assessed. Cells were incubated in microtitre plates previously coated with a CD32L transfected mouse fibroblast monolayer to determine whether any effect of anti-Fas would be potentiated by the presence of the human Fc receptor expressed by the CD32L cells (see chapter 5).

Method

1. $5x10^5$ myeloma cells/well in 100µl of medium were added to triplicate wells of microtitre plates previously coated with a monolayer of irradiated (30,000 rads) CD32L cells (10^5 cells per well in 100µl medium supplemented with HAT and were allowed to adhere for at least 2 hours prior to addition of the myeloma cells).

2. Anti-Fas antibody at a final concentration of 200ng/ml was added and cells from triplicate wells were harvested at intervals up to 20 hours. One aliquot was fixed with a 1:10 dilution of "Cell Fix" (Becton Dickinson) and assessed by flow cytometry whilst the remainder was fixed and stained with acridine orange for subsequent analysis by fluorescence microscopy.

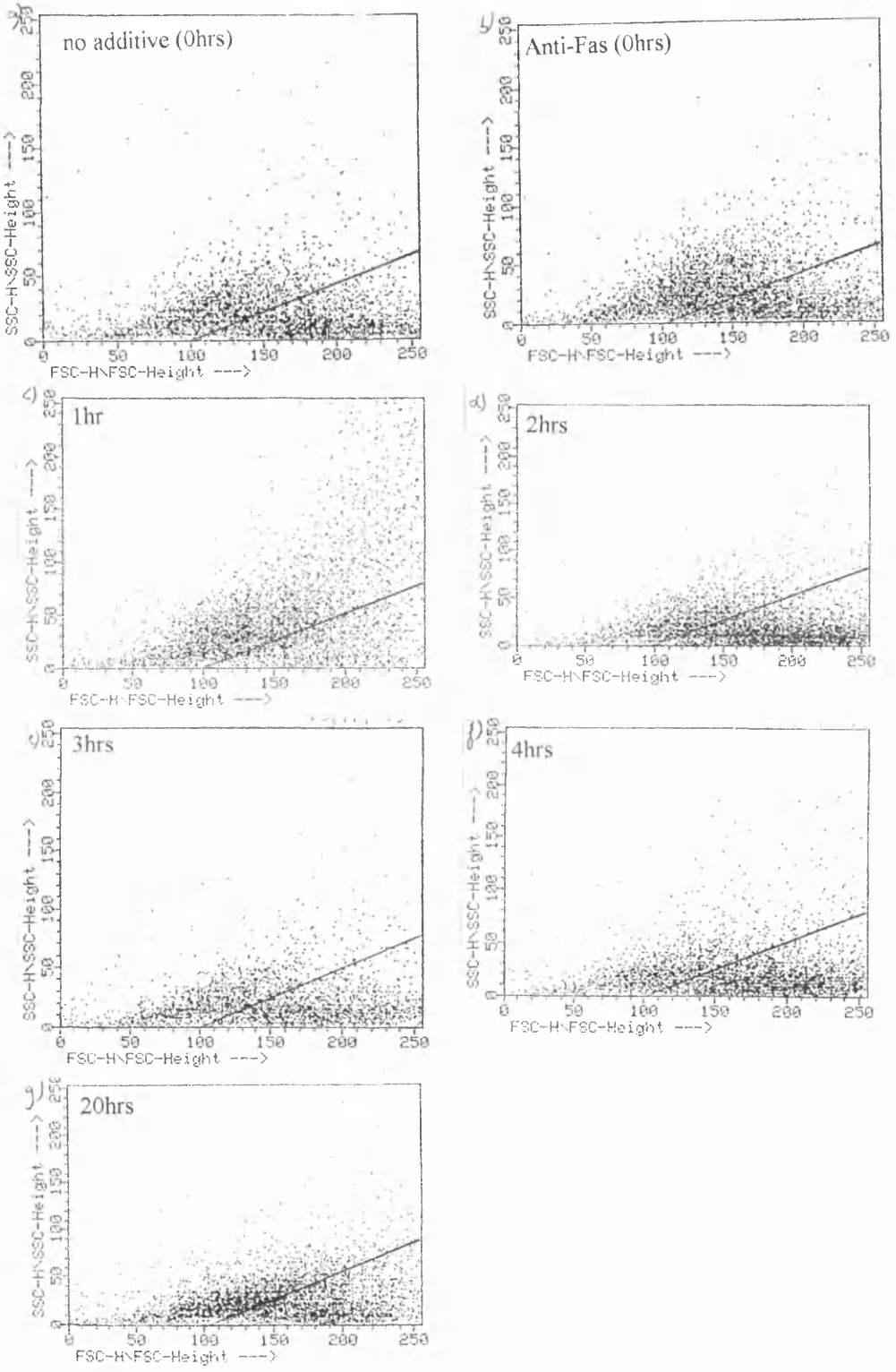
Figure 4.4a

Expression of Fas antigen on the surface of myeloma cell lines by flow cytometric analysis. Myeloma cells (5x10-5) were labeled with an FITC-conjugated mouse anti-human Fas monoclonal antibody. 10,000 cells per sample were analysed by FACS.

Fas antigen expression amongst myeloma cell lines	
Cell Line	**Fas expression by flow cytometric analysis**
	% positive (range)
JIM-1	15 (2-23)
JIM-3	66 (30-83)
U266	91 (88-93)
IM-9	100
ANBL-6 (IL-6 independent)	66 (43-97)

Figure 4.4

Flow cytometric analysis of the myeloma cell line IM-9 incubated over 20 hours with anti-Fas at a final concentration of 200ng/ml. Cells were incubated with either no additive or with anti-Fas and were harvested after 1,2,3,4 and 20 hours to assess any changes in side scatter.



Results Table 4.4

Degree of apoptosis

Time after addition of anti-Fas Cell line	0hrs	1hr	2hrs	3hrs	4.5hrs	20hrs
JIM-1	-	-	-	-	-	-
JIM-3	-	-	-	-	-	-
U266	-	-	-	-	-	-
IM-9	-	-	-	-	-	-
ANBL-6 (IL-6 independent)	-	-	-	-	-	-

See also **Figure 4.4** - flow cytometric analysis of the myeloma cell line IM-9 incubated over 20 hours with anti-Fas. This figure is representative of all the myeloma lines tested.

Conclusion

Incubation of myeloma cell lines with the agonistic anti-Fas antibody (CH-11) at a concentration of 200ng/ml, up to a period of 20 hours, does not result in the induction of programmed cell death.

4.4.1 Dose effect of anti-Fas on myeloma cell lines over a 20 hour incubation period.

The same panel of myeloma cell lines (as in 4.4) were incubated with an increasing concentration of anti-Fas antibody to establish whether an increased dosage of anti-Fas would result in apoptosis of these cell lines over the same period of incubation.

Method

1. $5x10^5$ myeloma cells/well in 1ml were added to 24 well plates with (CD32L) or without (in suspension) a confluent monolayer of irradiated (30,000 rads) CD32L cells.
2. Anti-Fas antibody at a final concentration of 0-500ng/ml was added and cells were harvested after 20 hours incubation. One aliquot was fixed with a 1:10 dilution of "Cell Fix" and assessed by flow cytometry whist the remainder was fixed and stained with acridine orange for subsequent analysis by fluorescence microscopy.

Summary of results Table 4.4.1 Degree of apoptosis

Anti-Fas (ng/ml) Cell line		0	100	200	300	400	500
JIM-1	a) in suspension	-	-	-	-	-	-
	b) CD32L cells	-	-	-	-	-	-
JIM-3	a) in suspension	-	-	-	-	-	-
	b) CD32L cells	-	-	-	-	-	-
IM-9	a) in suspension	-	-	-	-	-	-
	b) CD32L cells	-	-	-	-	-	-
U266	a) in suspension	-	-	-	-	-	-
	b) CD32L cells	-	-	-	-	-	-
ANBL-6	a) in suspension	-	-	-	-	-	-
IL-6 indep.	b) CD32L cells	-	-	-	-	-	-

See also **Figure 4.4.1** - dose effect of anti-Fas on the myeloma cell line JIM-1 over 20 hours (in suspension). This experiment is representative of all lines tested (± CD32L cells).

Conclusion

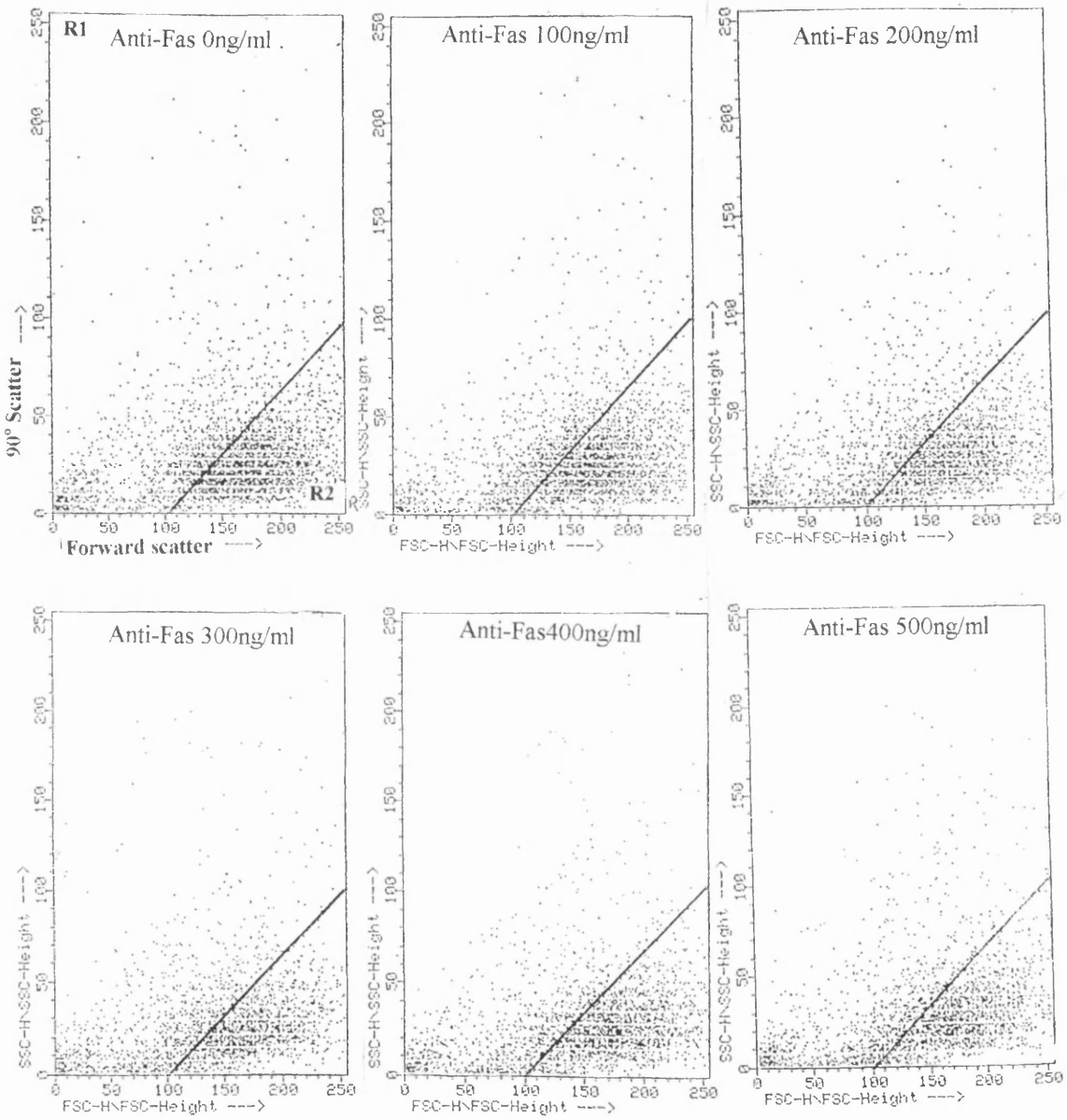
Incubation of these myeloma cell lines for 20 hours with anti-Fas monoclonal antibody up to a final concentration of 500ng/ml does not induce programmed cell death, nor is any change in effect observed by incubating the cells on a CD32L transfected mouse fibroblast monolayer.

4.4.2 Effect of prolonged incubation (3 days) of anti-Fas with myeloma cell lines

Two myeloma cell lines were chosen to observe the effect of Fas activation on the onset of activation induced cell death over a period of 3 days.

Figure 4.4.1

Dose effect of anti-Fas (0-500ng/ml) on the myeloma cell line JIM-1 over 20 hours. Cells were harvested after a 20 hour incubation with either no additive or anti-Fas at a concentration of 100, 200, 300, 400 or 500ng/ml and 4000 cells were analysed by flow cytometric light scatter analysis. Similar profiles were observed for all the myeloma cell lines tested in this experiment.



Note: This experiment was done retrospectively after further preliminary experiments using the CD40 system of cell culture (Chapter 5) had revealed that these two myeloma lines were susceptible to Fas-mediated apoptosis.

Method

1. The cell lines ANBL-6 (Passage 27.2.95, IL-6 independent) and IM-9 were used in this assay. Cells @ $5x10^4$/well were plated in triplicate wells of a Greiner microtitre plate in 200µl medium.

2. Anti-Fas monoclonal antibody (CH-11) was added at a final concentration of 100ng/ml and the plate incubated in a humidified atmosphere containing 5% carbon dioxide at 37°C.

3. Triplicate wells were harvested at 6 hours, 24 hours, 48 hours and 72 hours after addition of anti-Fas and assesssed, by a combination of cell viability (trypan blue dye exclusion), flow cytometry (Forward vs Side scatter) and physical characteristics of cytospins stained with Wright's stain, for signs of apoptosis.

Results Table 4.4.2

- = no apoptosis, + = 50-70% apoptosis, ++ = 70-90% apoptosis, +++ = 100% apoptosis

Length of incubation (anti-Fas @ 100ng/ml) **Cell line**		**6hrs**	**24hrs**	**48hrs**	**72hrs**
IM-9	**% viability**	81	69	0	0
	(FACS) apoptosis	-	+	++	+++
ANBL-6	**% viability**	89	50	0	0
	(FACS) apoptosis	-	+	++	+++

Note: Control experiments for both cell lines incubated without antibody were set up concurrently and showed 90-100% viability by trypan blue dye exclusion and flow cytometric analysis (results not shown).

See also **Figures 4.4.2a** and **4.4.2b** - flow cytometric analysis of these cell lines.

Conclusion

Both of these myeloma cell lines are killed by incubation with anti-Fas antibody although an incubation of at least 48 hours is required for all the cells to be effectively killed. Apoptosis commences between 20-24 hours after addition of anti-Fas (see experiments 4.4 and 4.4.1) but a longer incubation is required for comprehensive cell death.

4.4.3 Effect of prolonged incubation and increasing doses of anti-Fas on myeloma cell lines.

Two myeloma cell lines were chosen to observe the effect of an increasing concentration of anti-Fas antibody on the onset of programmed cell death after a period of incubation of 3 days.

Method

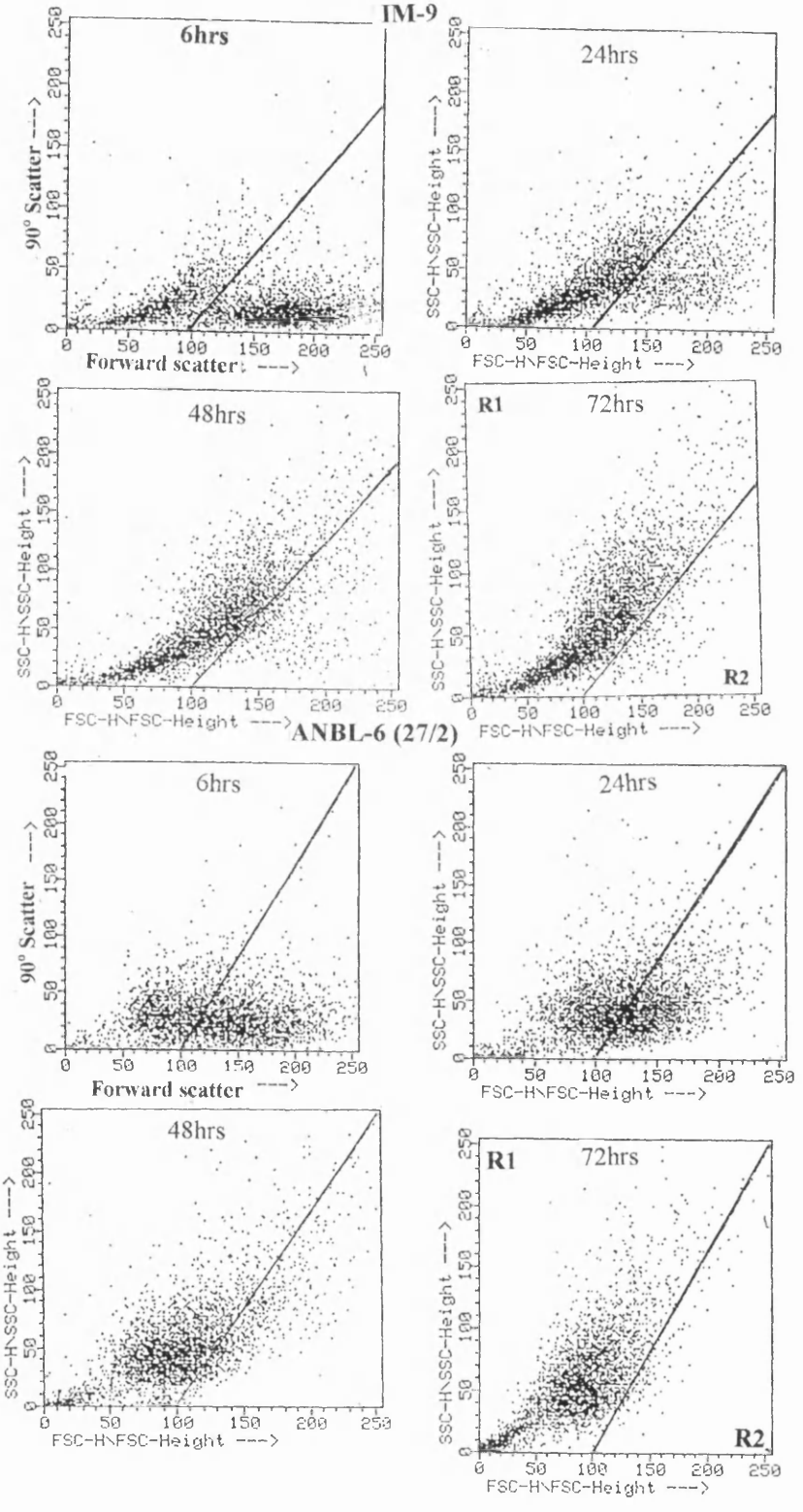
1. The same myeloma cell lines as in the previous experiment (4.4.2), ANBL-6 (27.2) and IM-9 @ $5x10^4$cells/well were plated in triplicate wells of a Greiner microtitre plate in 200ul medium.

2. Anti-Fas monoclonal antibody (CH-11) was added to triplicate wells at a final concentration of 0, 100, 200, and 300ng/ml and the plate incubated in a humidified atmosphere containing 5% carbon dioxide at 37°C for 3 days.

3. Triplicate wells were harvested 72 hours after the addition of anti-Fas and assesssed, by a combination of cell viability (trypan blue dye exclusion), flow cytometry (Forward vs Side scatter) and physical characteristics of cytospins stained with Wright's stain, for signs of apoptosis.

Figure 4.4.2a and **Figure 4.4.2b**

Flow cytometric analysis of the myeloma cell lines IM-9, Figure 4.4.2a and ANBL-6 (27/2), Figure 4.4.2b, over a 72 hour incubation with anti-Fas at a final concentration of 100ng/ml. Cells were harvested after 6, 24, 48 and 72 hours. Regions R1 and R2 distinguish between apoptotic and live cells respectively.



Only background (0-15%) apoptosis was observed at 0 hours

Results Table 4.4.3

Anti-Fas (ng/ml, 72 hr incubation) Cell line		0	100	200	300
IM-9	% viability	97	14	7	0
	(FACS) apoptosis	-	++	+++	+++
ANBL-6	% viability	92	12	0	0
	(FACS) apoptosis	-	++	+++	+++

- = no apoptosis, ++ = 70-90% apoptosis, +++ = 100% apoptosis

See also **Figures 4.4.3a and 4.4.3b** - flow cytometric analysis of these cell lines.

Conclusion

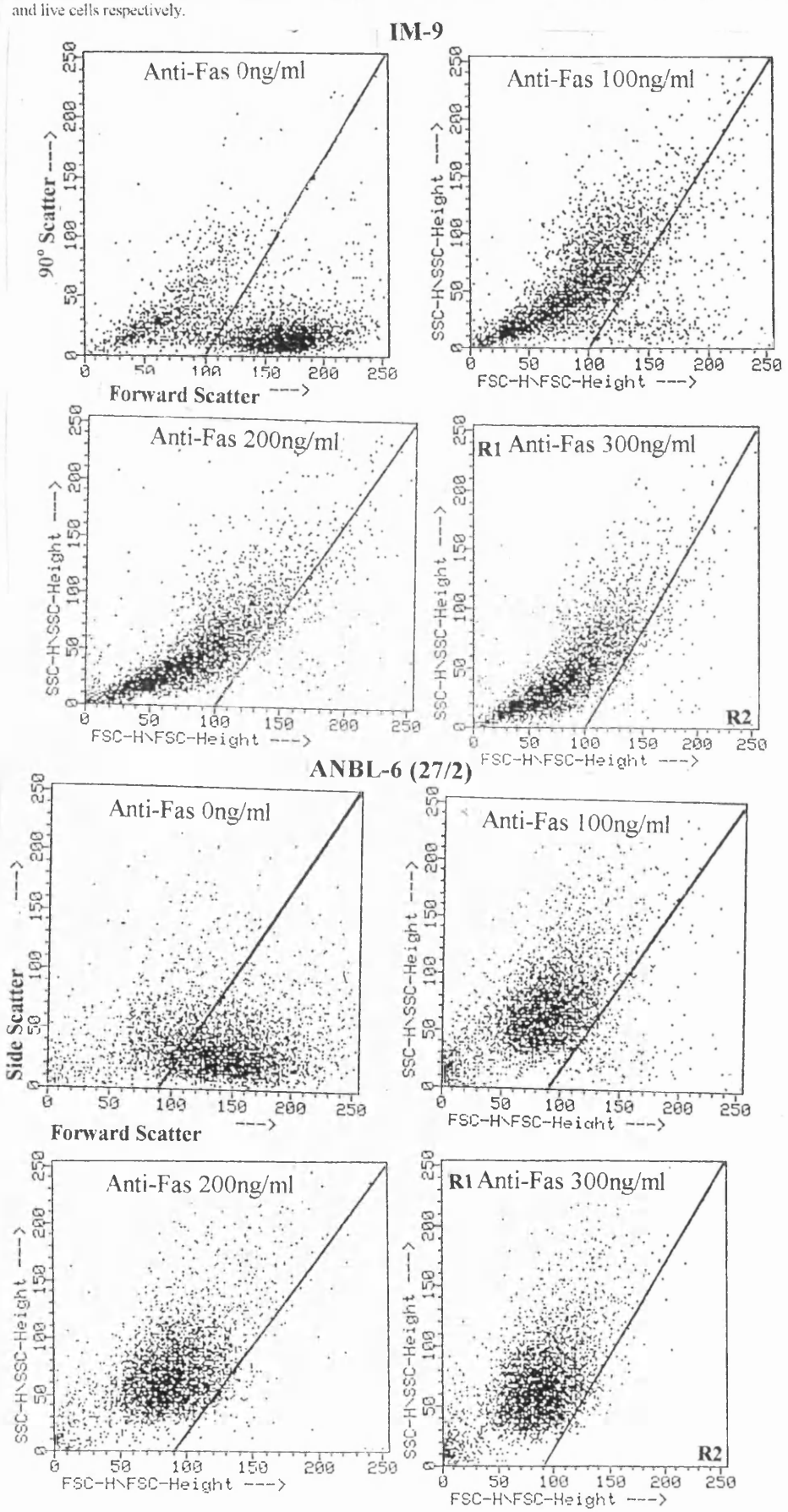
Both of these cell lines are killed by anti-Fas after a 72 hour incubation. A concentration of 100ng/ml anti-Fas is sufficient to kill at least 85% of the cells with concentrations above 200ng/ml resulting in 100% cell death.

Summary of results:

Following standardisation of the method of observation and assessment of the onset of programmed cell death using the cell line L3055 (4.2), it was discoverd that the myeloma cell lines tested could all be induced to die by AICD after treatment with 1μg/ml of calcium ionophore but this death was not initiated until an incubation of at least 24 hours had elapsed (4.3). Agonistic anti-Fas antibody up to a final concentration of 500ng/ml was unable to induce apoptosis in the myeloma cell lines tested when incubation was allowed to proceed for up to 20 hours (4.4, 4.4.1). Incubation periods for at least 24 hours however, with an anti-Fas concentration of 100ng/ml, resulted in the onset of cell death (4.4.2). This programmed cell death was most effective if the myeloma cells were incubated for at least 48 hours (Figures 4.4.2a and 4.4.2b). Incubation of these cell lines with concentrations of anti-Fas at concentrations of 100-300ng/ml for 72 hours resulted in comprehensive cell death (4.4.3).

Figure 4.4.3a and **Figure 4.4.3b**

Flow cytometric analysis of the myeloma cell lines IM-9, Figure 4.4.3a and ANBL-6 (27/2), Figure 4.4.3b, following a 72 hour incubation with anti-Fas at final concentrations of 0,100, 200 and 300ng/ml. Regions R1 and R2 distinguish between apoptotic and live cells respectively.



CHAPTER 5

ESTABLISHMENT OF THE CD40 AND CD40Lig-L CULTURE SYSTEM ASSAYS

5.1 Introduction

Activation of CD40 has been shown to be crucial in normal B cell differentiation and activation (105-115) with one of it functions being to rescue cells from apoptosis when activated (105,112,113) and another being the induction of low-level IL-6 secretion by cross-linkage of CD40 in normal B cells with MoAb (90). It has also been found to be expressed on malignant plasma cells (88) although it has been reported as being absent on normal plasma cells (89,240).

Cross-linking of cell surface CD40 on human B cells with a monoclonal antibody to CD40 (90,91,92), or cross-linking of CD40 with it ligand (CD40L) which is normally found on activated T cells, (90,93-96) results in B cell activation and IL-6 secretion. This effect has been similarly observed in myeloma cells, myeloma cell lines (88) bone marrow myeloma cells, bone marrow stromal cells (BMSC) and BMSC-derived cell lines (57). It has also been shown that CD40 engagement, using either a recombinant human CD40 Ligand or a cross-linked anti-CD40 MoAb, induces resting B cells to express high levels of Fas antigen and that ligation of such, using anti-Fas mAb (CH-11), leads to apoptotic cell death (120,122).

Since myeloma is a tumour of the B cell lineage it was decided to investigate whether similar mechanisms of cell activation and induction of, or protection from, activation induced cell death may be employed in this disease. All the myeloma cell lines tested were found to express CD40 and Fas antigen at different intensities (Table 5.1). It was thus attempted to activate these antigens and observe whether they too, would be sensitive to a) proliferate further due to CD40 activation; b) the induction of Fas-mediated apoptosis and c) co-activation of CD40 and Fas resulting in any potentiation of effect. Previous studies of Fas expression in fresh myeloma plasma cells as well as established myeloma cell lines has revealed that Fas is constitutively expressed in both although the intensity of expression is variable (126,236).

Two culture systems originally established in an effort to generate factor-dependent B cell lines (108,120) were adapted for the study of myeloma cell activation of proliferation via CD40 (and the cytokines IL-4 and IL-6) and induction of apoptosis. Although myeloma cell

Table 5.1

Flow cytometric analysis of CD40 and Fas antigen expression in a panel of myeloma cell lines. Cells were labeled with a combination of FITC-conjugated mouse-anti-human Fas antigen and PE-conjugated mouse-anti-human CD40. A dual labeled mouse IgG was used as negative control. 10,000 cells per sample were analysed.
Results are representative of at least 3 experiments.

Fas and CD40 protein expression amongst myeloma cell lines

	Flow cytometric expression by FACS % positive (range)	
Cell Line	**Fas**	**CD40**
JIM-1	15 (2-23)	15 (4-25)
JIM-3	66 (30-83)	13 (10-16)
JJN-3	82 (70-94)	17(4-34)
U266	91 (88-93)	17 (7-28)
IM-9	100	100
RPMI-8226	79 (56-88)	94 (93-94)
ANBL-6 (P32)	44	80
ANBL-6 (26/12)	40 (21-58)	70 (59-81)
ANBL-6 (16/1)	40 (38-42)	36 (11-60)
ANBL-6 (17/2)	64 (43-84)	10 (9-10)
ANBL-6 (27/2)	91 (84-97)	26 (9-43)
ANBL-6 (6/3)	51 (33-68)	11 (8-14)
ANBL-6 (22.5)	58 (39-90)	10 (3-14)
JT	66 (40-100)	51 (10-96)

ANBL-6 (26/12 and 16/1) are IL-6 dependent and were passaged from the original sample of ANBL-6 (P32), also IL-6 dependent , which was a generous gift from Dr Diane Jelinek of the Mayo Clinic. The remaining passages of ANBL-6 (17/2, 27/2, 6/3 and 22/5) are all IL-6 independent and were derived from ANBL-6 (P32). JT is a myeloma cell line derived by myself from a patient who developed plasma cell leukaemia in Glasgow Royal Infirmary.(results not published).

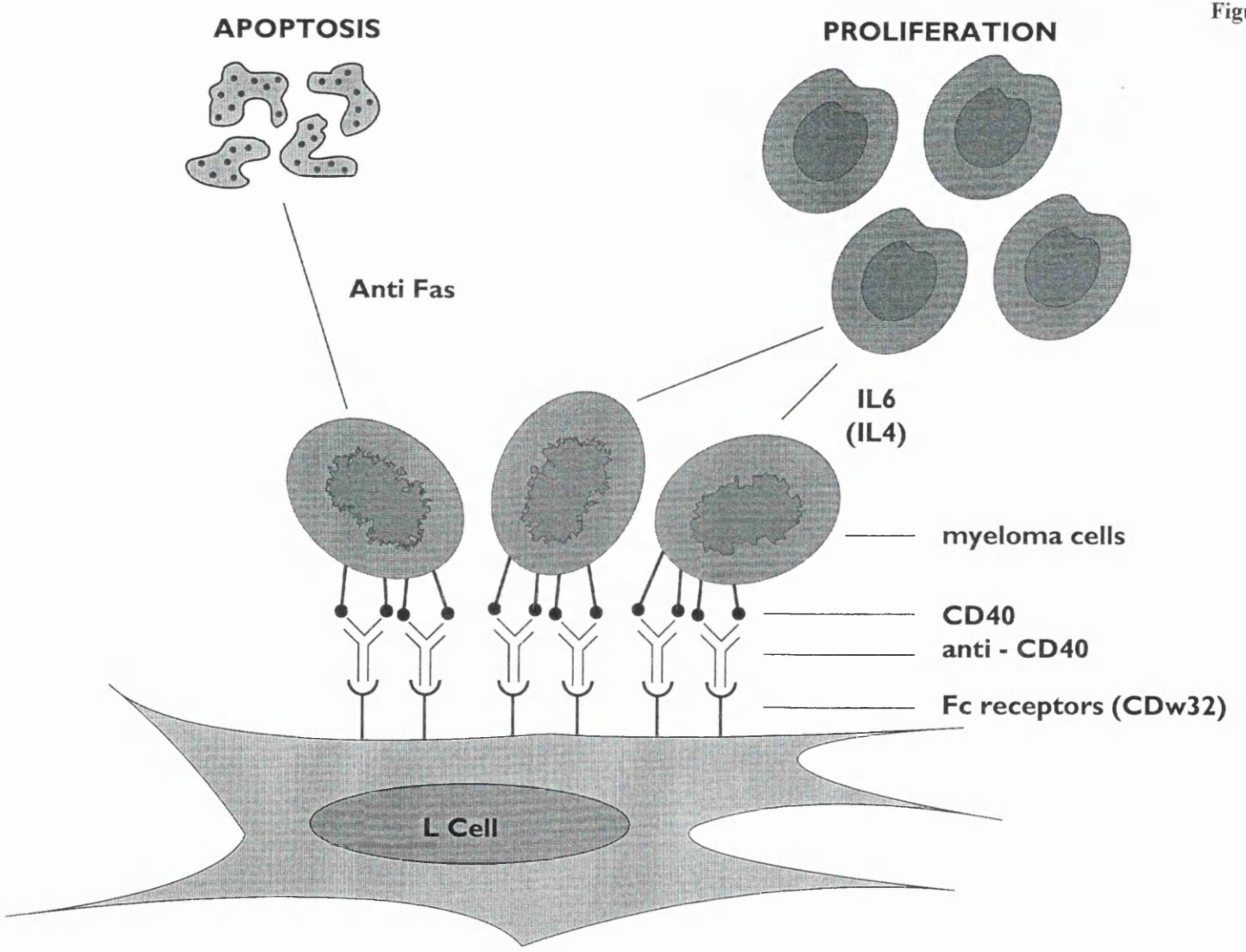
lines are obviously no longer dependent on stromal layers for their propogation it was decided that the use of the CD40 (108) and CD40Lig-L (120) culture systems may potentiate the response to CD40 activation. Cross-linkage of this antigen on the surface of myeloma cells using mouse fibroblasts transfected with either the human immunoglobulin Fc receptor (FcγRII/CDw32) or with the human CD40 ligand constitute the CD40 and CD40Lig-L culture systems respectively. Interleukin-4 was used to test for proliferation as a comparison to interleukin-6 in these studies since it is a potent B cell stimulation factor (108). Both the CD32L and CD40Lig-L transfected mouse fibroblast cell lines were a generous gift of Dr. J. Banchereau, Schering Plough Laboratories, Dardilly, France.

The experiments detailed in this chapter consist of a series of pilot experiments aimed at determination of the extent of CD40-, cytokine- and Fas- mediated activation possible using established myeloma cell lines and to determine the optimum conditions required for the assays. Chapter 6 describes the results of replicate, standardised assays using the conditions described in this method section (5.3).

5.2 CD40 culture system (Figure 5.2a)

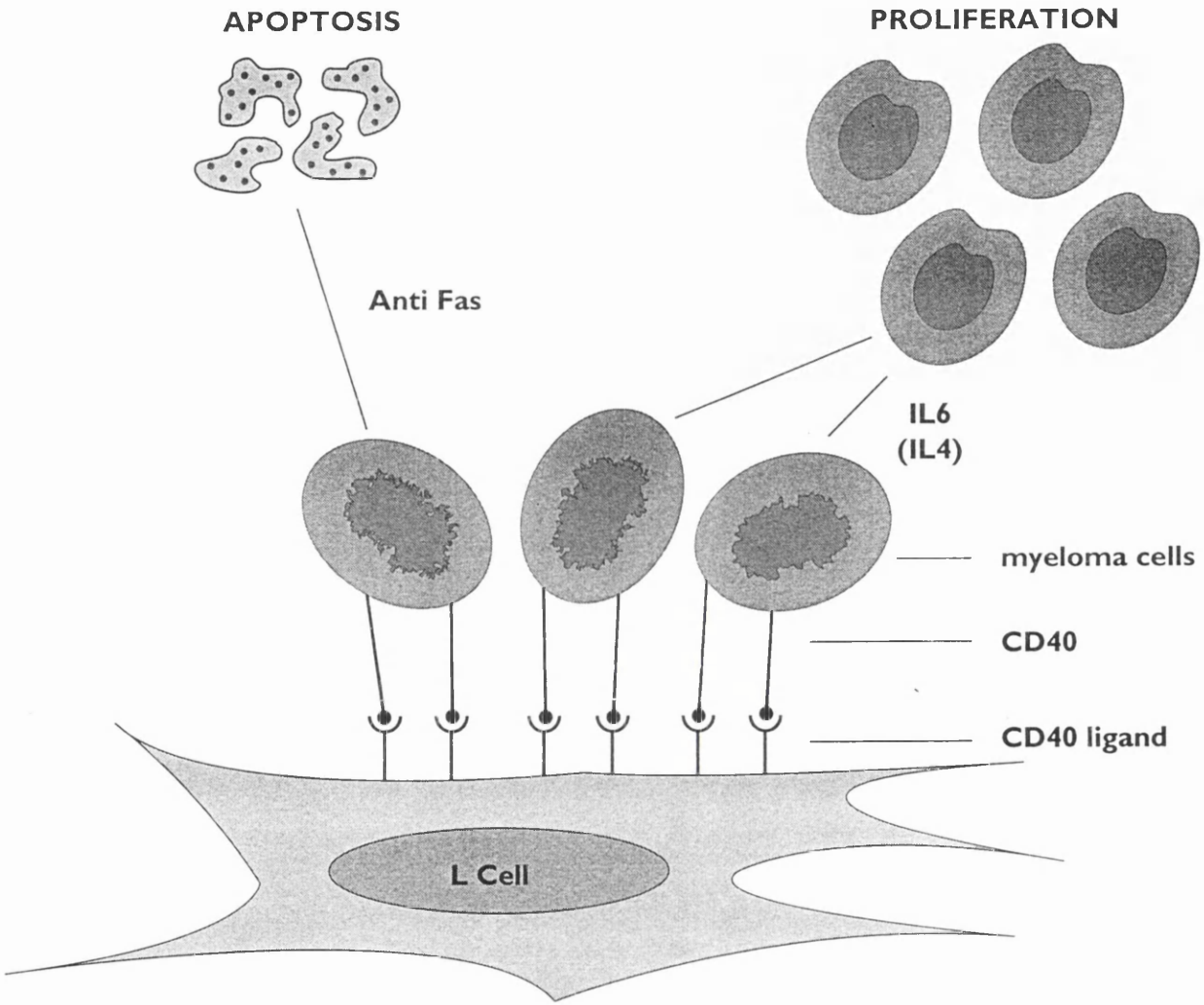
As has been already stated, this culture system was developed by Banchereau et al. (108) in an effort to reproducibly generate factor-dependent human B cell lines. It was hypothesised that the immobilisation of anti-CD40 monoclonal antibody (MoAb) by cross-linking it on a mouse fibroblastic cell line (Ltk^-) transfected with the human immunoglobulin Fc receptor, FcγRII/CDw32 (273) would represent an *in vitro* system as close as possible to the situation which occurs *in vivo* when B cells interact with cellular partners through surface antigens in germinal centres (274). Interleukin-4 (IL-4) and monoclonal antibodies to CD40 (anti-CD40) are presented in a cross-linked fashion by these cells and this has enabled the establishment of factor-dependent, long-term human B cell lines. The stable CDw32L transfectants were prepared by cloning FcγRII cDNA into the XhoI restriction site of the mammalian cell expression vector pSRα296 and transfecting them into mouse Ltk^- cells using the calcium phosphate precipitation technique. Successful transfectants were selected using HAT medium, subcloned by limiting dilution and assayed for FcγRII surface expression by immunofluorescence staining.

Figure 5.2a



Characteristics of the CD40 Culture system.

Figure 5.2b



Characteristics of the CD40 LigL Culture system.

5.2.1 The CD40 ligand-L cell culture system (Figure 5.2b)

In order to abrogate the requirement for anti-CD40 in the culture system by directly cross-linking B cells with the human CD40 ligand, Garrone et al. (120) transfected mouse L cells with the CD40 ligand gene (CD40Lig-L cells). Briefly, human CD40 ligand cDNA (243) was amplified by PCR. The product was then digested, gel purified and cloned into the expression plasmid pME18S-neo, which contains the neomycin resistance gene. Mouse L cells were then transfected with the resulting expression plasmid pME18S-neo-CD40Lig by electroporation. Neomycin-resistant L cells were selected in medium containing 0.5mg/ml G418 (Neomycin) and CD40Lig-expressing cells were further isolated by three rounds of sorting by FACS™ after staining with a CD40-Fc chimeric molecule (275).

5.3 Materials and Methods

a) **Cytokines and culture medium:**

CD32L cells were maintained in RPMI-1640 medium with 10% heat-inactivated FCS, 2% L-Glutamine, 1% Penicillin/Streptomycin, supplemented with 2% HAT. CD40Lig-L cells were maintained in the same culture medium excluding HAT. Purified recombinant rIL-4 was used at a final concentration of 100U/ml.

b) **Antibodies:**

Purified anti-human CD40 monoclonal antibody, (clone G28-5) a generous gift from Professor John Gordon, Dept. of Cellular Immunology, The Medical School, University of Birmingham, was used at a final concentration of 0.5μg/ml, unless otherwise stated. Mouse-anti-human anti-Fas MoAb (IgM, clone CH-11, Immunotec) was routinely used in assays at a final concentration of 100ng/ml. For flow cytometric analysis; a non-apoptotic FITC-conjugated mouse anti-human Fas MoAb (IgG_1, clone UB2), PE-conjugated mouse-anti-human CD40 MoAb (IgG_1, clone B-B20,), PE-conjugated mouse IgG and dual-labelled FITC- and PE-conjugated mouse IgG negative control MoAbs, were used.

c) **Myeloma cell lines:**

The cell lines JIM-1, JIM-3, JJN-3, U266, RPMI-8226, IM-9 and IL-6 independent passages of ANBL-6 (17/2, 27/2, 6/3 and 22/5) were studied.

d) **Proliferation assays:**

For proliferation assays myeloma cells in flat-bottomed 96 well microtitre plates (Greiner Labs.) were seeded at the specified number of cells/well in the presence of gamma-irradiated (15,000 rads. unless otherwise stated) CDw32L or CD40LigL cells (numbers varied in different experiments). L cells were allowed to adhere for at least 2 hours prior to the addition of myeloma cells. Cytokine and antibodies were added at the initiation of culture in a final volume of 200µl/well. Plates were incubated for, on average, 72 hours in a humidified incubator with 5% CO_2 at 37°C. Each culture condition was performed in triplicate and proliferation was determined by uptake of tritiated thymidine, [^{3}H]-TdR, after pulsing cells with 1µCi/well (Specific activity 1x37MBq) during the last 16 hours of culture. After harvesting cells on glass-fibre filters (Canberra Packard Harvester), [^{3}H]-TdR incorporation was measured by standard liquid scintillation counting techniques using a Packard Matrix 96 Direct Beta counter.

e) **Measurement of cell viability and apoptosis:**

Triplicate wells were harvested after culture in the absence or presence of additives; IL-4, anti-CD40 or anti-Fas as described. Viable cells and cell counts were enumerated by Trypan Blue dye exclusion. Cell death was assessed as described in chapter 4 (271). Briefly, triplicate wells were harvested and the cells washed in PBS prior to flow cytometric light scatter analysis. An aliquot of the same cells was also used to prepare cytospins which were either stained with Wrights stain and examined under light microscopy, or with acridine orange and observed by fluorescence microscopy. The percentage of viable cells observed by counting at least 100 cells per cytospin was compared with the flow cytometric picture after drawing an arbritary axis to distinguish between viable and non-viable cells.

f) **Flow cytometric analysis: see section 2.2.22**

For dual colour cytometric analysis of cell surface Fas and CD40 antigen expression, triplicate wells were harvested and labeled with FITC-conjugated anti human-Fas antigen and PE-conjugated anti human-CD40, using an isotype-matched FITC- and PE-conjugated negative control. Labeled cells were analysed using LysisII software on a FACScan flow cytometer (Becton Dickinson).

5.4 Initial stimulation of myeloma cell lines using the CD40 culture system

In order to determine whether myeloma cell lines could be stimulated to proliferate in the CD40 system, the cell lines JIM-1, JIM-3, JJN-3, U266, RPMI-8226 and IM-9 were incubated in triplicate wells of microtitre plates on irradiated CDw32L cells in the presence or absence of anti-CD40 MoAb.

Method

CD32L cells were gamma irradiated (30,000 rads.) and plated into microtitre wells at 10^5cells/well. Myeloma cells at $2.5x10^4$/well were plated into triplicate wells in a total volume of 200µl/well with either a) no additive or b) anti-CD40 (G28-5) at a final concentration of 1µg/ml. Plates were incubated at 37°C for three days. Tritiated thymidine (1µCi/well) was added during the last 16 hours of culture after which, the cells were harvested and [^{3}H]-TdR incorporation measured by liquid scintillation.

Results Table 5.4

Conclusion

Significant increase in proliferation induced by CD40 stimulation was only observed for JJN-3 and JIM-1 amongst myeloma cells incubated on CD32L cells. The cell line IM-9 proliferates at a much greater rate than the other myeloma lines tested with or without CD40 activation as observed by the magnitude of tritiated thymidine uptake.. The extent of proliferation observed amongst these cell lines did not relate to the degree of CD40 expression detected by flow cytometry in this experiment.

Table 5.4

Results expressed as mean (+/- % variation) of triplicate determinants

^{3}H-thymidine uptake over the last 16 hours of a 3 day culture of myeloma cell lines ($2.5x10^{4}$/well) on irradiated CD32L cells (10^{5}/well). Results expressed as counts per minute (cpm). Baseline (blank) measured from ^{3}H-thymidine incorporation in wells containing irradiated CD32L cells in medium only was 220cpm (subtracted).

Additive / **Cell line**	**CD40 expression (see Table 5.1)**	**None (-)** cpm	**Anti-CD40** cpm	**% proliferation**
JIM-1	15%	1161 ± 16%	2295 ± 36%	98
JIM-3	13%	9777 ± 17%	8669 ± 30%	0
JJN-3	17%	11197 ± 1%	18238 ± 3%	63
U266	17%	7764 ± 7%	8821 ± 6%	14
RPMI-8226	94%	4821 ± 5%	5693 ± 5%	18
IM-9	100%	472K ± 7%	439K ± 2%	0

% proliferation = - [1-^{3}H-TdR incorporation with anti-CD40/(-)] x 100

5.4.1 Effect of CD40, CD40+IL-4 and Fas stimulation of myeloma cell lines in the CD40 system

Microtitre plates containing irradiated (30,000 rads) CD32L cells (10^5/well) were set up and the cell lines JIM-1, JIM-3, JJN-3, U266 and IM-9 (@ $2.5x10^4$/well) were added with or without additives: anti-CD40 (1μg/ml), anti-CD40+IL-4 (100U/ml) or anti-Fas (100ng/ml). Plates were incubated for either 3 or 4 days and the effect of additives on stimulation or inhibition of proliferation was assessed by tritated thymidine uptake.

Results Table 5.4.1

Conclusion

Background (CD32L cells only) counts were very high most probably due to the use of too many L cells/well and incomplete irradiation of the L cells so that their proliferation overrided that of the myeloma cells and exhausted all the nutrients (especially after a four day incubation). There was no significant increase in proliferation observed amongst myeloma cells incubated with anti-CD40 with or without IL-4 with the exception of IM-9. There was, however, a significant relative decrease in uptake of tritiated thymidine observed for JIM-3 (3 day incubation) and IM-9 (3 and 4 day incubation) incubated with anti-Fas MoAb.

Table 5.4.1

Results expressed as mean of triplicate determinants

^{3}H-thymidine uptake and percentage proliferation or inhibition of proliferation during the last 16 hours of culture of myeloma cell lines ($2.5x10^{4}$/well) on irradiated CD32L cells (10^{5}/well). Baseline ^{3}H-thymidine incorporation (blank) was not subtracted from the test results.

Additive	None (-)	Anti-CD40		Anti-CD40 IL-4		Anti-Fas		Blank (Not Sub.)
Cell line		cpm	% proliferation	cpm	% proliferation	cpm	% inhibition	
JIM-1 72hrs	1902	2098	10	1714	0	1293	32	4860
96hrs	3204	1907	0	793	0	2998	6	9337
JIM-3 72hrs	7894	7355	0	6531	0	2593	67	4860
96hrs	1482	2273	53	1441	0	3886	0	9337
JJN-3 72hrs	4987	5234	5	4774	0	4691	0	4860
96hrs	402	387	0	312	0	287	0	9337
U266 72hrs	5632	5179	0	4903	0	4967	12	4860
96hrs	2167	2204	2	2418	12	1420	34	9337
IM-9 72hrs	240K	323K	35	354K	48	23,578	90	4860
96hrs	200K	257K	29	256K	28	30,112	85	9337

% proliferation = - [1-^{3}H-TdR incorporation with anti-CD40 MoAb/(-)]x100

% inhibition = [1-^{3}H-TdR incorporation with anti-Fas MoAb/(-)]x100

5.4.2 Standardisation of assays with respect to ratio of L cells to myeloma cells and concentration of anti-CD40 required for stimulation.

In an attempt to standardise this assay after consultation with Dr John Pound of the Department of Immunology, The Medical School, University of Birmingham, it was decided to use anti-CD40 MoAb at a concentration of 0.5μg/ml and to use 10-fold less CD32L cells than myeloma cells per well. The myeloma cell lines (10^5/well), JIM-1, JIM-3, U266, RPMI-8226, IM-9, ANBL-6 (6/3) and mononuclear cells isolated from the peripheral blood of a normal control (MNC) were incubated in triplicate wells of microtitre plates. The plates were seeded at least 2 hours previously with irradiated (30,000 rads) CD32L cells (@ 10^4/well). Myeloma cells were added, with or without additives (anti-CD40 @ 0.5μg/ml, IL-4 @ 100U/ml, anti-Fas @ 100ng/ml), and the plates were then incubated for 72 hours at 37°C.

Results Table 5.4.2

Conclusion

The background counts in the blank were lower by using 10x fewer CD32L cells/well than in previous assays. Fresh mononuclear cells were stimulated to proliferate extensively by the addition of anti-CD40 antibody without any increase in effect induced by co-incubation with IL-4. No marked increase in proliferation amongst myeloma cell lines was observed with anti-CD40 ± IL-4. Incubation with anti-Fas MoAb had no apoptotic effect on JIM-1 and a proliferative effect on the MNC control. Fas ligation had some inhibitory effect (16-82% inhibition of proliferation) on the other cell lines tested. The strongest Fas-mediated inhibition of proliferation was observed in the myeloma cell line IM-9 ± anti CD40 (77-82% inhibition).

Table 5.4.2

Results expressed as mean (± % variation) of triplicate determinants

^{3}H-thymidine uptake and percentage proliferation or inhibition during the last 16 hours of culture of myeloma cell lines and MNC control (10^{5}/well) on irradiated CD32L cells (10^{4}/well). Baseline (blank) thymidine incorporation was not subtracted from the test results.

Additive	None (-)	Anti-CD40		Anti-CD40+IL-4		Anti-CD40+Anti-Fas		Anti-Fas		Blank
Cell line	cpm	cpm	% proliferation	cpm	% proliferation	cpm	% inhibition	cpm	% inhibition	(Not sub.)
JIM-1	4943±14%	5131±9%	4	6213±12%	26	4696±8%	0	5451±5%	0	737
JIM-3	7178±39%	6120±38%	0	6944±40%	0	3896±20%	46	4786±26%	33	737
U266	7673±40%	5418±26%	0	4631±22%	0	4095±8%	47	4889±28%	36	737
RPMI-8226	5840±3%	6335±14%	8	6334±20%	8	3965±17%	32	5583±30%	4	737
IM-9	360K±21%	365K±3%	0	380K±6%	6	81,475±24%	77	65,393±25%	82	1633
ANBL-6 (6/3)	5375±11%	6533±11%	22	6729±5%	25	6268±12%	0	4541±18%	16	1633
MNC (AS)	2729±28%	14,524±10%	400	14,068±18%	400	11,955±25%	0	5928±34%	0	1633

% proliferation = - [1-^{3}H-TdR incorporation with anti-CD40 MoAb/(-)]x100

% inhibition = [1-^{3}H-TdR incorporation with anti-Fas MoAb/(-)]x100

5.4.3 Standardisation of time of irradiation required to reduce background thymidine uptake in CD40 system assays.

In order to determine the optimum dosage of radiation required for CD32L cells not to provide significant background thymidine uptake, duplicate plates were set up with CD32L cells at 10^4/well and myeloma cells U266, RPMI-8226, IM-9 and mononuclear cell control cells at 10^5/well. One plate was seeded with CD32L cells irradiated for 39 minutes (15,000 rads) and the second plate seeded with CD32L cells which had been irradiated for 78 minutes (30,000 rads). Additives were as in experiment 5.4.2. Culture was continued for 72 hours at 37°C.

Results Table 5.4.3

Conclusion

Irradiation of CD32L cells for 39 minutes (15,000 rads) was sufficient to result in low background for proliferation assays. Mononuclear cells under both conditions proliferated markedly when incubated with anti-CD40 and IL-4 but these cells were not found to be significantly inhibited by Fas activation. The myeloma cell lines IM-9 and RPMI-8226 were stimulated to proliferate by anti-CD40 with or without IL-4 whereas U266 was not except when incubated for 3 days on a monlayer of CD32L cells (irradiated at 30,000 rads) in the presence of anti-CD40+IL-4. Proliferation of IM-9 and RPMI-8226 was substantially inhibited when these lines were incubated with either a combination of anti-Fas and anti-CD40 or with anti-Fas alone. although the effect was potentiated by CD40 and Fas coligation. U266 showed a less obvious inhibition of proliferation induced by Fas activation of 0-33% and this could be background.

Table 5.4.3

Results expressed as mean (± % variation) cpm of triplicate wells.

^{3}H-thymidine uptake and percentage proliferation or inhibition of proliferation during the last 16 hours of culture.of myeloma cell lines and MNC control (10^{5}/well) on irradiated CD32L cells (10^{4}/well). Baseline (blank) thymidine incorporation was not subtracted from the readings.

Additive / Cell line	None (-)	Anti-CD40		Anti-CD40+IL-4		Anti-CD40+Anti-Fas		Anti-Fas		Blank
	cpm	cpm	% proliferation	cpm	% proliferation	cpm	% inhibition	cpm	% inhibition	cpm
U266										
15,000rad	5026±16%	4706±22%	0	4522±21%	0	4401±30%	12	3371±16%	33	455
30,000rad	3677±13%	3791±30%	0	5753±4%	56	3722±22%	0	3256±4%	11	500
RPMI-8226										
15,000rad	3427±13%	5127±12%	50	5097±3%	49	1188±21%	65	1900±19%	45	455
30,000rad	2394±26%	3611±7%	50	4922±2%	100	602±8%	75	1213±10%	49	500
IM-9										
15,000rad	96,997±15%	572K±30%	500	572K±6%	500	4102±26%	100	24,983±25%	74	455
30,000rad	351K±16%	492K±3%	40	555K±5%	58	4564±30%	100	11,406±25%	97	500
MNC (JR)										
15,000rad	7777±9%	10,373±12%	33	24,373±11%	100	6502±28%	16	6648±15%	15	455
30,000rad	942±16%	3035±8%	100	18,256±14%	1800	3751±32%	0	1502±28%	0	500

% proliferation = - [1-^{3}H-TdR incorporation with anti-CD40 MoAb/(-)]x100

% inhibition = [1-^{3}H-TdR incorporation with anti-Fas MoAb/(-)]x100

5.5 Effect of Fas activation on CD40 antigen expression over time

A panel of myeloma cell lines was examined for the effect of Fas activation on CD40 antigen expression over time using a phycoerythrin labeled-anti-CD40 (clone B-B20) monoclonal antibody to detect CD40 on the cell surface of the myeloma cells.

Method

1. The IL-6 independent myeloma cell lines JIM-1, JIM-3, JJN-3, IM-9, ANBL-6 (Passages 17/2, 27/2, 6/3 and 22/5), @ 10^5/ml were plated into triplicate wells of a Greiner microtitre plate in 200µl medium/well and incubated in the presence of anti-Fas (CH-11) monoclonal antibody at a final concentration of 100ng/ml.

2. Cells were harvested after 0hrs, 6hrs, 20hrs, 43hrs and 64hrs incubation at 37°C, washed x2 in PBS and labelled with PE-conjugated anti-CD40 monoclonal antibody using mouse-IgG-PE as a negative control.

3. 10,000 cells were analysed by FACS, results were plotted as histograms with CD40 expression (FL-1) versus side scatter.

Results Table 5.5 and Figures 5.5 (a-d)

Conclusion

Incubation with anti-Fas over 3 days resulted in an up-regulation of CD40 protein expression which was initially manifested after 20-43 hours in the myeloma cell lines JIM-3 and IL-6 independent passages of ANBL-6 (17/2, 27/2, 6/3, 22/5). JIM-1 CD40 expression was slightly down-regulated after incubation with anti-Fas. IM-9 maintained 100% expression of CD40 which was unaffected by Fas activation and JJN-3 CD40 expression was negative throughout this experiment and was unaffected by Fas-activation.

Table 5.5

CD40 protein expression by FACS™ following a 3 day incubation with anti-Fas

Intensity of CD40 protein expression with time on the surface of myeloma cell lines by flow cytometric analysis is shown during a three day incubation with anti-Fas MoAb. 10^5 myeloma cells/well were incubated in triplicate wells of a microtitre plate in the presence of anti-Fas at a final concentration of 100ng/ml. Cells were harvested after 0,6,20,43 and 64 hours, labelled with PE-conjugated mouse anti-human-CD40 MoAb and analysed by flow cytometry using a mouse IgG-PE as negative control.

Time after addition of anti-Fas Cell line	0 hrs	6 hrs	20 hrs	43 hrs	64 hrs
JIM-1	++	+	+	+	+
JIM-3	-	-	+	++	+++
JJN-3	-	-	-	-	-
IM-9	+++	+++	+++	+++	+++
ANBL-6 (17/2)	-	-	+/-	+	++
ANBL-6 (27/2)	-	-	+	++	+++
ANBL-6 (6/3)	-	-	+/-	+	++
ANBL-6 (22/5)	-	-	+/-	+	++

5.5.1 Effect of CD40 activation on Fas antigen expression over time

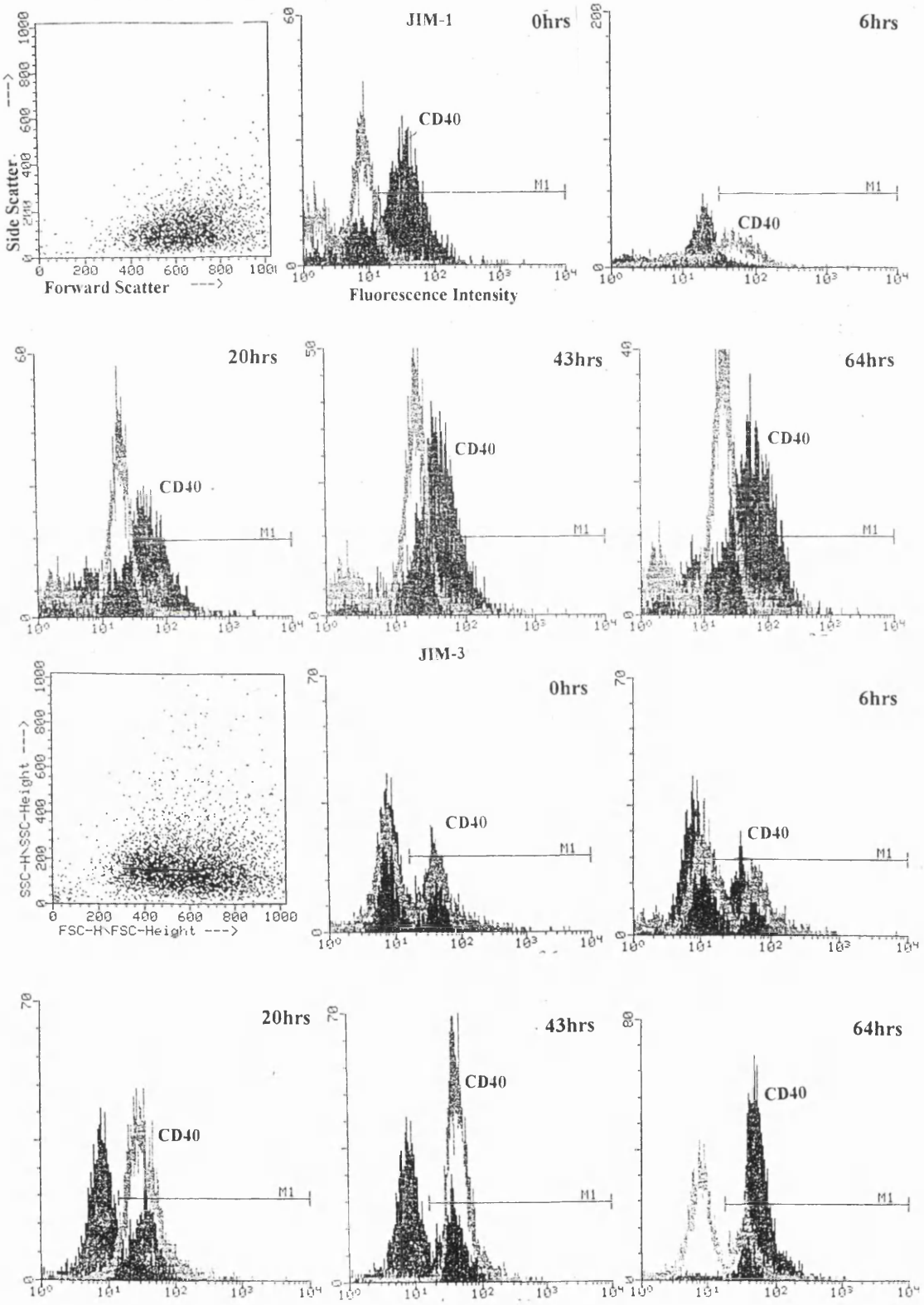
The same panel of myeloma cell lines was also examined using the same conditions as in experiment 5.5 to examine the effect of CD40 activation on Fas antigen expression over time using a fluorescein labeled-anti-Fas (clone UB2) monoclonal antibody to detect cell surface Fas expression.

Results

No change in Fas antigen expression over time was observed for any of these myeloma cell lines incubated with anti-CD40 monoclonal antibody over a three day period with samples tested after 0, 6, 20, 43 and 64 hours incubation (results not shown).

Figure 5.5a **CD40 protein expression following Fas activation in the myeloma cell lines JIM-1 and JIM-3**

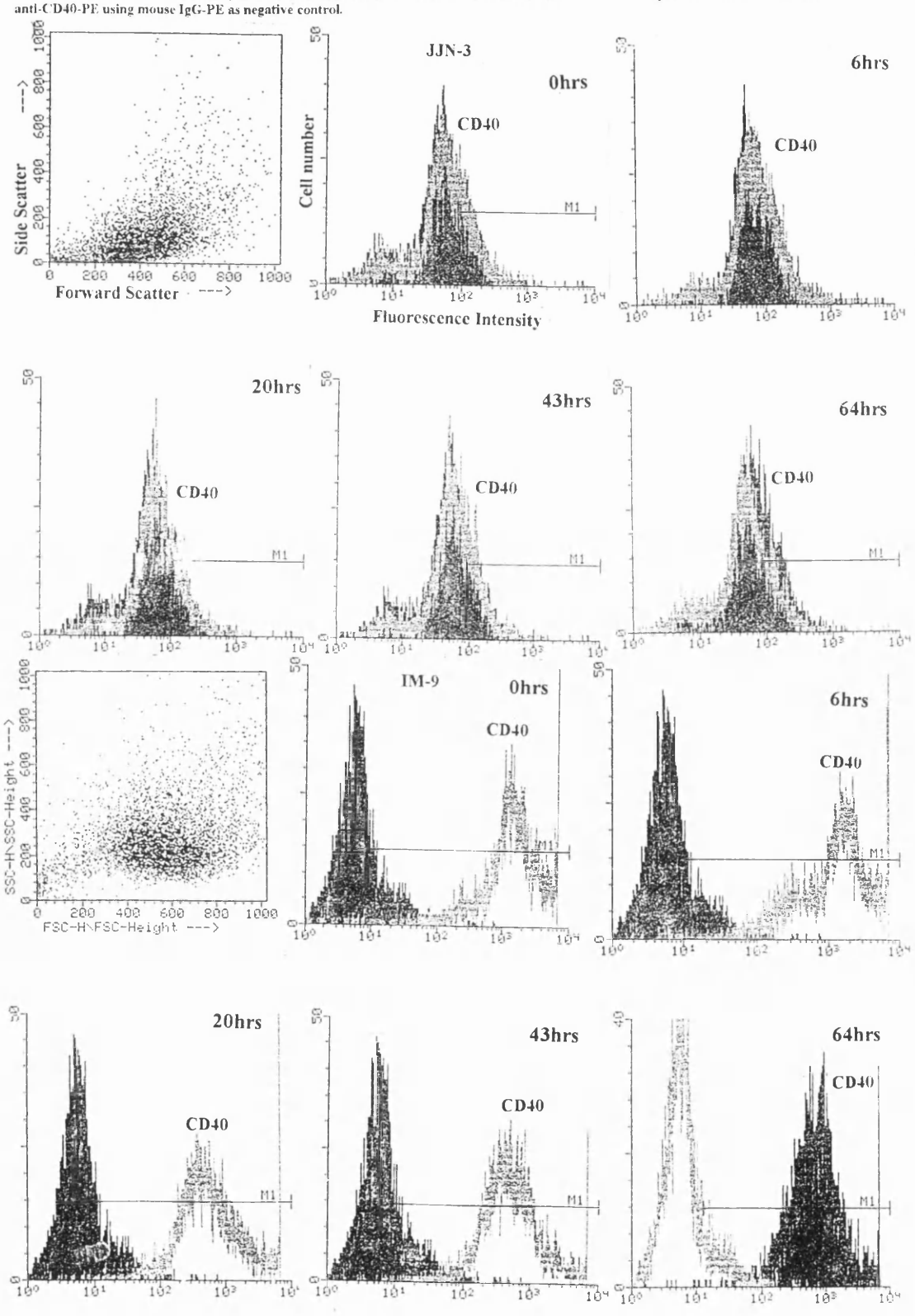
CD40 expression in myeloma cell lines following activation of Fas antigen. Cells were incubated in the presence of anti-Fas (100ng/ml) for 64 hours, harvested at intervals (0hrs,6hrs,20hrs,43hrs and 64hrs) and 10,000 cells analysed for CD40 expression after labeling with anti-CD40-PE using mouse IgG-PE as negative control.



Marker (M1) indicates end of negative control peak

Figure 5.5b **CD40 protein expression following Fas activation in the myeloma cell lines JJN-3 and IM-9**

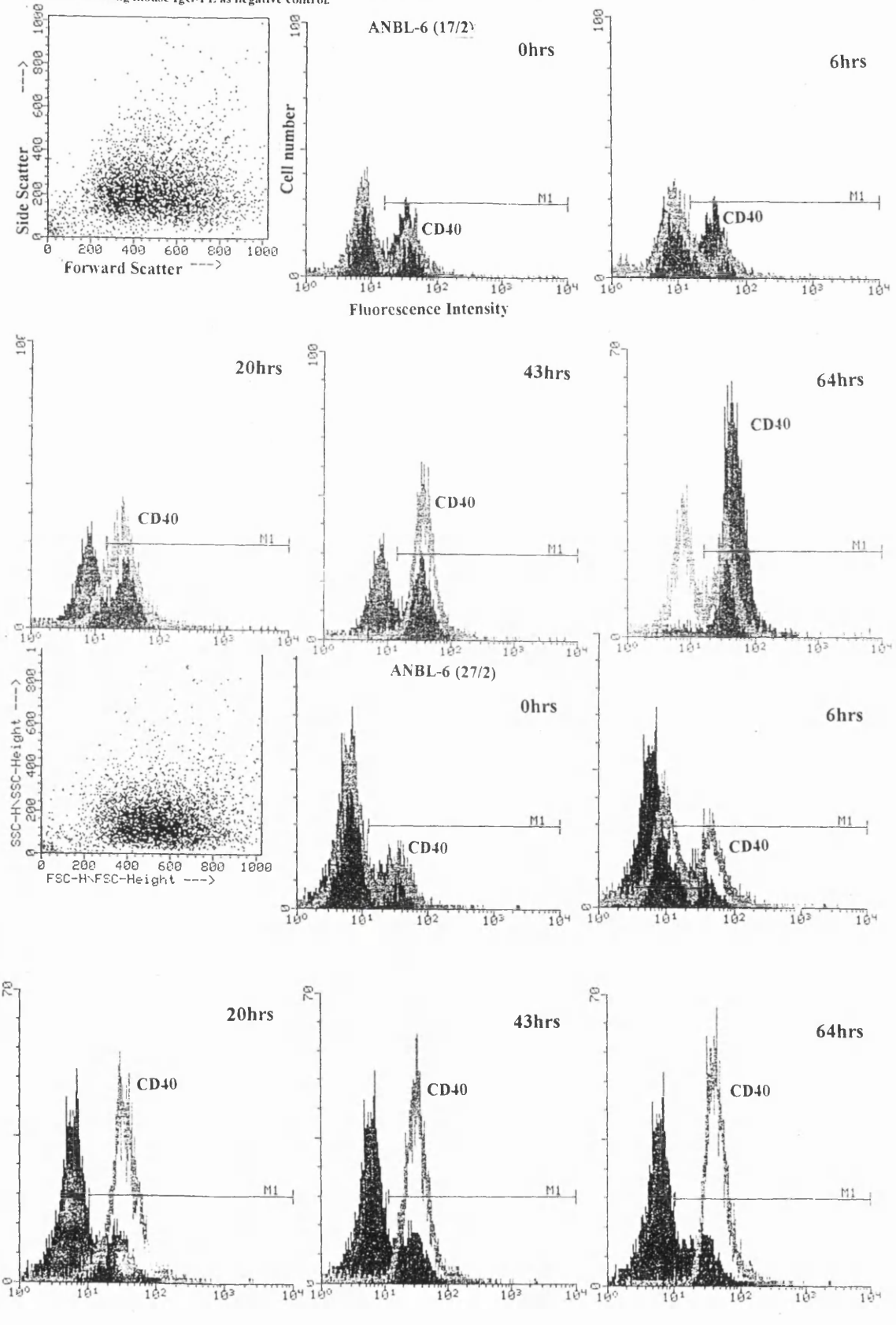
CD40 expression in myeloma cell lines following activation of Fas antigen. Cells were incubated in the presence of anti-Fas (100ng/ml) for 64 hours, harvested at intervals (0hrs,6hrs,20hrs,43hrs and 64hrs) and 10,000 cells analysed for CD40 expression after labeling with anti-CD40-PE using mouse IgG-PE as negative control.



Marker (M1) indicates end of negative control peak

Figure 5.5c **CD40 protein expression following Fas activation in the myeloma cell lines ANBL-6 passages 17/2 and 27/2**

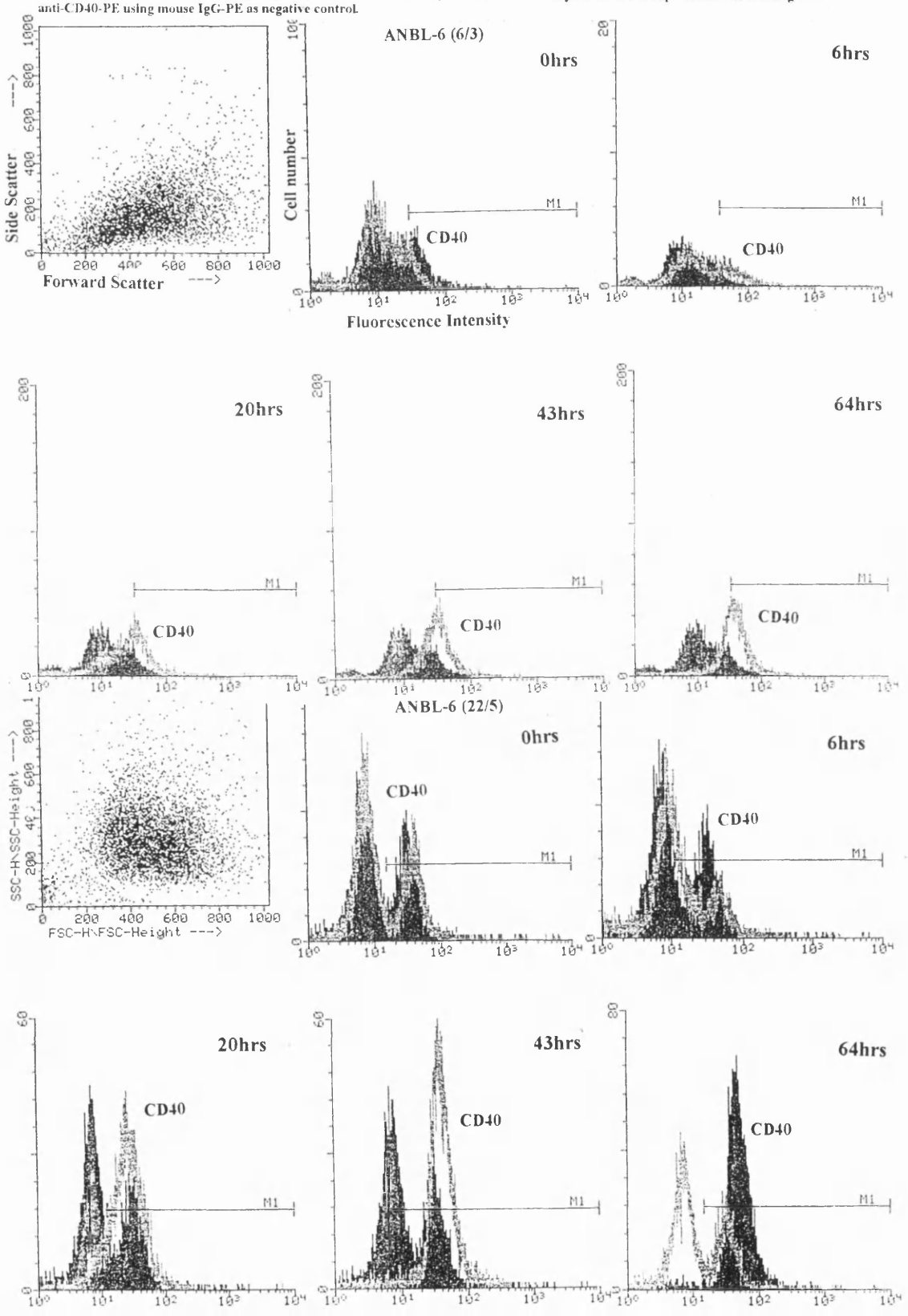
CD40 expression in myeloma cell lines following activation of Fas antigen. Cells were incubated in the presence of anti-Fas (100ng/ml) for 64 hours, harvested at intervals (0hrs,6hrs,20hrs,43hrs and 64hrs) and 10,000 cells analysed for CD40 expression after labeling with anti-CD40-PE using mouse IgG-PE as negative control.



Marker (M1) indicates end of negative control peak

Figure 5.5d **CD40 protein expression following Fas activation in the myeloma cell lines ANBL-6 passages 6/3 and 22/5**

CD40 expression in myeloma cell lines following activation of Fas antigen. Cells were incubated in the presence of anti-Fas (100ng/ml) for 64 hours, harvested at intervals (0hrs,6hrs,20hrs,43hrs and 64hrs) and 10,000 cells analysed for CD40 expression after labeling with anti-CD40-PE using mouse IgG-PE as negative control.



Marker (M1) indicates end of negative control peak

CHAPTER 6

CD40 AND CD40LIG-L CULTURE SYSTEM ASSAYS - EFFECT OF ACTIVATION OF CD40 AND FAS ANTIGEN IN MYELOMA CELL LINES ON PROLIFERATION, APOPTOSIS AND BCL-2 EXPRESSION.

6.1 Introduction

Pilot experiments having been completed, a number of replicate assays were then performed using the standardised method adopted as a result of the experiments performed in chapter 5. A comprehensive panel of IL-6-dependent and -independent myeloma cell lines were tested for their susceptibility to Fas-mediated apoptosis and CD40 activation with or without the cytokines IL-4 and IL-6 using the CD40- and CD40Lig-L- culture systems as a comparison. It was intended to ascertain which myeloma cell lines, if any, were repeatedly sensitive to CD40 stimulation and/or the induction of Fas-mediated apoptosis, whether this would be potentiated by CD40 and Fas coligation and whether any lines were completely resistant.

The product of the proto-oncogene *bcl-2* affords protection against apoptosis (105,136). Comparison of *bcl-2* mRNA and protein expression in germinal centres and in myeloma cell lines, by *in situ* hybridisation, immunohistochemistry and flow cytometric techniques indicates that *bcl-2* mRNA is expressed throughout the germinal centre albeit in varying amounts, and in most myeloma cell lines tested but the protein product is only manifested in the follicular mantle and in some myeloma cell lines (155,156 and personal findings). It has been demonstrated that rescue from apoptosis by CD40 cross-linking in germinal centre B cells is usually (105,130), but not always (112) accompanied by up-regulation of the *bcl-2* gene therefore experiments were carried out to examine Bcl-2 protein expression in myeloma cell lines before and after CD40 and Fas activation (6.6).

6.2 Materials and Methods

a) Cytokines and culture medium:

CD32L cells and CD40Lig-L cells were maintained in the culture conditions described in section 5.3. Purified recombinant human rIL-4 and rIL-6 were used at a final concentration of 100U/ml and 1ng/ml respectively.

b) Antibodies:

Antibodies were as described in section 5.3 with the addition of: FITC-conjugated mouse-anti-human Bcl-2 (IgG_1, DAKO clone 124) and FITC-conjugated mouse IgG negative control.

c) **Myeloma cell lines:**

The cell lines JIM-1, JIM-3, JJN-3, U266, IM-9, RPMI-8226 and both IL-6 dependent (passages 26/12 and 16/1) and independent passages of ANBL-6 (passages 17/2, 27/2, 6/3 and 22/5) were studied. The original clone (P32) of ANBL-6 (which was a generous gift of Dr Diane Jelinek of the Mayo Clinic, Rochester, Minnesota, also IL-6 dependent) and 'JT', a myeloma cell line derived by myself from a patient with plasma cell leukaemia were also tested in these assays. The results of experiments using P32 and JT are found in Appendices 1.12 and 1.13 respectively.

d) **Proliferation assays:**

For proliferation assays myeloma cells in flat-bottomed 96 well microtitre plates (Greiner Labs.) were seeded at $5x10^4$cells/well in the presence of $5x10^3$ gamma-irradiated (15,000 rads) of either CDw32L or CD40Lig-L cells/well. L cells were allowed to adhere for at least 2 hours prior to the addition of myeloma cells. Cytokines and antibodies were added at the initiation of culture with a final volume of 200µl/well. Plates were incubated for 72 hours in a humidified incubator with 5% CO_2 at 37°C. Each culture condition was performed in triplicate and proliferation was determined by uptake of tritiated thymidine after pulsing cells with 1µCi/well during the last 16 hours of culture as described in section 5.3.

e) **Measurement of cell viability and apoptosis:**

Triplicate wells were harvested after 72 hours culture in the absence or presence of additives; IL-4, IL-6, anti-CD40 or anti-Fas as described. Viable cells and cell counts were enumerated by Trypan Blue dye exclusion. Cell death was assessed as described in section 5.3).

f) **Flow cytometric analysis:**

Analysis was as described in section 5.3. For examination of Bcl-2 expression, cells were permeabilised (2.2.22) before labeling with an FITC-conjugated anti human-Bcl-2 mAb using an FITC-conjugated IgG negative control.

g) Standardised culture system assays

Duplicate or triplicate plates were set up and incubated for 72 hours at 37°C. One plate was pulsed with tritiated thymidine during the last 16 hours of culture and then the cells were harvested as described (5.3) with results expressed as mean counts per minute of triplicate wells. The second replicate plate was used to determine:

a) cell viability and cell counts (Trypan blue dye exclusion)
b) the extent of apoptotic cell death by acridine orange staining and fluorescence microscopy (or, alternatively, cells were stained with Wright's stain and examined by light microscopy)
c) flow cytometric light-scatter analysis

The cells harvested from the third replicate plate were used to perform cytospins for *in situ* hybridisation analysis of CD40, *bcl-2* or *fas* mRNA expression. A comparison was made of all these parameters by stimulating myeloma cells using:

1) no L cells (termed as "in Suspension")
2) CD32L cells ± anti-CD40 MoAb (the CD40 system)
3) CD40Lig-L cells (no anti-CD40 required, termed the CD40Lig-L system).

The summarised results shown are the mean results of replicate experiments using myeloma cell lines cultured in the CD40Lig-L system. Results of tritiated thymidine uptake in individual experiments for each myeloma cell line and the comparative results for tritiated thymidine uptake, cell counts, percentage proliferation or inhibition of proliferation and percentage viability in replicate experiments performed using no L cells, CD32L cells and CD40Lig-L cells for each myeloma cell line are shown in Appendix 1. Representative flow cytometric light scatter analyses for all the myeloma cell lines tested using the CD32L culture system are also shown in Appendix 2a (Figures 7.1a, 7.1b, 7.3a and 7.3b).

Note: The experiments detailed in the following two sections (6.3 and 6.3.1) were all performed with no knowledge of which myeloma cell lines, if any, would be repetitively susceptible to Fas-mediated apoptosis. The results have been split retrospectively into two sections 6.3 and 6.3.1 according to their non-susceptibility or otherwise to the onset of AICD.

6.3 CD40Lig-L culture system assays in myeloma lines non-susceptible to Fas-mediated apoptosis.

Myeloma cell lines were incubated in microtitre plates as described (6.2) for 72 hours on irradiated CD40Lig-L cells with either no additive, IL-4, IL-6 or anti-Fas MoAb. Two duplicate plates were set up per experiment. The first plate was used to assess cell proliferation through tritiated thymidine uptake (6.2d). The second plate was used to assess:

- The percentage viability by morphology (Wright's stain or Acridine Orange) and trypan blue dye exclusion.
- The percentage increase in proliferation by CD40 and cytokine stimulation or inhibition of proliferation by Fas activation (determined by cell counts).
- The extent of apoptotic cell death by flow cytometric light scatter analysis.

Results are summarised in Table 6.3, Figure 6.3 and Figures 6.3(a-c)

Figures 6.3a and 6.3b are representative photographs (JIM-1 and ANBL-6 passage 26/12, respectively) of the cell lines non-susceptible to Fas-mediated apoptosis.

Conclusion

The myeloma cell lines JIM-1, U266 (both IL-6 independent) and the IL-6-dependent passages of ANBL-6 proved consistently to be resistant to Fas-mediated apoptosis as shown by lack of inhibition of proliferation. This was indicated by:

- Tritiated thymidine uptake - no significant decrease in uptake was observed when these cell lines were incubated for 72 hours in the presence of anti-Fas MoAb. Raw data showing counts per minute due to tritiated thymidine uptake are depicted in graphical form in Figure 6.3. and are tabulated in appendix 1.
- Percentage viability by i) trypan blue dye exclusion and ii) morphology which remained normal in 85-100% of cells in spite of prolonged exposure to anti-Fas. Photographs (Figures 6.3a and 6.3b) revealed that the majority of cells were still healthy with only background signs of apoptotic cell death occurring (0-15%).

Table 6.3

Summary of CD40Lig-L culture system assays in myeloma cell lines non-susceptible to Fas-mediated apoptosis. $5x10^4$ myeloma cells were incubated in the presence of $5x10^3$ CD40Lig-L cells in triplicate wells of microtitre plates for 72hrs.

CULTURE CONDITIONS		No additive	IL-4	IL-6	Anti-Fas
CELL LINE	[1]TEST				
1) JIM-1[2]	a)Trypan blue % viability	90	91	88	86
	b)[^{3}H-TdR] % proliferation		6	5	
	% inhibition				15
	c)Morphology % viability	85	87	90	84
	d)Flow cyt. apoptosis +/-	-	-	-	-
2) U266[3]	a)Trypan blue % viability	62	83	83	57
	b)[^{3}H-TdR] % proliferation		14	16	26
	% inhibition				
	c)Morphology % viability	65	79	84	65
	d)Flow cyt. apoptosis +/-	-	-	-	-
3) ANBL-6[4]	a)Trypan blue % viability	82	79	86	78
(IL-6 dependent)	b)[^{3}H-TdR] % proliferation		15	280	
	% inhibition				8

[1] Summary of percentage viability by trypan blue dye exclusion and morphology (either by acridine orange or Wright's staining), percentage proliferation or inhibition of proliferation by tritiated thymidine uptake and cell death assessed by flow cytometric light scatter analysis.

[2] Incubation for three days with the cytokines IL-4 or IL-6 has no effect on proliferation. Incubation with anti-Fas has no significant inhibitory effect on proliferation since background apoptosis is usually 0-15%.

[3] The cytokines IL-4 and IL-6 result in a 15% increase in proliferative effect. Incubation with anti-Fas results in an increase in tritiated thymidine uptake with no decrease in cell viability observed by morphological or flow cytometric analysis.

[4] Whilst IL-4 has no proliferative effect, IL-6 stimulation results in a potent increase in proliferation. Anti-Fas has no inhibitory effect.

Figure 6.3

Tritiated thymidine uptake (cpm) in myeloma cell lines <u>non-susceptible</u> to Fas-mediated apoptosis after a 3 day incubation with various additives in the CD40Lig-L culture system.

Error bars depict the standard error of the mean (S.E.M.) of replicate experiments

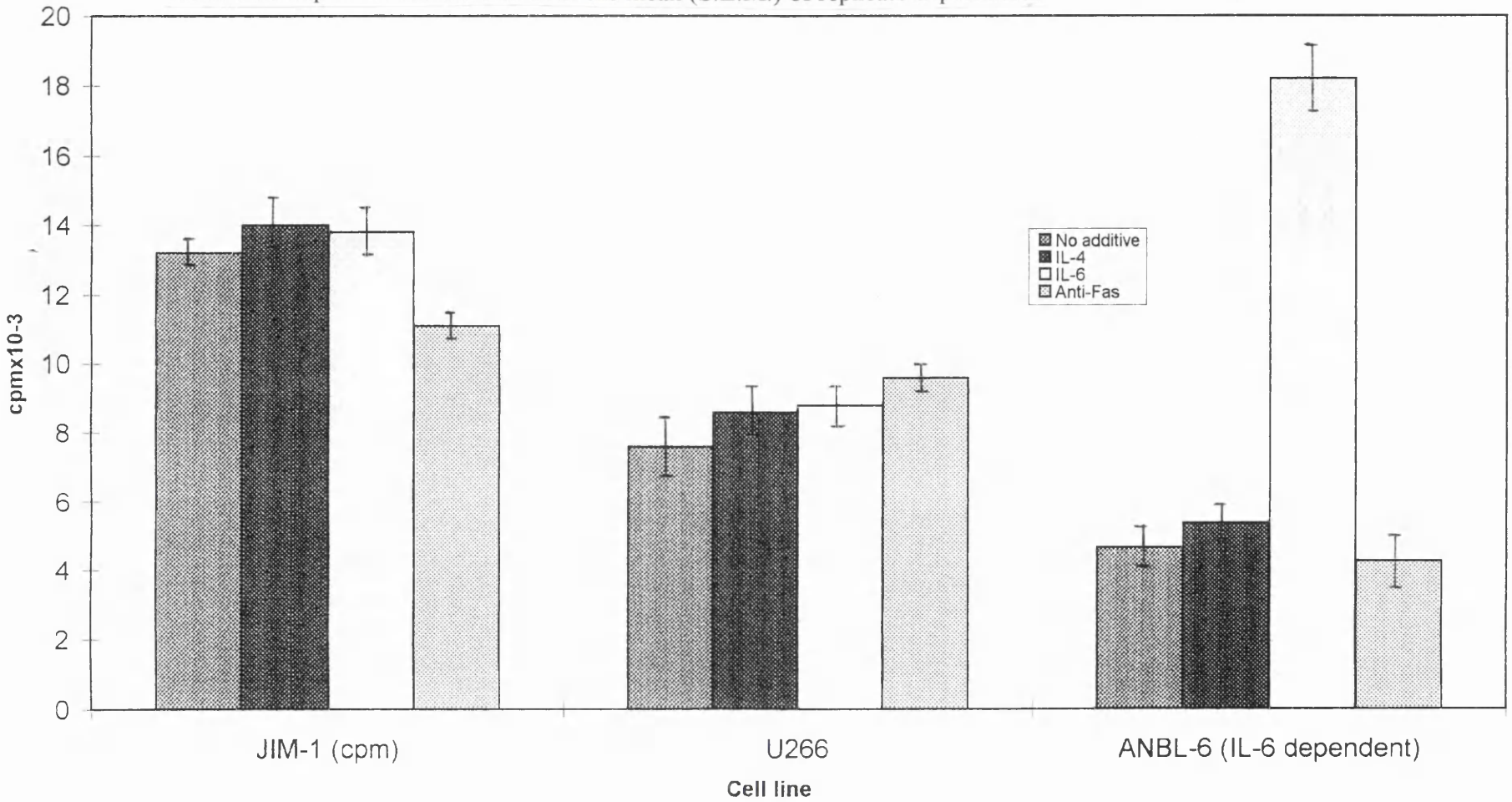
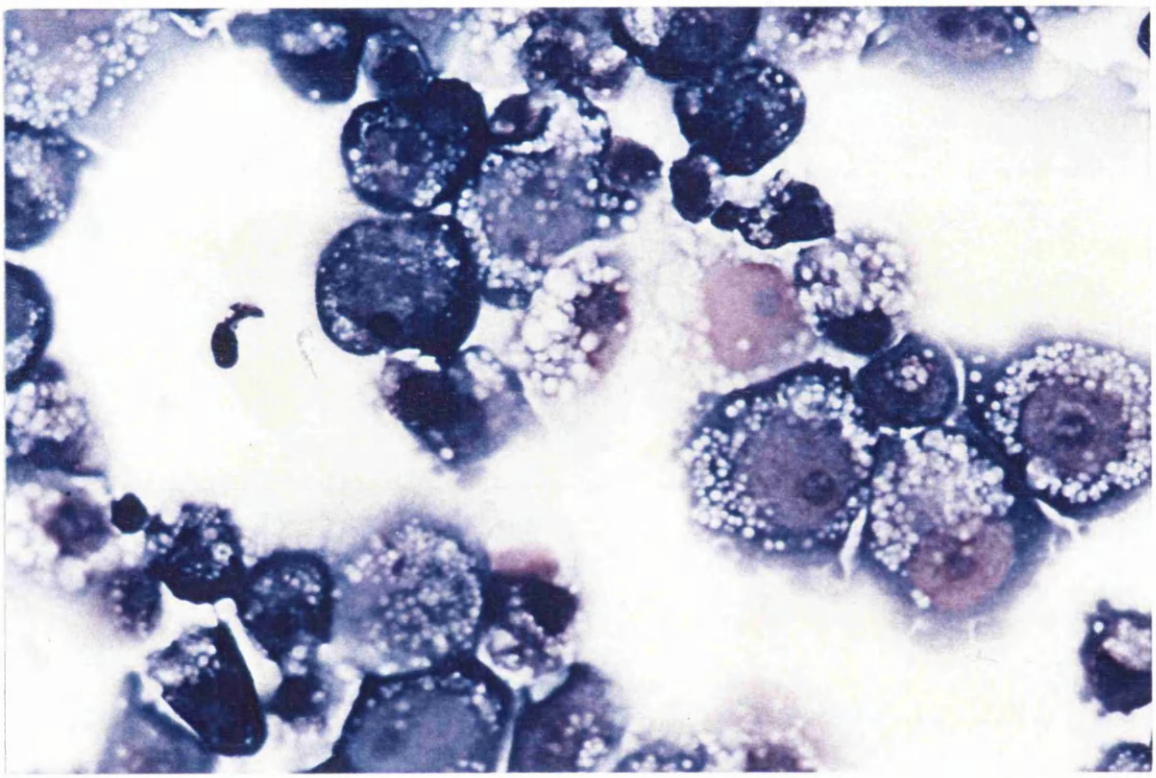


Figure 6.3a

Wright's staining of the myeloma cell line JIM-1 under light microscopy (magnification x 40) following a three day culture in the CD40LigL system with additives a) IL-6 (1ng/ml) or b) anti-Fas (100ng/ml). Only background apoptotic cell death (< 15%) was observed under both conditions.

a)



b)

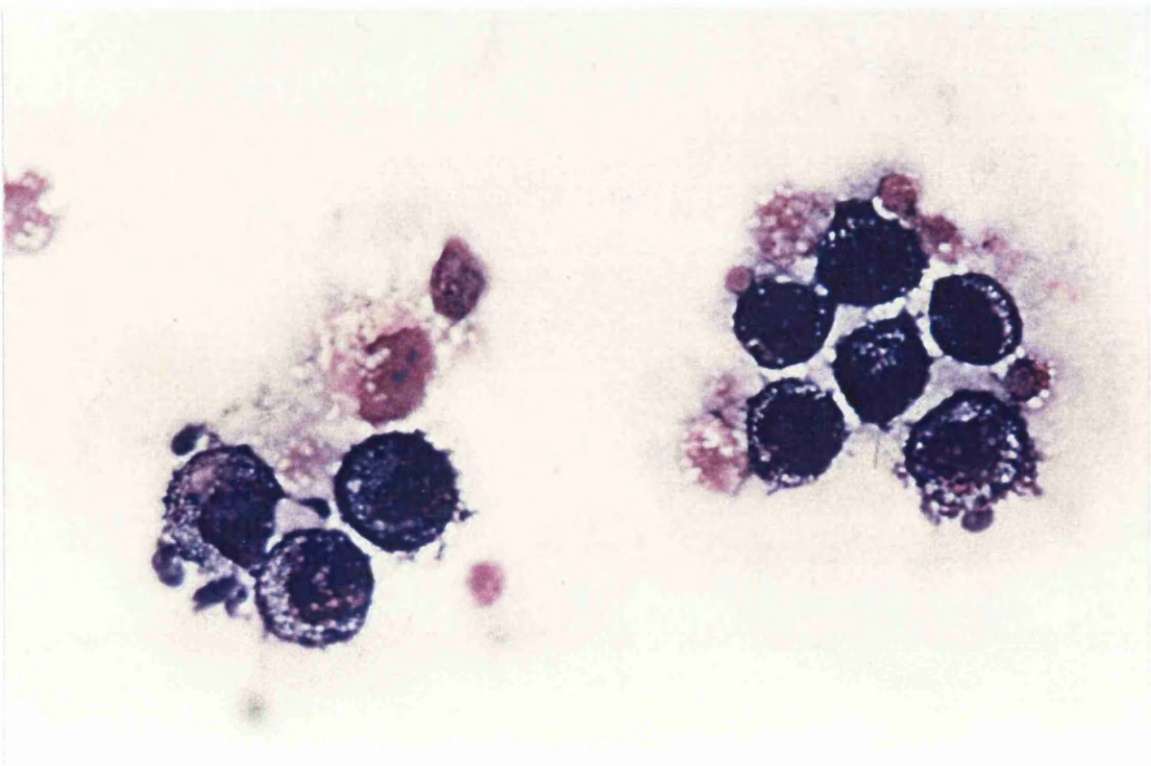
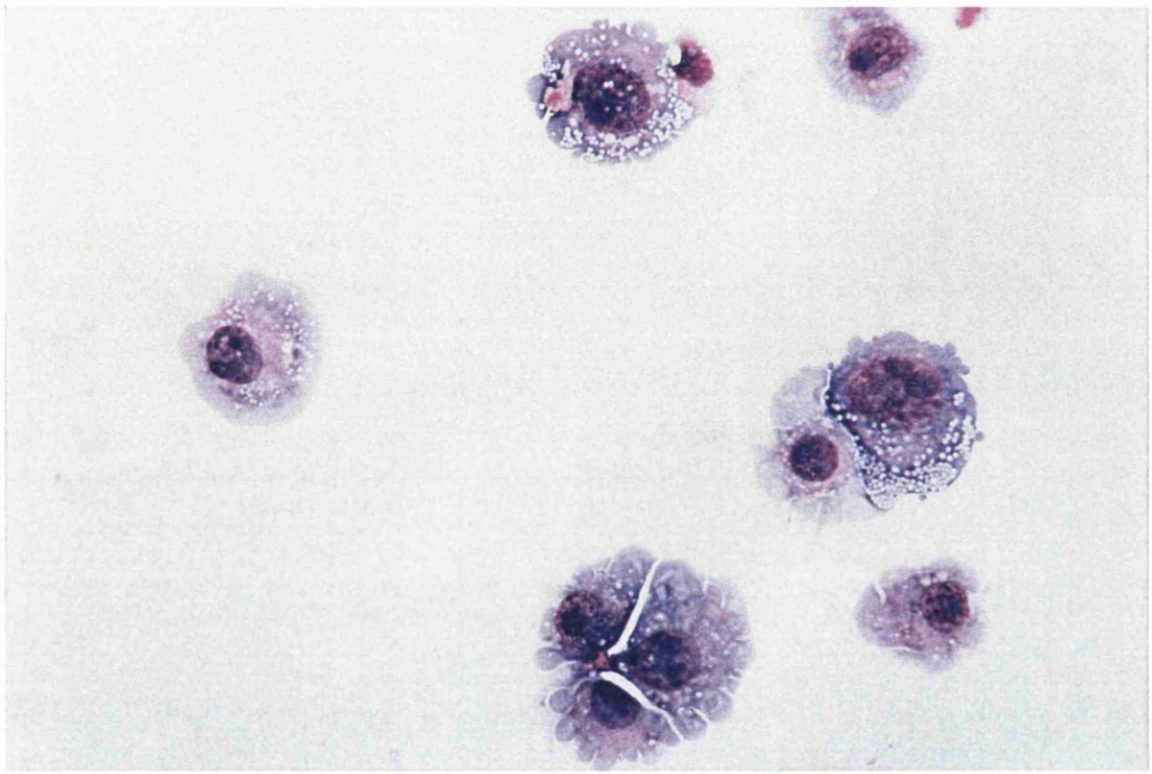


Figure 6.3b

Wright's staining of the myeloma cell line ANBL-6 (26/12, IL-6 dependent) under light microscopy (magnification x 40) following a three day culture in the CD40Lig-L system with additives a) IL-6 (1ng/ml) and b) anti-Fas (100ng/ml). only background apoptosis was observed in both cases.

a)



b)

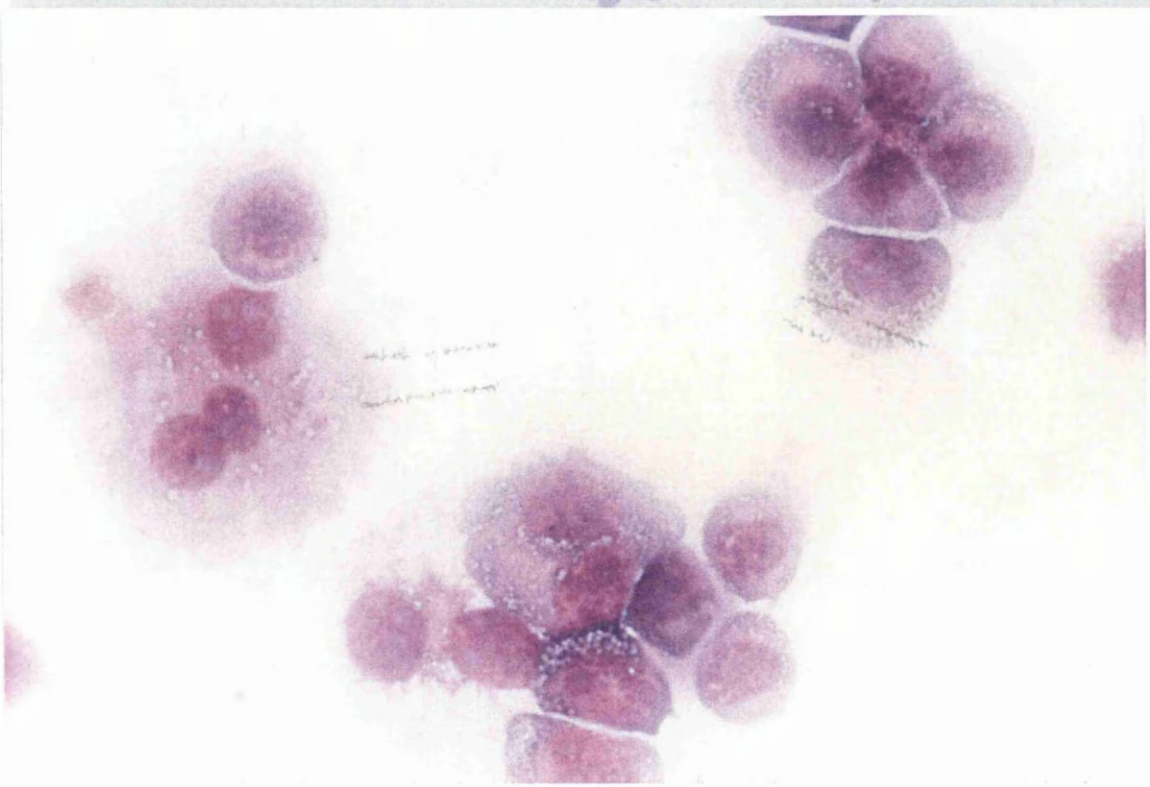
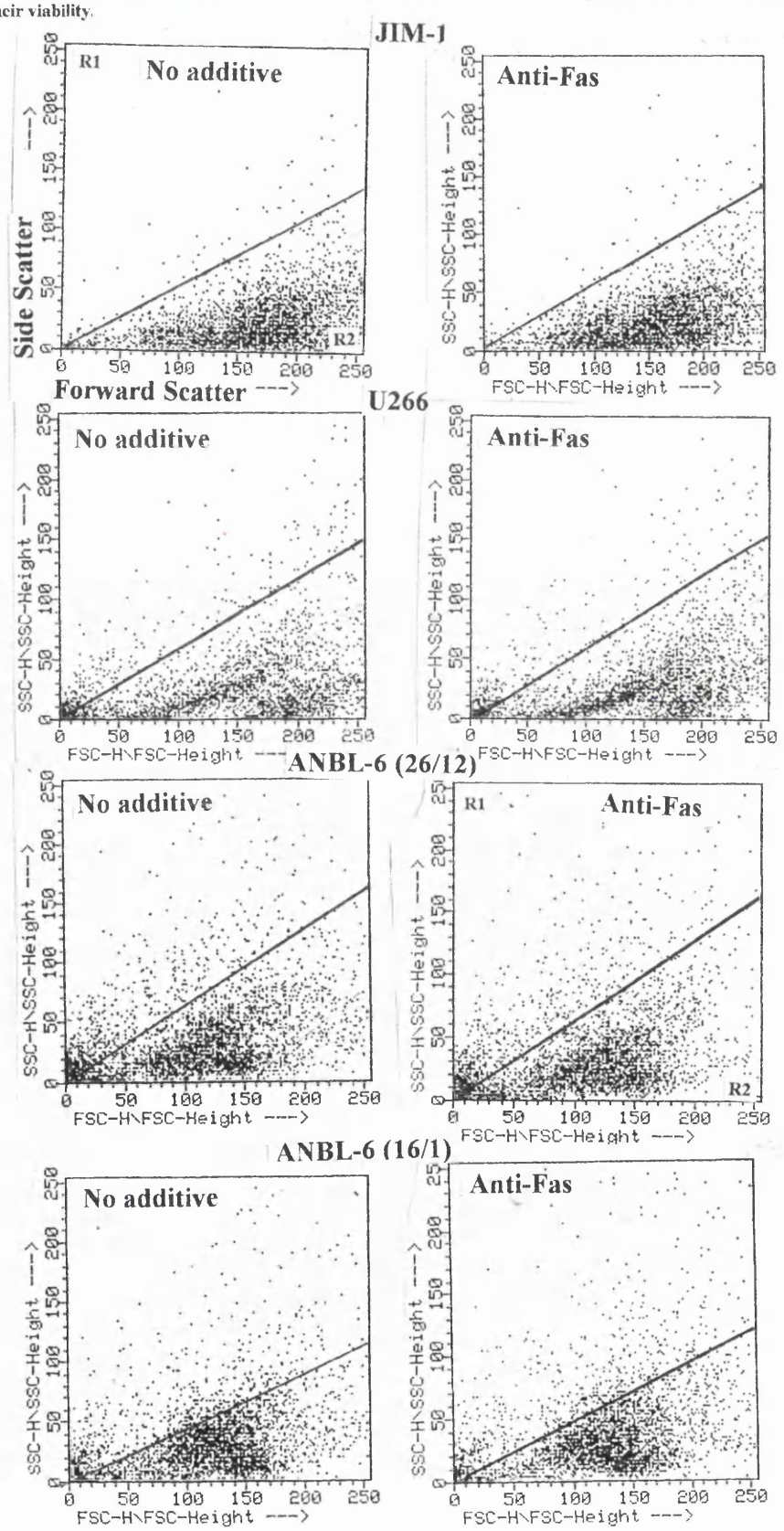


Figure 6.3c

Flow cytometric light scatter analysis of the myeloma cell lines JIM-1, U266, ANBL-6 (passages 26/12 and 16/1) cultured in the CD40Lig-L system following a 3 day incubation with either no additive, IL-4, IL-6 or anti-Fas MoAb. Results for cytokine stimulation are not shown since they were the same as those for cells incubated with no additive. An arbritary axis was drawn to distinguish between live (R2) and apoptotic (R1) cells. 4000 cells per experiment were analysed. No shift in scatter was observed in any of these cell lines indicating that Fas activation had no effect on their viability.



- As demonstrated by flow-cytometric light scatter analysis (Figure 6.3c) where no shift in scatter was observed between cells incubated with no additive or anti-Fas. There was no shift in scatter in any of the cell lines tested with either of the cytokines IL-4 or IL-6 compared with cells incubated in the same experiment with no additive (results not shown).

The original clone of ANBL-6, P32, which is IL-6 dependent, was also cultured using the CD40Lig-L and CD32L systems with additives (IL-6+anti-Fas) and (IL-6+anti-CD40+anti-Fas) respectively. Tritiated thymidine uptake and percentage viability was found to be as great as, or greater than, that observed when these cells were incubated with IL-6±CD40 ligation (Appendix 1.12). This indicates that, certainly in this IL-6-dependent cell line, activation of Fas antigen has no apoptotic effect either in the presence or absence of IL-6.

6.3.1 CD40Lig-L culture system assays in myeloma lines <u>susceptible</u> to Fas-mediated apoptosis.

Duplicate microtitre plates were set up with the myeloma cell lines JIM-3, JJN-3, IM-9, RPMI-8226 and the IL-6 independent passages of ANBL-6 (17/2, 27/2, 6/3, 22/5). These were incubated for 72 hours and harvested as described in section 6.3

<u>Results are summarised in Table 6.3.1 and Figures 6.3.1(a-e)</u>

Figures 6.3.1b and 6.3.1c are representative photographs (IM-9 and JJN-3 respectively) of the cell lines susceptible to Fas-mediated apoptosis.

<u>Conclusion</u>

The myeloma cell lines JIM-3, JJN-3, RPMI-8226, IM-9 and the IL-6 independent passages of ANBL-6 proved to be consistently susceptible to Fas-mediated apoptosis as shown by inhibition of proliferation indicated by:

- Tritiated thymidine uptake - a significant decrease in thymidine uptake was observed when cells were incubated for 72 hours in the presence of anti-Fas MoAb. Results are shown as percentage inhibition of proliferation compared with thymidine uptake in wells

Table 6.3.1

Summary of CD40Lig-L culture system assays in myeloma cell lines susceptible to Fas-mediated apoptosis.

$5x10^4$ myeloma cells were incubated in the presence of $5x10^3$ CD40Lig-L cells in triplicate wells of microtitre plates for 72hrs.

CULTURE CONDITIONS		No additive	IL-4	IL-6	[2]Anti-Fas
CELL LINE	[1]TEST				
1) JIM-3	a)Trypan blue % viability	79	81	86	0
	b)[^{3}H-TdR] % proliferation		0	13	
	uptake % inhibition				38
	c)Morphology % viability	75	79	89	10
	d)Flow cyt. [3]apoptosis +/-	-	-	-	++
2) JJN-3	a)Trypan blue % viability	82	84	92	30
	b)[^{3}H-TdR] % proliferation		0	0	
	uptake % inhibition				22
	c)Morphology % viability	78	76	82	15
	d)Flow cyt. apoptosis +/-	-	-	-	+
3) RPMI-8226	a)Trypan blue % viability	92	95	91	20
	b)[^{3}H-TdR] % proliferation		7	45	
	uptake % inhibition				47
	c)Morphology % viability	84	92	90	16
	d)Flow cyt. apoptosis +/-	-	-	-	+
4) IM-9	a)Trypan blue % viability	95	94	94	6
	b)[^{3}H-TdR] % proliferation		0	2	
	uptake % inhibition				92
	c)Morphology % viability	91	96	94	6
	d)Flow cyt. apoptosis +/-	-	-	-	++
5) ANBL-6	a)Trypan blue % viability	89	90	93	0
(IL-6 independent)	b)[^{3}H-TdR] % proliferation		0	0	
	uptake % inhibition				90
	c)Morphology % viability	92	97	86	5
	d)Flow cyt. apoptosis +/-	-	-	-	++

[1] Summary of percentage viability by trypan blue dye exclusion and morphology (either by acridine orange or Wright's staining), percentage proliferation or inhibition of proliferation by tritiated thymidine uptake and cell death assessed by flow cytometric light scatter analysis.

[2] None of these cell lines showed a marked increase in proliferation when incubated with either IL-4 or IL-6 compared to those incubated with no additives.All cell lines were inhibited (22-92%) when incubated in the presence of anti-Fas as observed by a decrease in viability and thymidine uptake and observation of flow cytometric analysis. Decreased uptake of thymidine was not always indicative of apoptosis as seen by the morphological picture (see discussion, chapter 7)

[3] Scale - to ++ indicates extent of deviation in light scatter observed by cells treated with various additives (see Figures 6.3.1d and 6.3.1e).

Figure 6.3.1a

Tritiated thymidine uptake in myeloma cell lines susceptible to Fas-mediated apoptosis after a 3 day incubation with various additives in the CD40Lig-L culture system.

*The cpm for IM-9 are x10-4, (not x10-3) in order to fit results into scale.

Error bars depict the standard error of the mean (S.E.M.) of replicate experiments

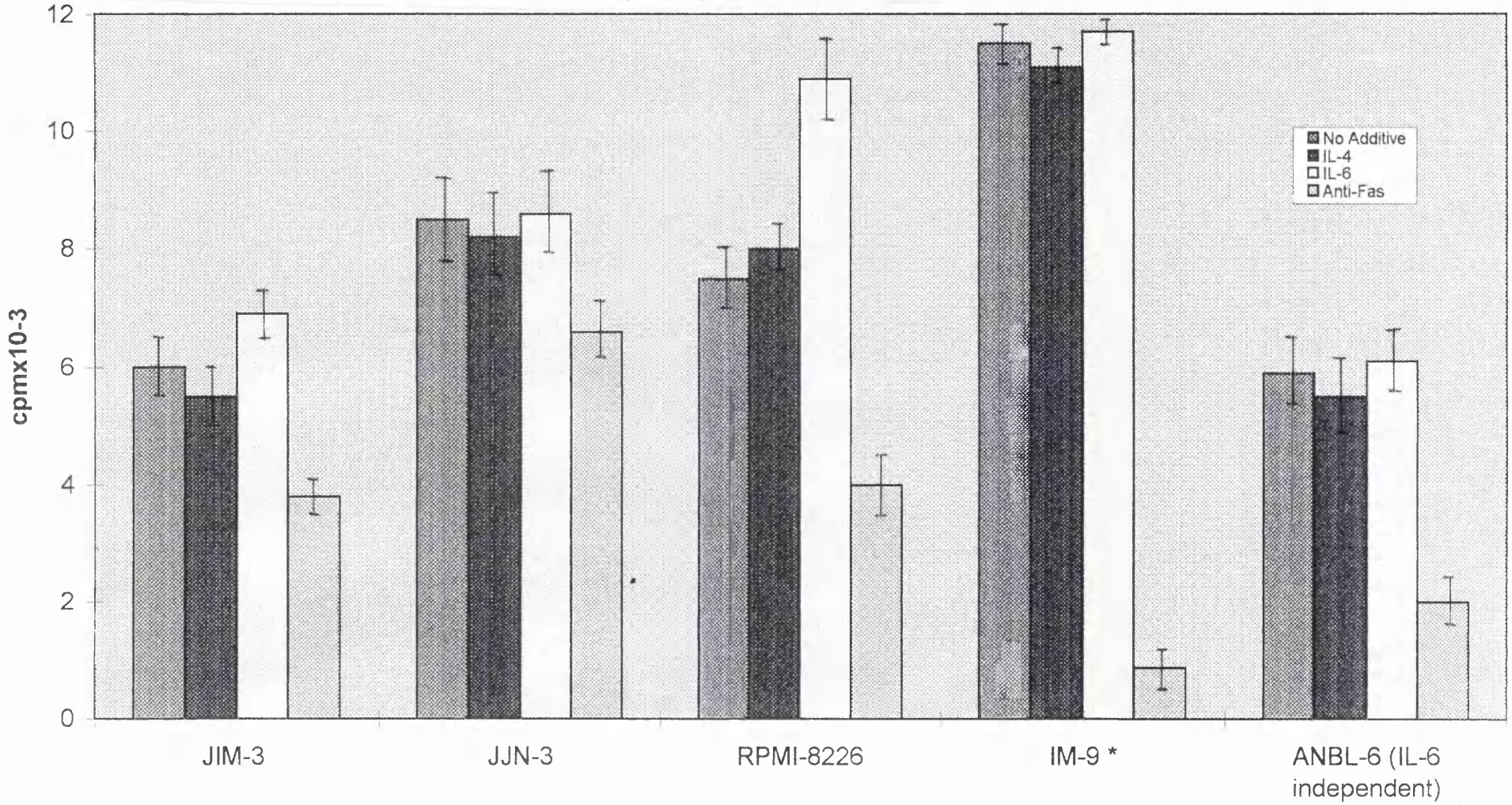
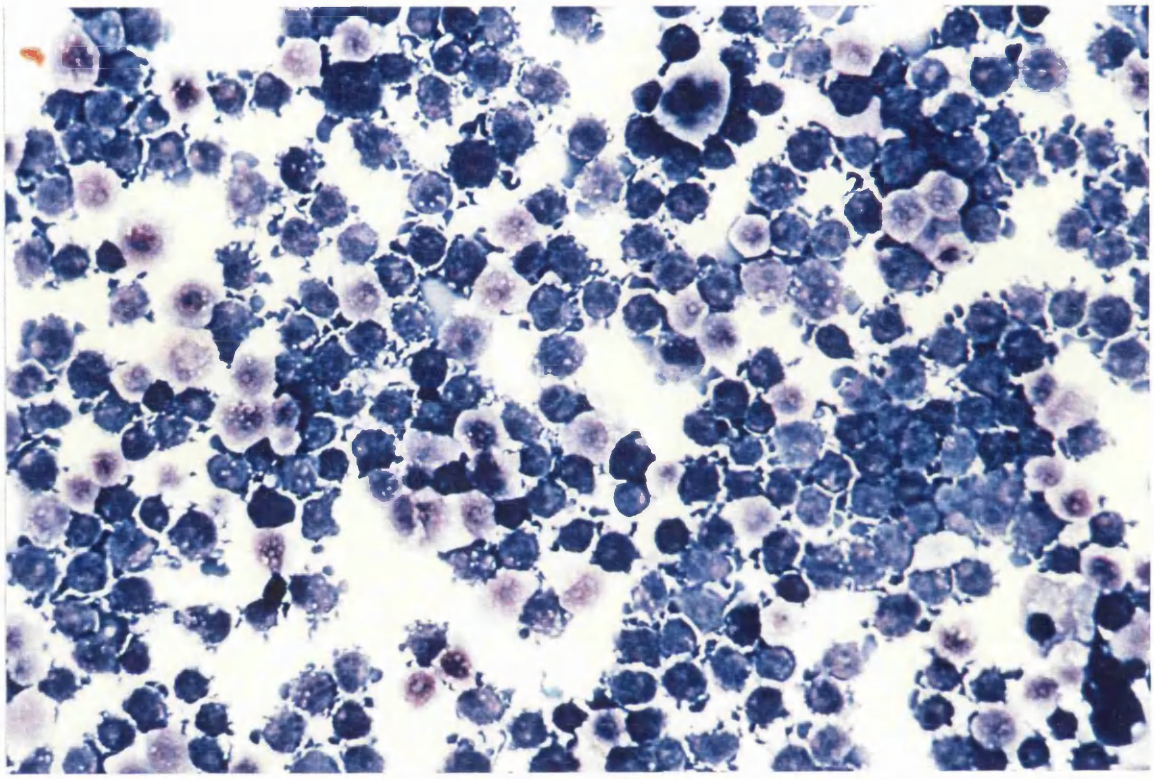


Figure 6.3.1b

Wright's staining of the myeloma cell line IM-9 under light microscopy (magnification x 40) following a three day culture in the CD40LigL system with additives a) IL-6 (1ng/ml) or b) anti-Fas (100ng/ml). Most of the Fas activated cells are dead by apoptosis as seen by their shrunken size, membrane blebbing and condensed chromatin.

a)



b)

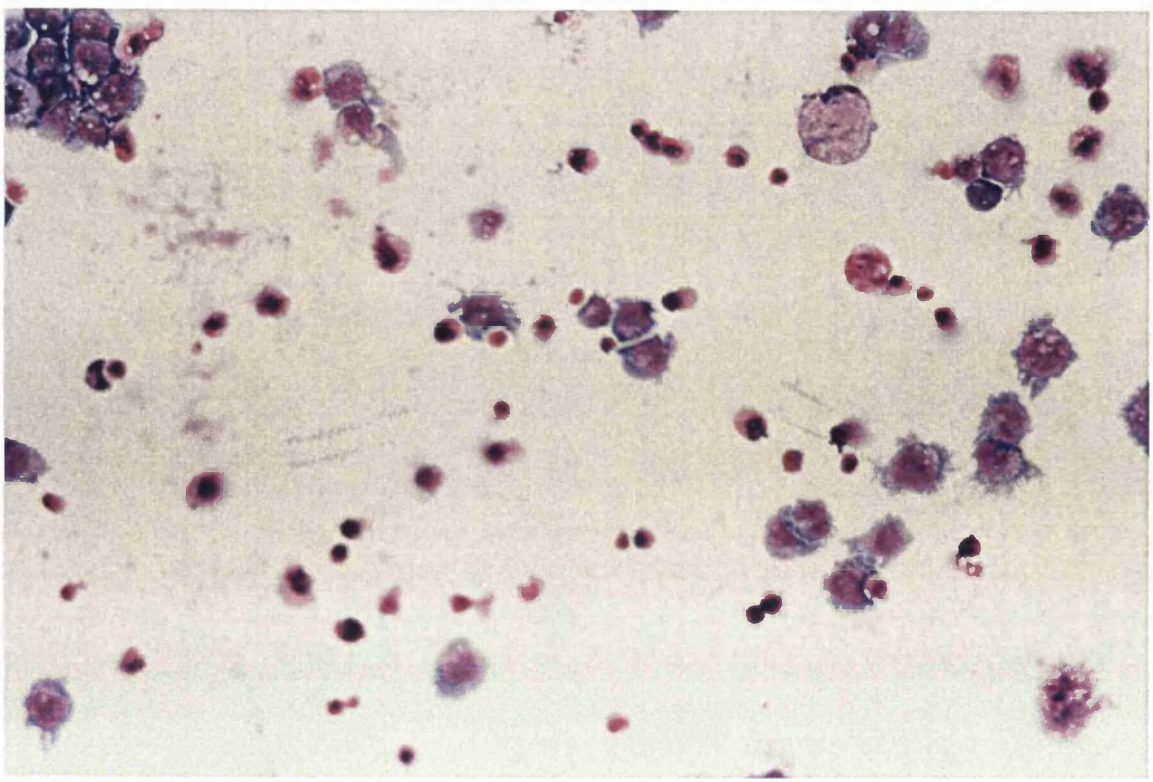
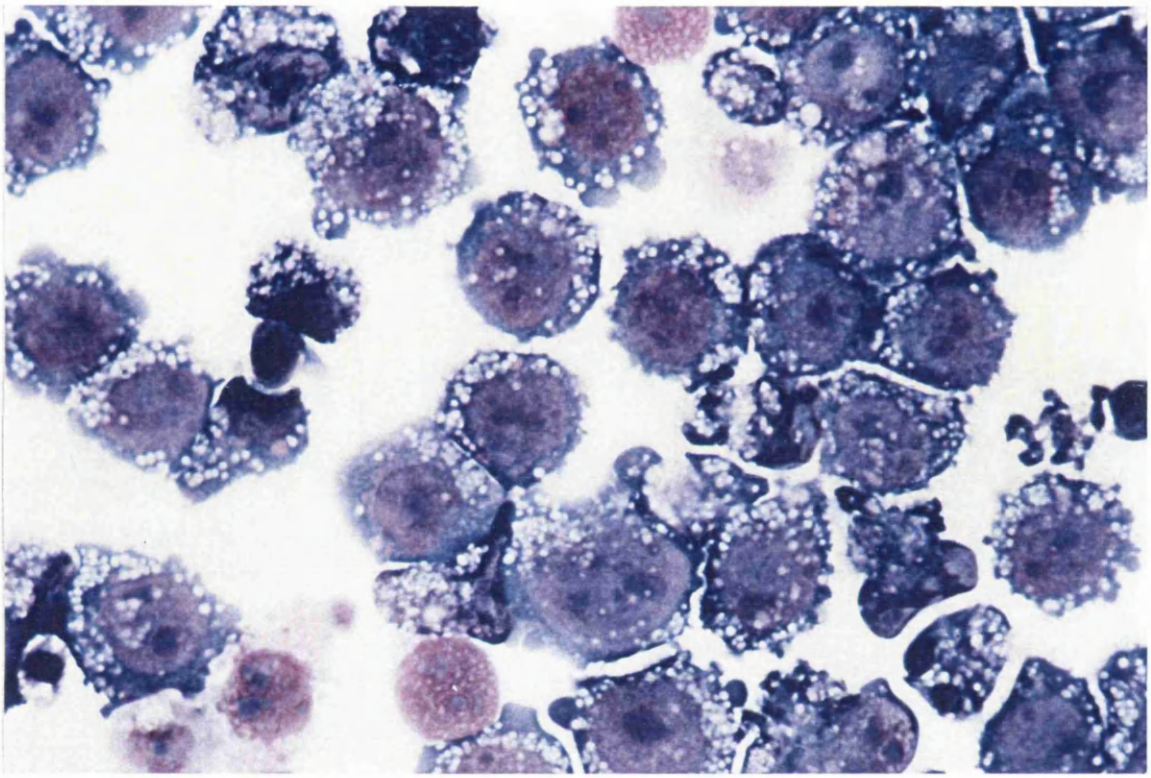


Figure 6.3.1c

Wright's staining of the myeloma cell line JJN-3 under light microscopy (magnification x 40) following a three day culture in the CD40Lig-L system with additives a) IL-4 (100U/ml) or b) anti-Fas (100ng/ml). Virtually all the Fas-activated cells are dead.

a)



b)

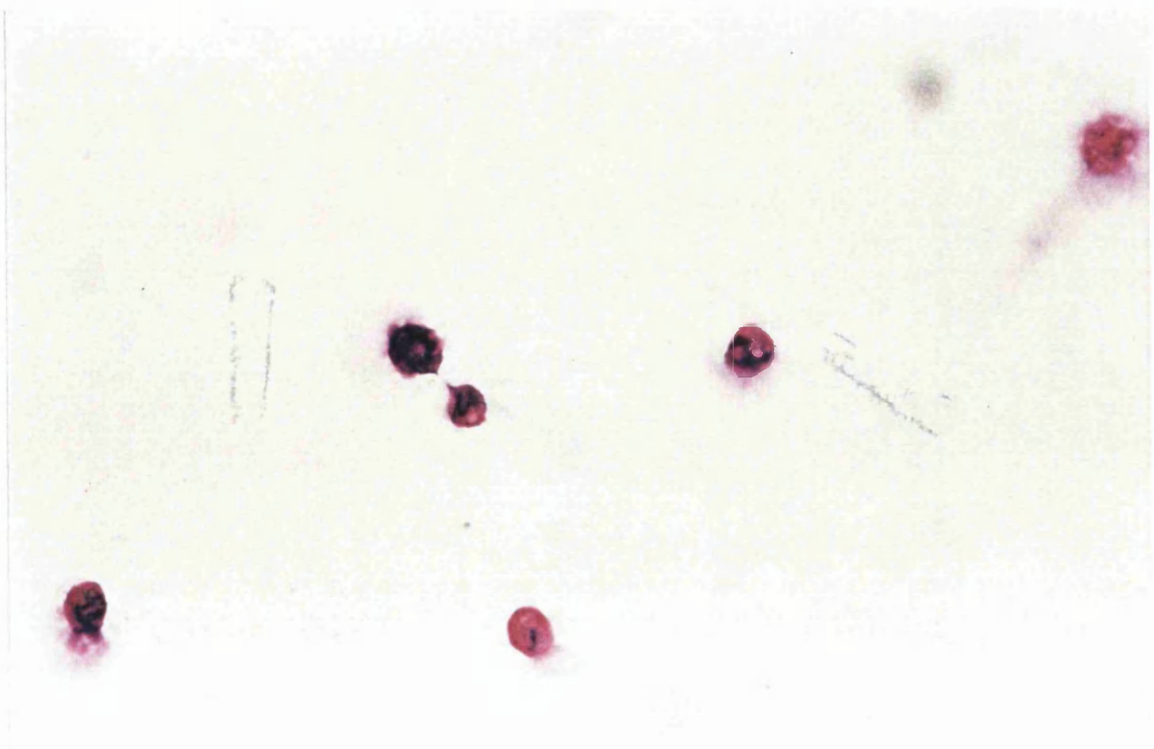


Figure 6.3.1d

Flow cytometric light scatter analysis of the myeloma cell lines JIM-3, JJN-3, IM-9, and RPMI-8226 cultured in the CD40Lig-L system following a 3 day incubation with either no additive, IL-4, IL-6 or anti-Fas MoAb. Results for cytokine stimulation are not shown since they were the same as those for cells incubated with no additive. An arbritary axis was drawn to distinguish between live (R2) and apoptotic (R1) cells. 4000 cells per experiment were analysed. A major shift in scatter, indicative of an increase in cell granularity and a decrease in cell size, was observed in all of these cell lines indicating that Fas activation had a potent apoptotic effect.

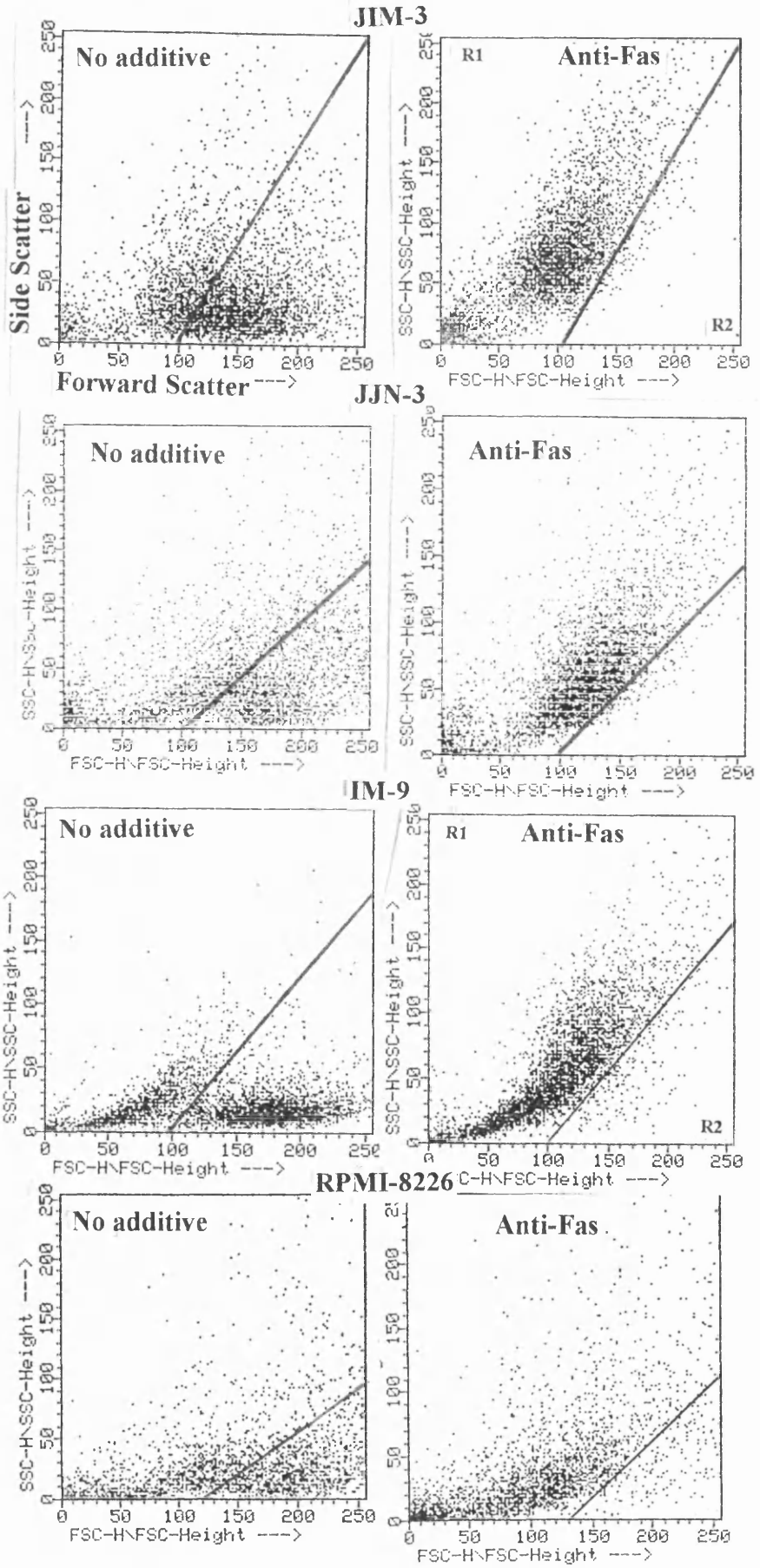
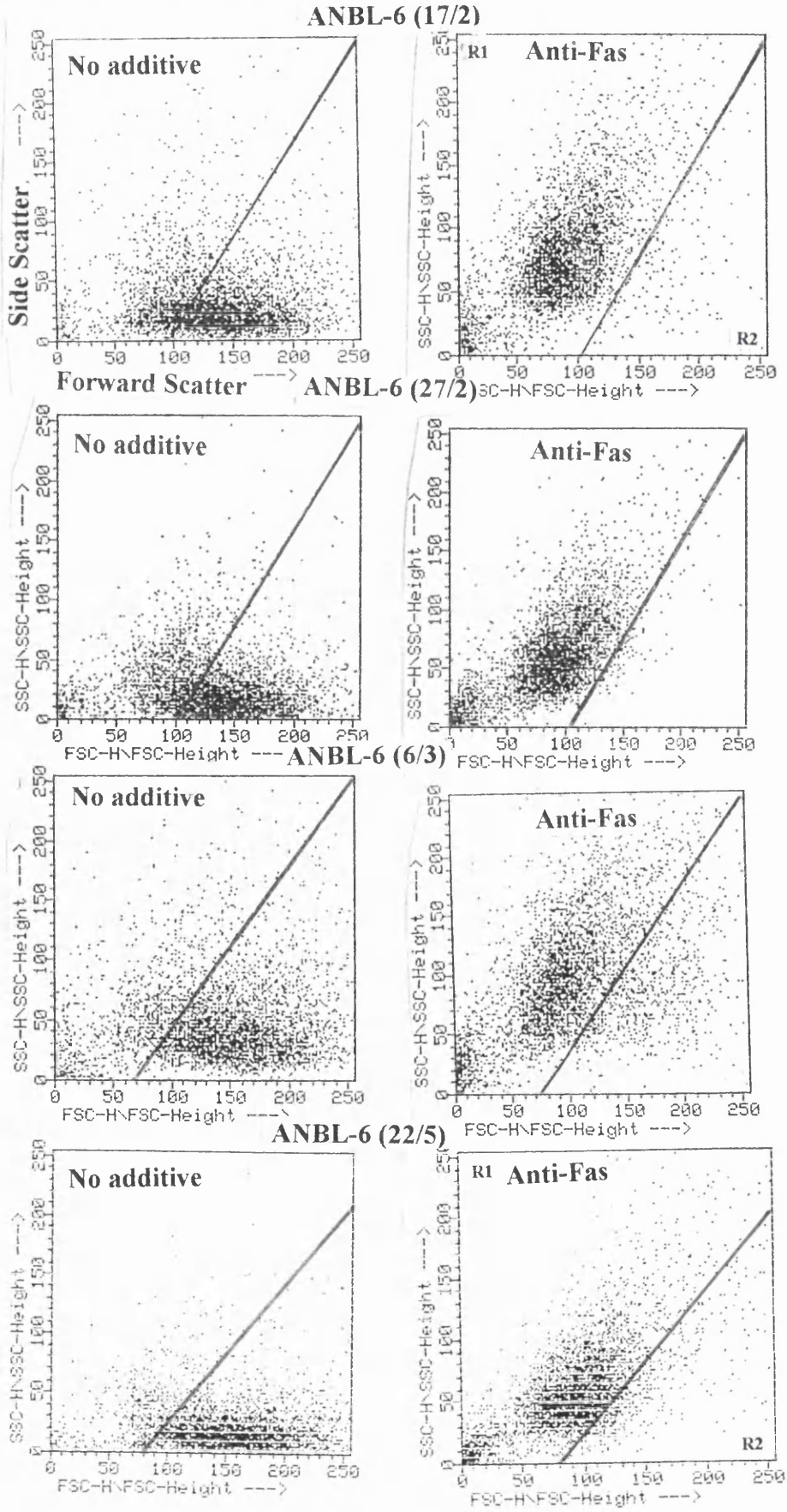


Figure 6.3.1e

Flow cytometric light scatter analysis of the IL-6 independent passages of ANBL-6 (17/2,27/2,6/3 and 22/5) cultured in the CD40Lig-L system following a 3 day incubation with either no additive, IL-4, IL-6 or anti-Fas MoAb. Results for cytokine stimulation are not shown since they were the same as those for cells incubated with no additive. An arbritary axis was drawn to distinguish between live (R2) and apoptotic (R1) cells. 4000 cells per experiment were analysed. A major shift in scatter, indicative of an increase in cell granularity and a decrease in cell size, was observed in all of these cell lines indicating that Fas activation had a potent apoptotic effect.



containing no additive. Raw data showing counts per minute due to tritiated thymidine uptake are depicted in graphical form in Figure 6.3.1a and are tabulated in appendix 1.

- A decrease in cell count and percentage viabilty by trypan blue dye exclusion and morphology following prolonged exposure to anti-Fas. Representative photographs of IM-9 and JJN-3 (Figures 6.3.1b and 6.3.1c) were consistent with the fact that the majority of cells incubated with anti-Fas were dying by apoptosis characterised by specific morphological changes that include shrinkage of the cells, nuclear condensation and condensation of chromatin and cytoplasmic organelle compaction.
- Flow-cytometric light scatter analysis (Figures 6.3.1d and 6.3.1e). A complete shift in scatter was observed in cells incubated with anti-Fas compared to those incubated with either no additive, IL-6 or IL-4 (analysis for cells incubated with IL-4 and IL-6 are not shown but were the same as that for cells incubated with no additive). This indicates that the cells incubated with anti-Fas had a marked increase in granularity (increased side scatter) and decrease in size (decreased forward scatter) characteristic of apoptotic cell death.

6.3.2 Summary of results for all myeloma cell lines cultured in the CD40Lig-L system (and CD40 system - see Appendix 1).

A summary of the results in sections 6.3 and 6.3.1 showing the percentage viability and extent of apoptosis following three days culture in the CD40Lig-L system of all the myeloma cell lines tested is shown in table 6.3.2.

Results Table 6.3.2

Conclusion

Both the CD40- (see Appendix 1) and CD40LigL- culture systems were found to be effective in potentiating CD40 activation in myeloma cell lines with a synergistic increase in proliferation achieved by the addition of either IL-4 or IL-6. IL-6 had the greatest proliferative effect on the IL-6-dependent passages of ANBL-6 as demonstrated by a marked increase in tritiated thymidine uptake.

Not all the myeloma cell lines tested were found to be susceptible to Fas-mediated apoptosis. All of the IL-6 dependent lines tested - passages 26/12, 16/1 and the original

Table 6.3.2

Summary of percentage viability and degree of apoptosis in myeloma cell lines following 3 days culture in the CD40LigL system

CULTURE CONDITIONS		No additive	IL-4	IL-6	Anti-Fas
CELL LINE	[1]**TEST**				
1) JIM-1	% viability	90	91	88	86
	[2]apoptosis +/-	-	-	-	-
2) JIM-3	% viability	79	81	86	0
	apoptosis +/-	-	-	-	++
3) JJN-3	% viability	82	84	92	30
	apoptosis +/-	-	-	-	+
4) U266	% viability	62	83	83	57
	apoptosis +/-	-	-	-	-
5) IM-9	% viability	95	94	94	6
	apoptosis +/-	-	-	-	++
6) RPMI-8226	% viability	92	95	91	20
	apoptosis +/-	-	-	-	+
7) ANBL-6	% viability	89	90	93	0
(IL-6 independent)	apoptosis +/-	-	-	-	++
8) ANBL-6	% viability	82	79	86	78
(IL-6 dependent)	apoptosis +/-	-	-	-	-
9) [3]ANBL-6 'P32'	% viability	85	82	87	73
	apoptosis +/-	-	-	-	-
10) [4]JT	% viability	84	80	80	77
	apoptosis +/-	-	-	-	-

[1] Summary of percentage viability by trypan blue dye exclusion and cell death assessed by a combination of flow cytometric light scatter analysis, morphological changes and tritiated thymidine uptake.

[2] Scale - to ++ indicates extent of deviation in light scatter observed by cells treated with various additives (see Figures 6.3.1d and 6.3.1e).
For discussion of results - see text.

[3] P32 is the original IL-6 dependent clone of ANBL-6 kindly donated by Dr D.F Jelinek (Mayo Clinic, Rochester, Minnesota) which was reported to be non-susceptible to Fas-mediated apoptosis.

[4] JT is an IL-6 dependent myeloma cell line recently established by MMD (unpublished).

clone of ANBL-6 (P32) as well as JT which is an IL-6 dependent myeloma line developed by myself from the peripheral blood of a patient with plasma cell leukaemia - were found to be non-susceptible to activation induced cell death (AICD). The results for P32 and JT are shown in appendix 1. The IL-6 independent myeloma cell lines U266 and JIM-1 were also resistant which is interesting since the subclone of JIM-1, JIM-3 is susceptible to Fas-mediated apoptosis. The cell lines JJN-3, IM-9, RPMI-8226 and all IL-6 independent subclones of ANBL-6 tested (passages 17/2, 27/2, 6/3 and 22/5) were susceptible to AICD. JJN-3 and RPMI-8226 exhibited the least marked apoptotic response as observed by flow cytometric light scatter analysis and the fact that 20-30% of the cells were still viable following a three day incubation with anti-Fas. It was decided therefore, to investigate the levels of CD40 and Fas antigen expression in all these cell lines by flow cytometric analysis both in normal culture conditions and following a three day culture in the CD40Lig-L system with either no additive, IL-6 or anti-Fas MoAb. Data were not collected for IL-4 stimulated cells due to financial shortages. It is worth noting that the intensity of Fas and CD40 expression in the cell lines grown under normal culture conditions will differ from that expressed in cell lines grown for three days in the CD40Lig-L culture system with no additives since the latter have obviously undergone CD40 activation during this period.

6.4 CD40 and Fas protein expression following three days culture in the CD40Lig-L culture system by flow cytometric analysis.

Baseline protein expression of CD40 and Fas antigen in unstimulated myeloma cell lines was assessed by performing at least three replicate experiments and the results were compared to antigen expression following a three day culture using the CD40LigL system. Flow cytometric analysis was as described in section 5.3.

Results Table 6.4a and Table 6.4b

Conclusion

Expression of the cell surface antigens CD40 and Fas amongst the eight myeloma cell lines examined in this study was found to range from negative (2-25%) to 100% positive prior to activation of the cells (Table 6.4a). The ensuing culture techniques then enabled the effect

Table 6.4a

Fas and CD40 protein expression amongst myeloma cell lines

Cell LIne	FACS expression % positive (range) Fas	CD40
JIM-1	15 (2-23)	15 (4-25)
JIM-3	66 (30-83)	13 (10-16)
JJN-3	82 (70-94)	29 (21-34)
U266	91 (88-93)	17 (7-28)
IM-9	100	100
RPMI-8226	79 (56-88)	94 (93-94)
ANBL-6 (26/12)	40 (21-58)	70 (59-81)
ANBL-6 (16/1)	40 (38-42)	36 (11-60)
ANBL-6 (17/2)	64 (43-84)	10 (9-10)
ANBL-6 (6/3)	51 (33-68)	11 (8-14)
ANBL-6 (22.5)	58 (39-90)	10 (3-14)
ANBL-6 (27/2)	91 (84-97)	26 (9-43)
JT	66 (40-100)	51 (10-96)
ANBL-6 (P32)	44	80

Table 6.4b

Summary of Fas and CD40 protein expression after 3 days in culture in the CD40LigL system.
(Average values after triplcate experiments, range +/- 10%).

Culture conditions	No additive		IL-6		anti-Fas	
Protein expression (FACS)	Fas	CD40	Fas	CD40	Fas	CD40
	% Positive		% Positive		% Positive	
Cell Line						
JIM-1	20	66	15	58	22	59
U266	60	18	70	26	30	17
ANBL-6 (26/12) [4]	32	34	16	31	24	29
ANBL-6 (16/1) [5]	63	64	63	60	49	27
IM-9	97	100	98	100	76	100
RPMI-8226	95	88	93	86	85	52
JIM-3	21	20	61	12	93	82
JJN-3	93	57	92	46	79	41
ANBL-6 (17/2) [1]	88	10	88	11	97	93
ANBL-6 (6/3) [2]	64	10	60	11	69	58
ANBL-6 (27/2) [3]	42	5	54	6	73	51

ANBL-6(1-3), IL-6 independent passages
ANBL-6 (4,5) IL-6 dependent passages

Cell lines not underlined are susceptible to Fas-mediated apoptosis.
Cell lines underlined are not susceptible to Fas-mediated apoptosis

of activation of these antigens on antigen expression, proliferation and induction of activation induced cell death to be established.

FACS analysis (Table 6.4b) of these lines following a three day incubation in the CD40Lig-L system with either no additive, IL-6 or anti-Fas demonstrated that, in those cell lines non-susceptible to Fas-mediated apoptosis, Fas protein expression was either low initially (no additive, 20-32%) and remained low following incubation with anti-Fas (22-24%) or was initially moderately high (no additive, 60-63%) and was down-regulated (30-49%) after incubation with anti-Fas. Similarly with CD40 antigen expression amongst these lines expression was either low (no additive, 18-34%) and remained low after stimulation with anti-Fas (17-29%) or was initially moderate (64-66%, no additive) and expression remained the same or was down-regulated following Fas activation (27-59%).

In contrast, in those cell lines susceptible to Fas-mediated apoptosis stimulation with anti-Fas antibody resulted in a marked up-regulation of CD40 and Fas protein expression unless their levels of expression were almost 100% before the introduction of additives, in which case expression remained high. An exception to this was that CD40 expression in JJN-3 and RPMI-8226 after incubation with anti-Fas was only moderate compared with pre-stimulation in these two cell lines and it is interesting to note that they also have the least marked apoptotic response of all the lines tested for their susceptibility to Fas-mediated apoptosis. Levels of Fas and CD40 expression following IL-6 stimulation were similar to those in unstimulated cells in both those lines susceptible as well as non-susceptible to apoptosis (Table 6.4b). It is therefore postulated from these results that a high level of expression of both cell surface CD40 and Fas is required for a cell to be susceptible to the onset of AICD with a high level of Fas alone being insufficient to initiate the process. It could be that Fas activation *in vivo* is mediated through CD40. Fas and CD40 are both members of the TNF receptor family and share considerable homology with each other, as do their ligands which are both members of the TNF family.

6.5 Transcription of CD40 and *fas* genes in myeloma cell lines tested by ISH following a three day culture in the CD40 system. Comparison with RT-PCR results.

As a comparison with CD40 and Fas protein expression by flow cytometric analysis, the RNA expression of these genes was assessed by *in situ* hybridisation as described in section

2.2.6 using CD40- and *fas*- sense and antisense riboprobes. Baseline expression of *fas* and CD40 mRNA by RT-PCR in myeloma lines is shown in section 2.2.11. All myeloma cell lines tested were positive by RT-PCR for *fas* mRNA expression using one set of sequence specific primers and all were also positive for CD40 mRNA expression after nested PCR, ie using two sets of sequence specific primers.

Myeloma cells, having been cultured in the CD40 system with or without various additives (IL-4, IL-6, anti-CD40±cytokines and anti-Fas±anti-CD40) were tested for CD40 and *fas* mRNA expression by ISH, and for any noticable changes in expression due to the presence of additive. All myeloma cell lines were found to express CD40 mRNA with the strongest intensity observed in the IM-9, RPMI-8226 and IL-6 dependent cell lines (RK, JT, ANBL-6 passages 26/12, 16/1 and P32) in unstimulated cells which is consistent with these lines exhibiting the highest protein expression of CD40 (Table 6.4a). Intensity of expression was stronger following incubation with anti-CD40 ± (IL-4 or IL-6) in all lines tested, with CD40 expression, but no change in intensity, observed after incubation with anti-Fas MoAb ± anti-CD40. *Fas* mRNA expression was observed by ISH also in all cell lines tested with the weakest intensity amongst JIM-1 and the IL-6-dependent passages of ANBL-6. No change in expression was noticed following incubation with anti-CD40 ± cytokines but an increase in intensity was observed following Fas activation ± anti-CD40. Representative results demonstrating CD40 and *fas in situ* hybridisation for the cell lines IM-9, U266, RPMI-8226 and ANBL-6 (22/5), IL-6 independent, are shown in Figures 6.5 (a-e).

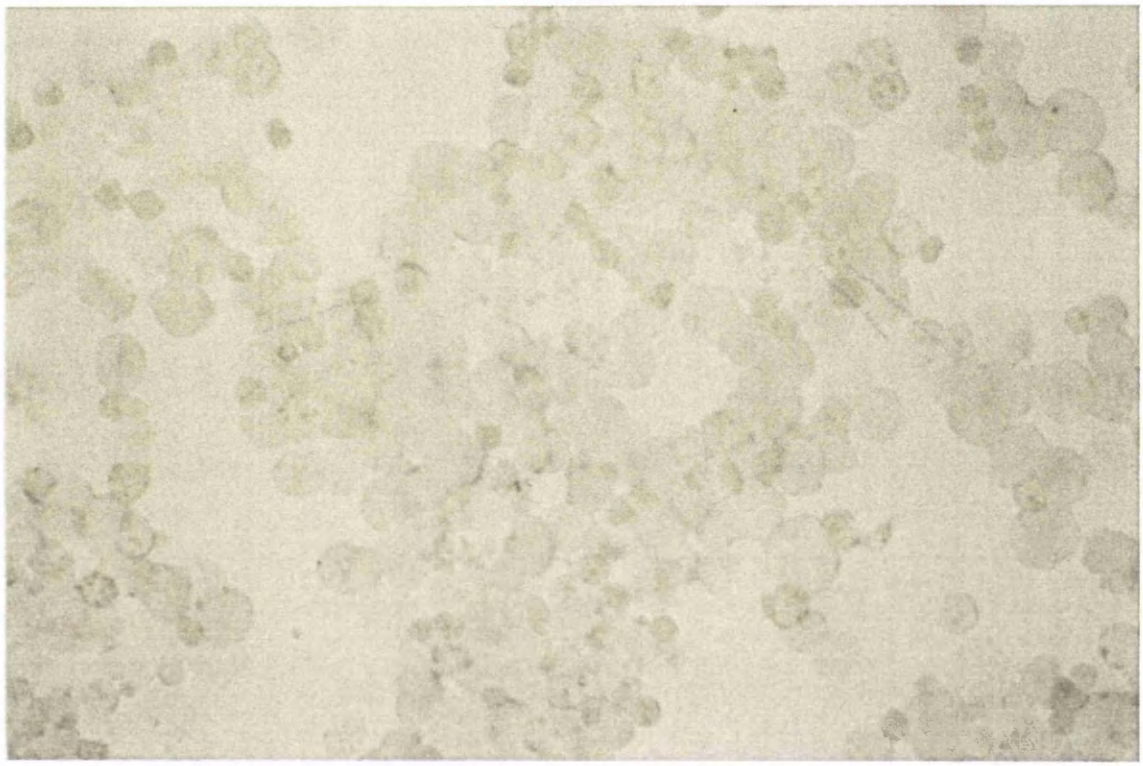
6.6 Changes in Bcl-2 expression in myeloma cell lines stimulated via CD40, Fas or with exogenous cytokines.

In an attempt to assess the relationship, if any, between Bcl-2 expression in myeloma cells and activation of CD40 or Fas antigens, an initial study was carried out examining baseline Bcl-2 protein expression in myeloma cell lines. This was demonstrated by flow cytometric analysis - cytoplasmic Bcl-2 protein was detected by permeabilisation of myeloma cells and labeling with an FITC-conjugated, monoclonal anti-human Bcl-2 antibody as described in section 2.2.22. Expression of Bcl-2 found in the myeloma panel tested is summarised in Table 2.2.22 with flow cytometric analysis shown in Figure 2.2.22b.

Figure 6.5a

In situ hybridisation showing CD40 mRNA expression in the myeloma cell line IM-9 using Digoxygenin-labeled sense (a) and antisense (b) riboprobes. Cells had been incubated for three days previously with anti-CD40 MoAb (0.5μg/ml) in the CD40 culture system. Counterstaining was with Light Green. Slides were viewed under light microscopy (magnification x 40). Antisense positivity indicates that these cells are positive for CD40 transcription.

a)



b)

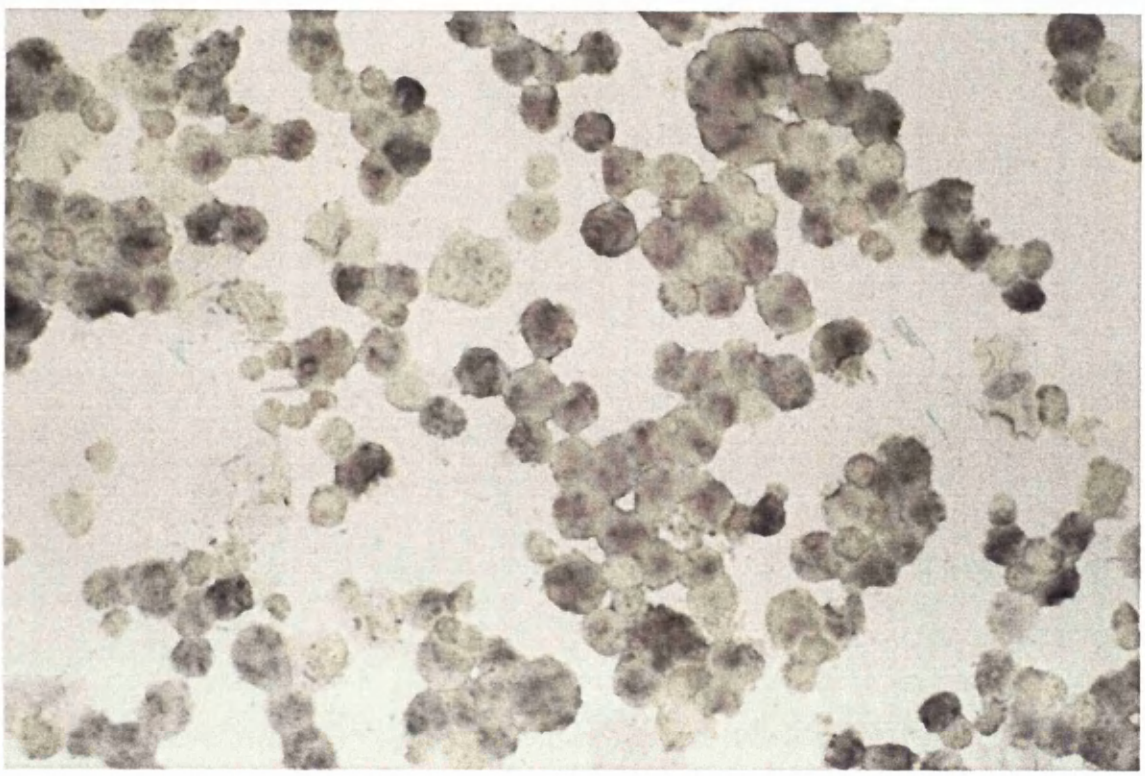
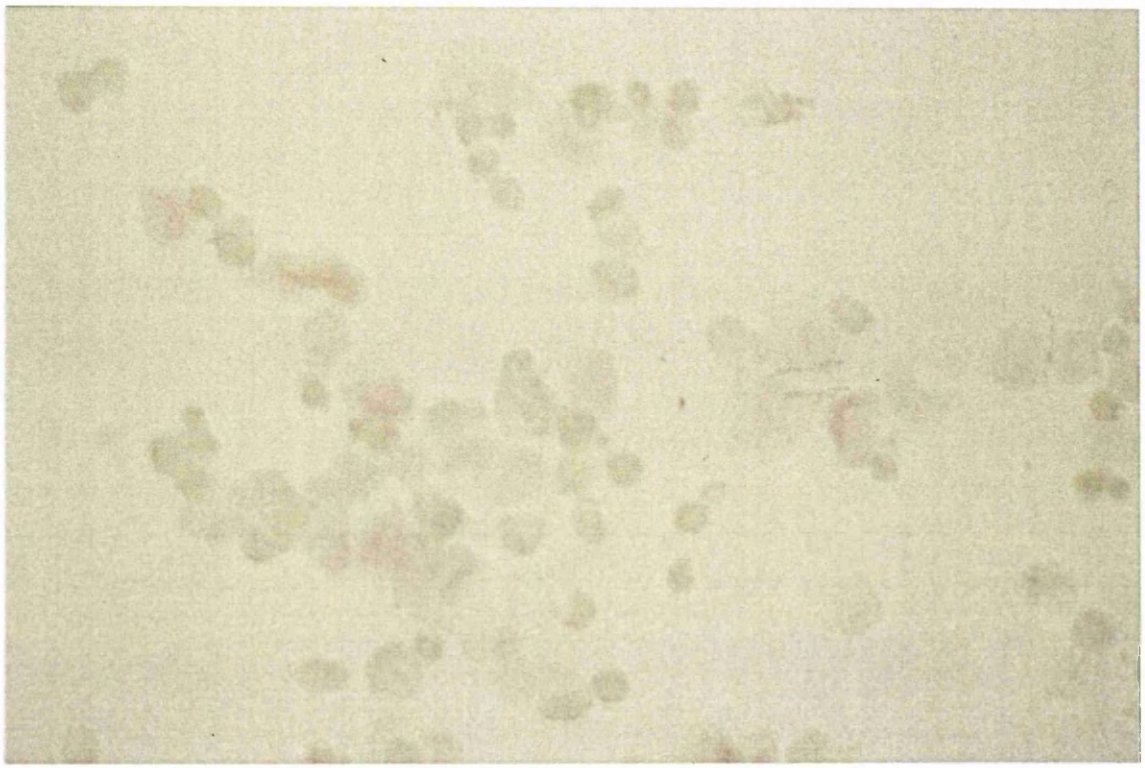


Figure 6.5b

In situ hybridisation showing *fas* mRNA expression in the myeloma cell line IM-9 using Digoxygenin-labeled sense (a) and antisense (b) riboprobes. Cells had been incubated for three days previously with anti-Fas MoAb (100ng/ml) in the CD40LigL culture system. Counterstaining was with Light Green. Slides were viewed under light microscopy (magnification x 40). Antisense positivity indicates that these cells are transcribing *fas* mRNA.

a)



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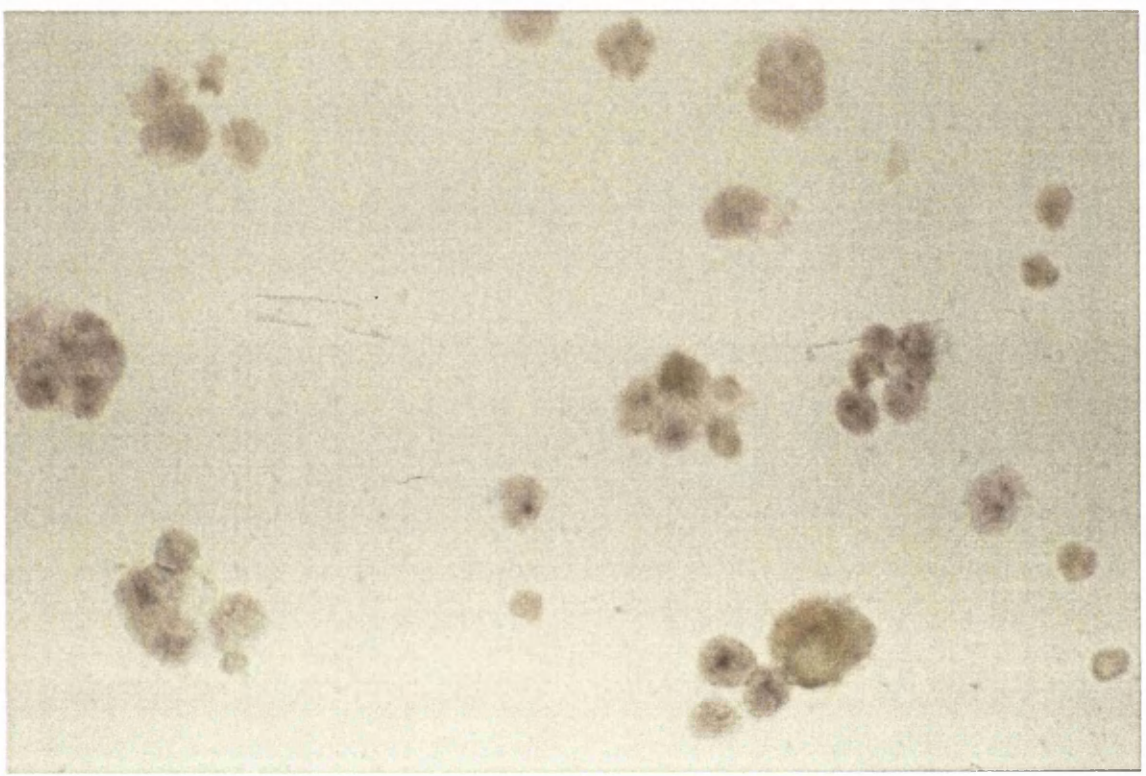
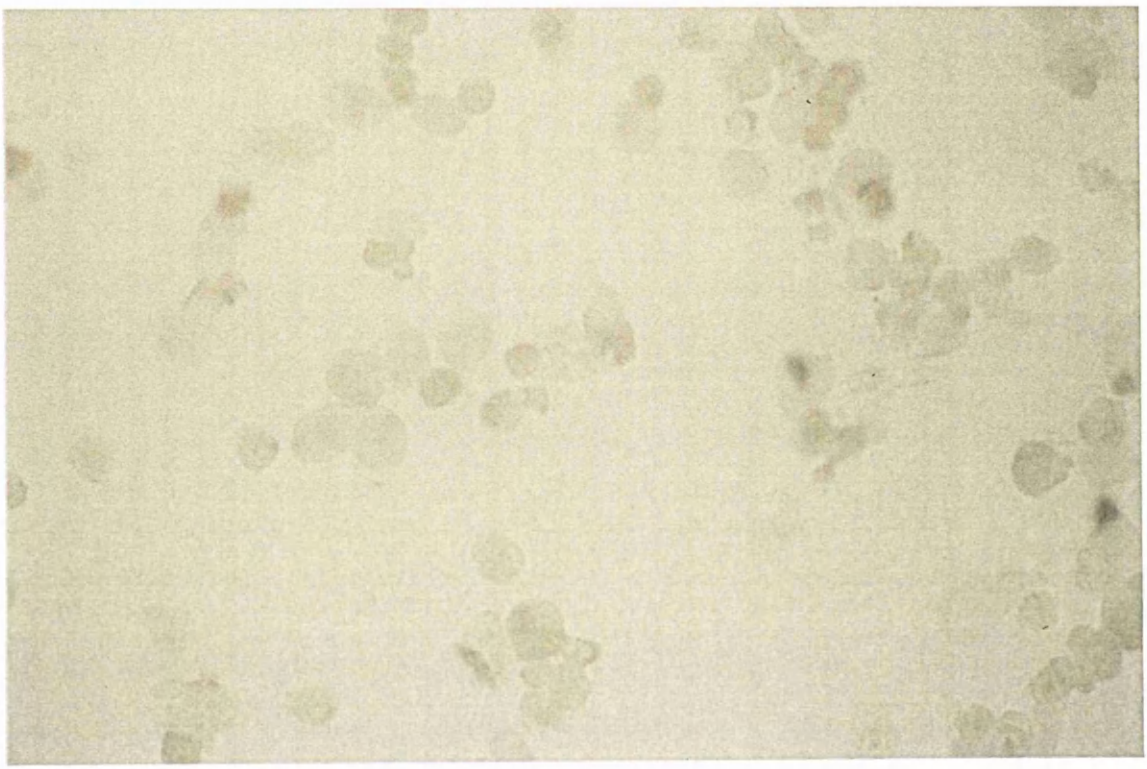


Figure 6.5c

In situ hybridisation showing CD40 mRNA expression in the myeloma cell line U266 using Digoxygenin-labeled sense (a) and antisense (b) riboprobes. Cells had been incubated for three days previously with anti-CD40 MoAb (0.5μg/ml)+ IL-4 (100U/ml) in the CD40 culture system. Counterstaining was with Light Green. Slides were viewed under light microscopy (magnification x 40). Antisense positivity indicates that these cells are weakly positive for CD40 transcription.

a)



b)

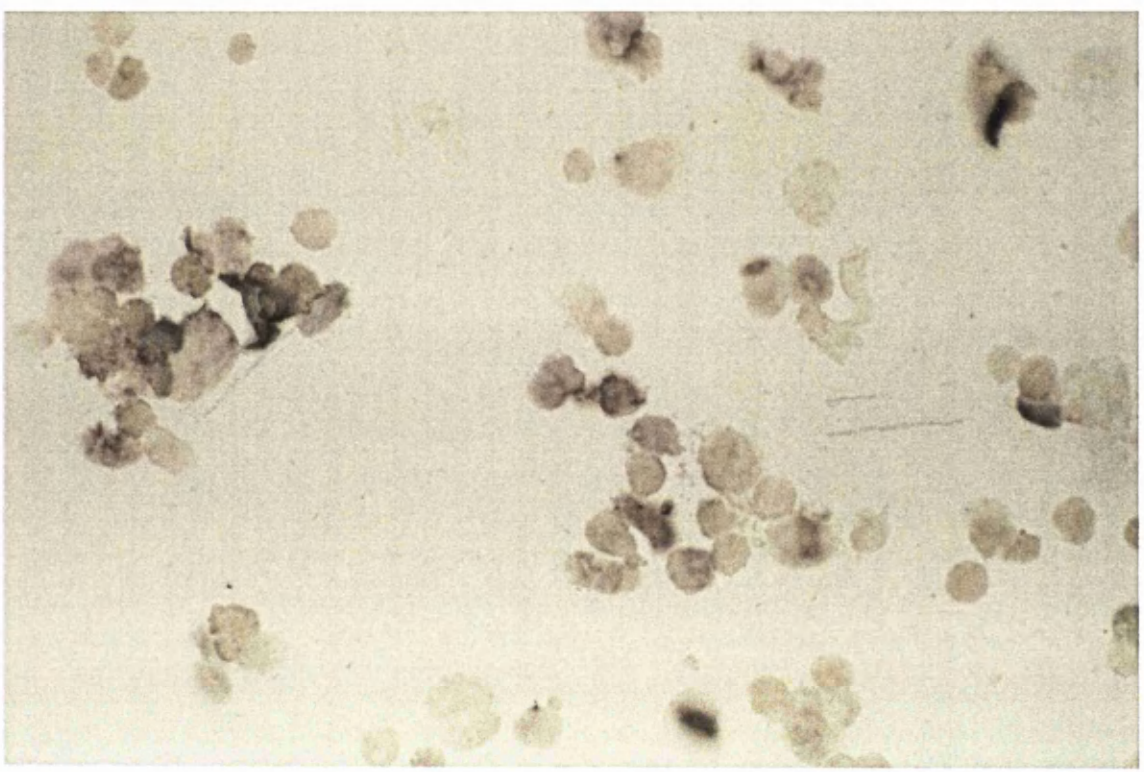
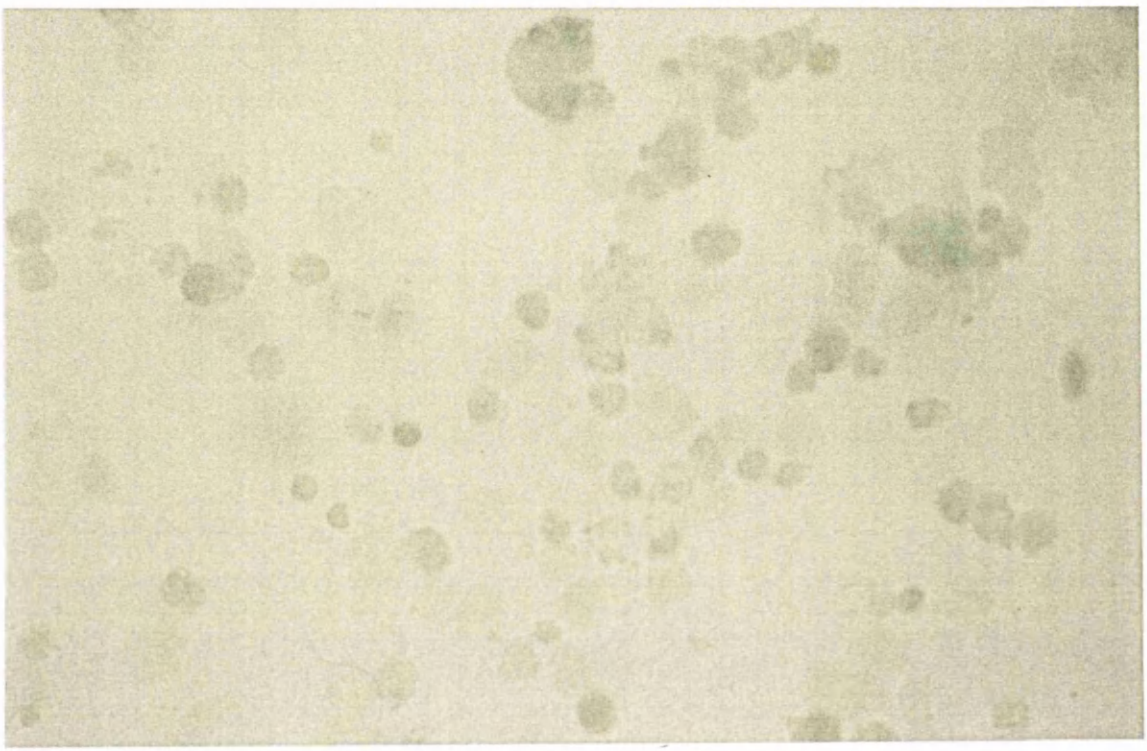


Figure 6.5d

In situ hybridisation showing CD40 mRNA expression in the myeloma cell line RPMI-8226 using Digoxygenin-labeled sense (a) and antisense (b) riboprobes. Cells had been incubated for three days previously with anti-CD40 MoAb (0.5μg/ml)+ IL-4 (100U/ml) in the CD40 culture system. Counterstaining was with Light Green. Slides were viewed under light microscopy (magnification x 40). Antisense positivity indicates that these cells are strongly positive for CD40 transcription.

a)



b)

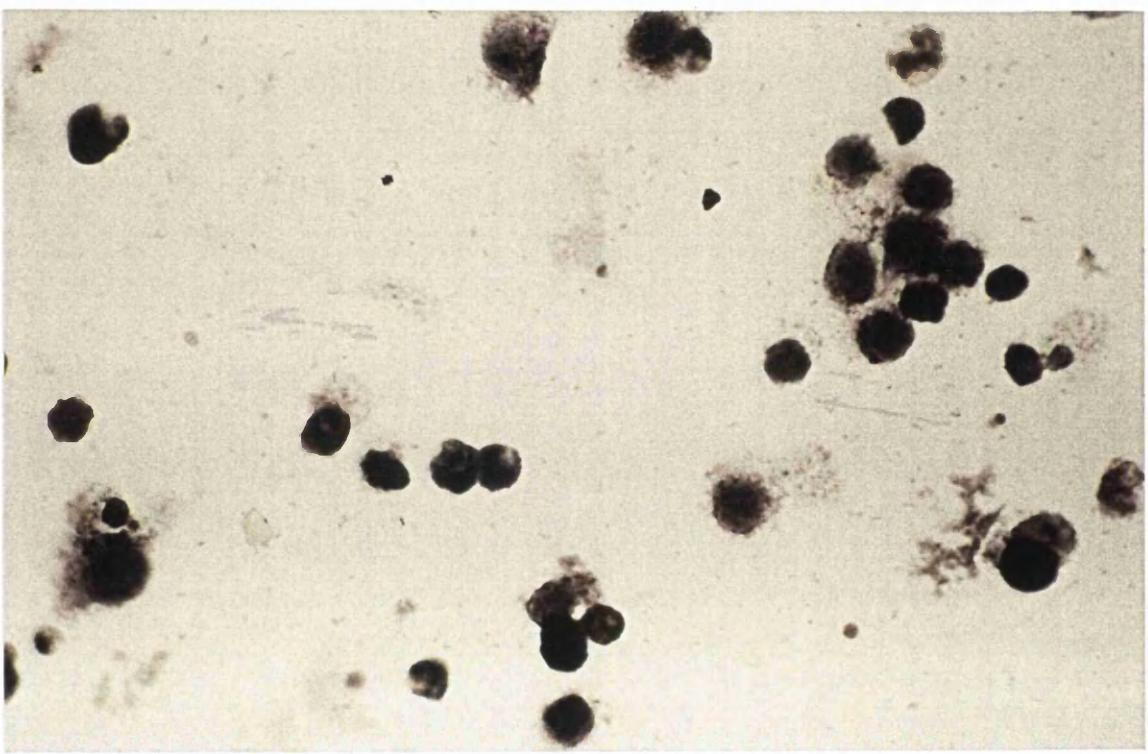
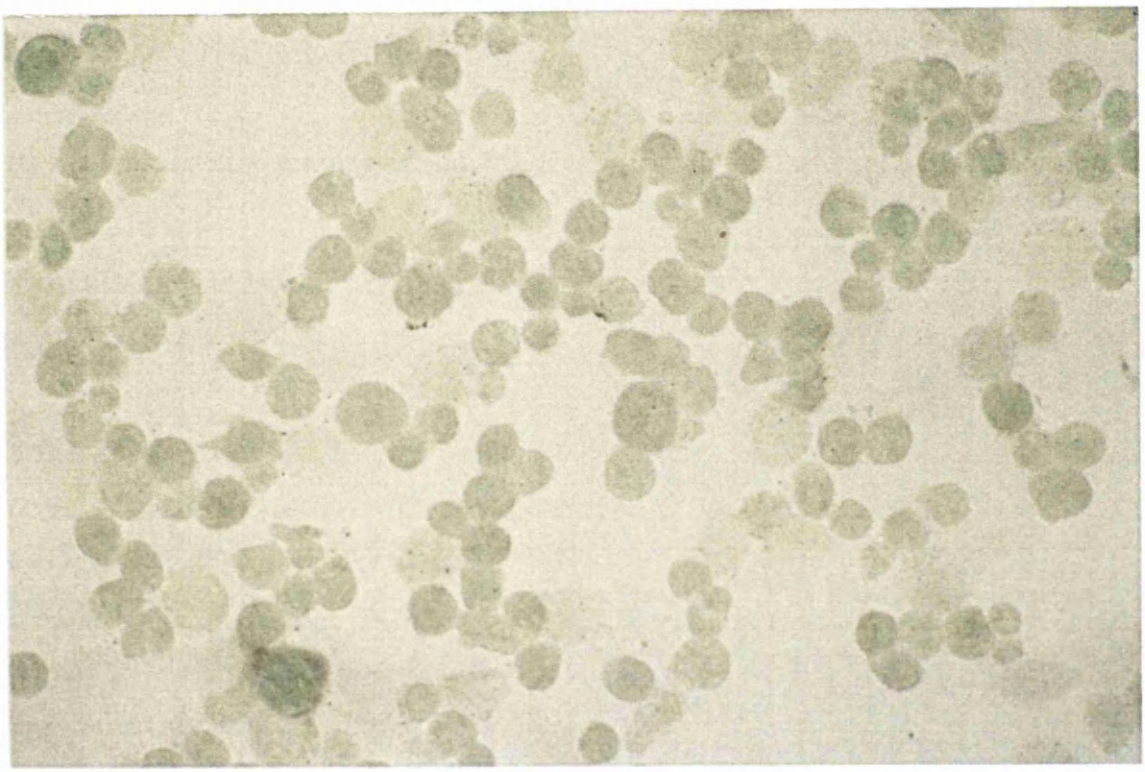


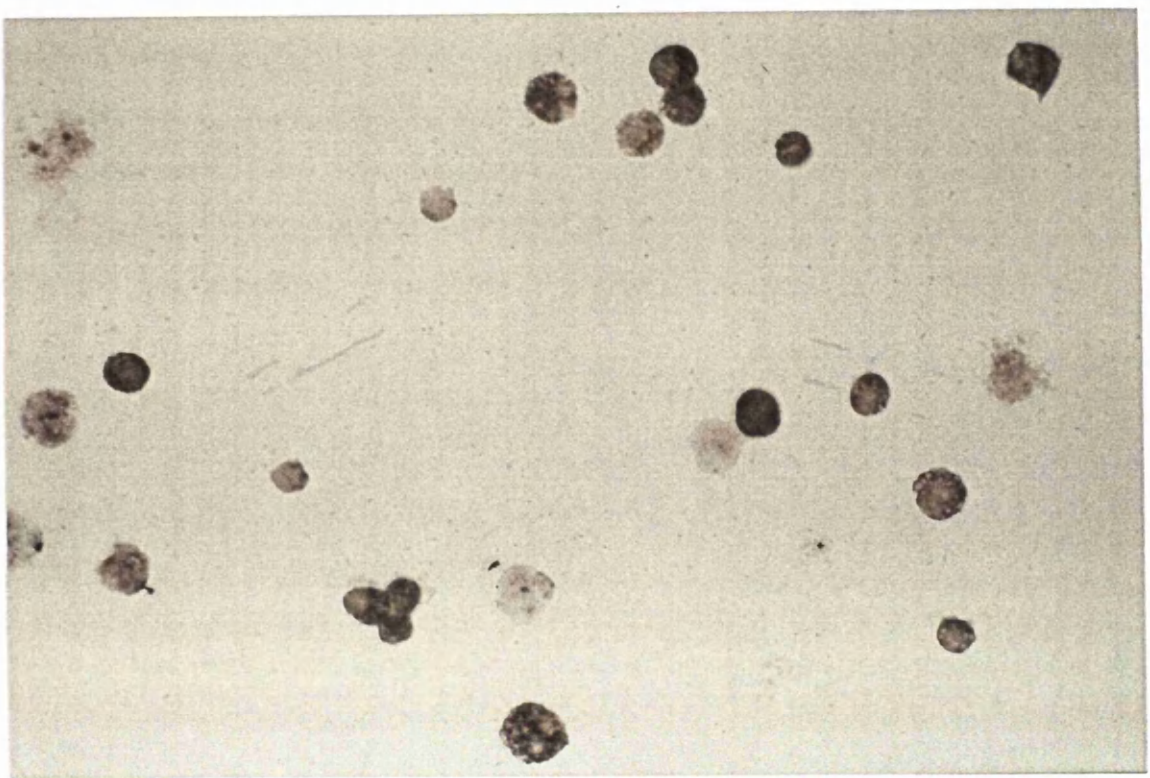
Figure 6.5e

In situ hybridisation showing *fas* mRNA expression in the myeloma cell line ANBL-6 (22/5) using Digoxygenin-labeled sense (a) and antisense (b) riboprobes. Cells had been incubated for three days previously in the CD40 culture system with no additives Counterstaining was with Light Green. Slides were viewed under light microscopy (magnification x 40). The sense probe shows background light green staining only whereas the antisense probe indicates that these cells are strongly positive for *fas* mRNA expression.

a)



b)



Changes in Bcl-2 expression were then observed after stimulation of a panel of myeloma cell lines with various additives. The myeloma cell lines JIM-1, JIM-3, JJN-3, U266, IM-9, RPMI-8226, ANBL-6 (26/12), ANBL-6 (16/1), ANBL-6 (17/2), ANBL-6 (27/2), ANBL-6 (6/3) and ANBL-6 (22/5) and were incubated for three days in either the CD40- or the CD40Lig-L culture systems with either:

a) no additve,

b) IL-6 (± anti-CD40, CD40 system only) or

c) anti-Fas

Cells were harvested, permeabilised and analysed by flow cytometry for Bcl-2 expression.

Results are summarised in Table 6.6 with representative flow cytometric analysis shown in Figures 6.6(a-c)

Conclusion

Fas activation in those cell lines susceptible to Fas-mediated apoptosis (JIM-3, JJN-3, IM-9, and all interleukin-6 independent passages of ANBL-6 (6/3,22/5,17/2,27/2) resulted in down-regulation of Bcl-2 protein expression assessed by flow cytometric analysis (Figures 6.6a and 6.6b show the results for IM-9 and ANBL-6 (6/3) as representative examples). Expression of Bcl-2 in RPMI-8226, also susceptible to Fas-mediated apoptosis however, remained negative throughout stimulation (Figure 6.6c). Bcl-2 expression in JIM-1 and U266, both non-susceptible to Fas-mediated apoptosis remained negative, ie unchanged, throughout stimulation. Bcl-2 expresssion in the IL-6 dependent passage of ANBL-6 (26/12) was negative when cultured in the CD40 system for three days with or without Fas activation. Bcl-2 expression in ANBL-6 (16/1), also IL-6 dependent, when cultured for three days in the CD40Lig-L system, was negative with no additive or with IL-6 added to the culture but became Bcl-2 positive when cultured for three days with anti-Fas. Results are summarised in Table 6.6.

Note: Figure 2.2.22b shows that JIM-1, U266 and RPMI-8226 are Bcl-2 positive by flow cytometric analysis of unstimulated cells. It is likely that expression of this gene changes through continual passage of cell lines.

From these results it looks as if expression of the proto-oncogene *bcl-2* is down-regulated in those myeloma cell lines which are susceptible to Fas-mediated apoptosis, or, in the case of RPMI-8226, Bcl-2 expression is negative and remains so following Fas activation. There are conflicting reports about whether Fas-mediated apoptosis is regulated by Bcl-2 (175,180). Strasser et al (175) state that the activation induced lymphocyte cell death induced by Fas activation is not regulated through Bcl-2 expression and Lee et al. (180) state that Bcl-2 protects against Fas-based T cell-mediated cytolysis. Two groups have examined Bcl-2 expression in myeloma cells with relation to Fas activation and both found no correlation between the expression of Bcl-2 and susceptibiliy to Fas-mediated cell death (126,236). It has recently been proposed that *bcl-2* plays a critical role in the growth of myeloma cell lines and in their death by spontaneous or Dexamethasone-induced apoptosis (161) but it remains to be proven whether or not this proto-oncogene protects myeloma cells from Fas-mediated AICD.

Table 6.6 Summary of Bcl-2 protein expression by flow cytometric analysis following a three day culture in either the CD40- or the CD40Lig-L -systems with or without additives: IL-6, anti-CD40 (CD40 system only) or anti-Fas. Permeabilised myeloma cells were labeled with an FITC-conjugated mouse anti-human-Bcl-2 MoAb using an FITC-conjugated mouse-IgG as negative control. 10,000 cells per experiment were analysed for fluorescence intensity relating to Bcl-2 expression which is expressed on a scale from - to ++. See conclusion (6.6).

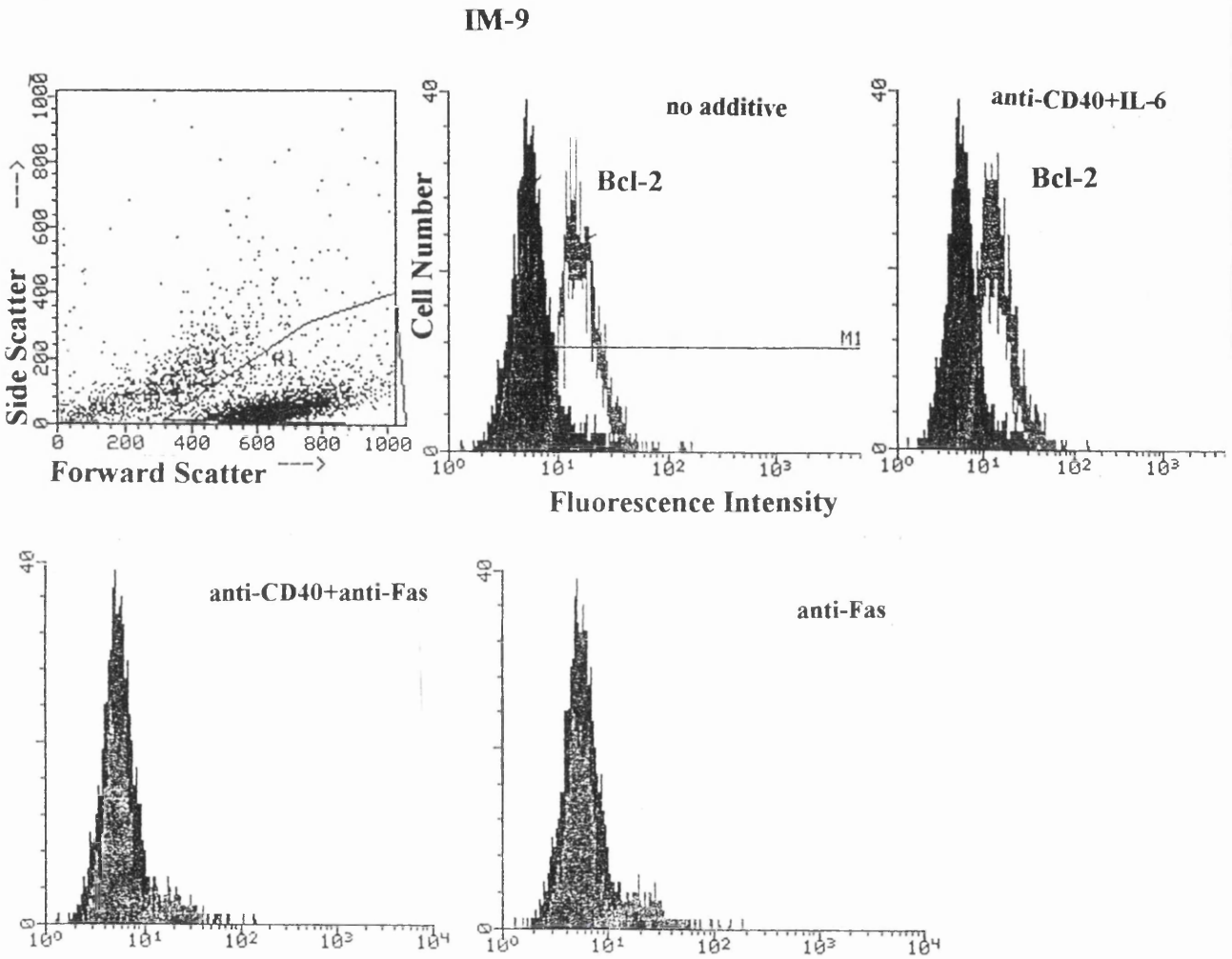
Additive	No additive	IL-6 ± anti-CD40	Anti-Fas ± anti-CD40
Cell line			
JIM-1	-	-	-
JIM-3	++	+	-
JJN-3	$+^{weak}$	$+^{weak}$	-
U266	-	-	-
IM-9	++	++	-
RPMI-8226	-	-	-
ANBL-6 (26/12)	-	-	-
ANBL-6 (16/1)	-	-	+
ANBL-6 (17/2)	+	+	-
ANBL-6 (27/2)	$+^{weak}$	$+^{weak}$	-
ANBL-6 (6/3)	+	+	-
ANBL-6 (22/5)	++	+	$+^{weak}$

6.7 Transcription of CD40 ligand, Fas ligand and interleukin-6 receptor by ISH in myeloma cell lines following culture in the CD40 or CD40Lig-L systems -comparison with RT-PCR results and protein expression of IL-6R by flow cytometric analysis.

It was attempted to assess whether Fas ligand, CD40 ligand or the interleukin-6 receptor were transcribed in myeloma cell lines by *in situ* hybridisation. No positive hybridisation indicating transcription of IL-6R, CD40L and FasL was detected in myeloma cell lines cultured with or without CD32L or CD40Lig-L cells despite repeated attempts at *in situ* hybridisation using specific Digoxygenin-labeled riboprobes. It had already been established (2.2.11) that RT-PCR was also negative for CD40L and FasL mRNA expression for all myeloma cell lines tested. CD40 ligand and Fas ligand expression has been reported to be restricted mainly to activated T lymphocytes so the failure to detect these transcripts by ISH or RT-PCR is not altogether surprising. RT-PCR analysis of IL-6R transcription, however (2.2.11), has shown that the mRNA is transcribed in the myeloma cell lines JIM-1, JIM-3, U266 and RPMI-8226 and IL-6 independent passages of ANBL-6 (IL-6 dependent passages were not tested). Expression was absent by RT-PCR for JJN-3 and IM-9. It could be that ISH was not sensitive enough to detect non-amplified IL-6R transcripts or that the copy number was too low for detection. IL-6R protein expression has been detected by flow cytometry in U266, RPMI-8226 and IL-6-dependent passages of ANBL-6 and was negative for JIM-1, JIM-3, JJN-3 and EJM (Figure 2.2.22). Transcription of IL-6 by RT-PCR was only detected in the myeloma cell line U266 of all the lines tested (2.2.11). Failure to detect IL-6 transcription amongst the other IL-6-independent cell lines tested does not support the theory that these lines have developed an autocrine pathway of IL-6 production although nested PCR may reveal the presence of IL-6 transcription and this was not tested.

Figure 6.6a

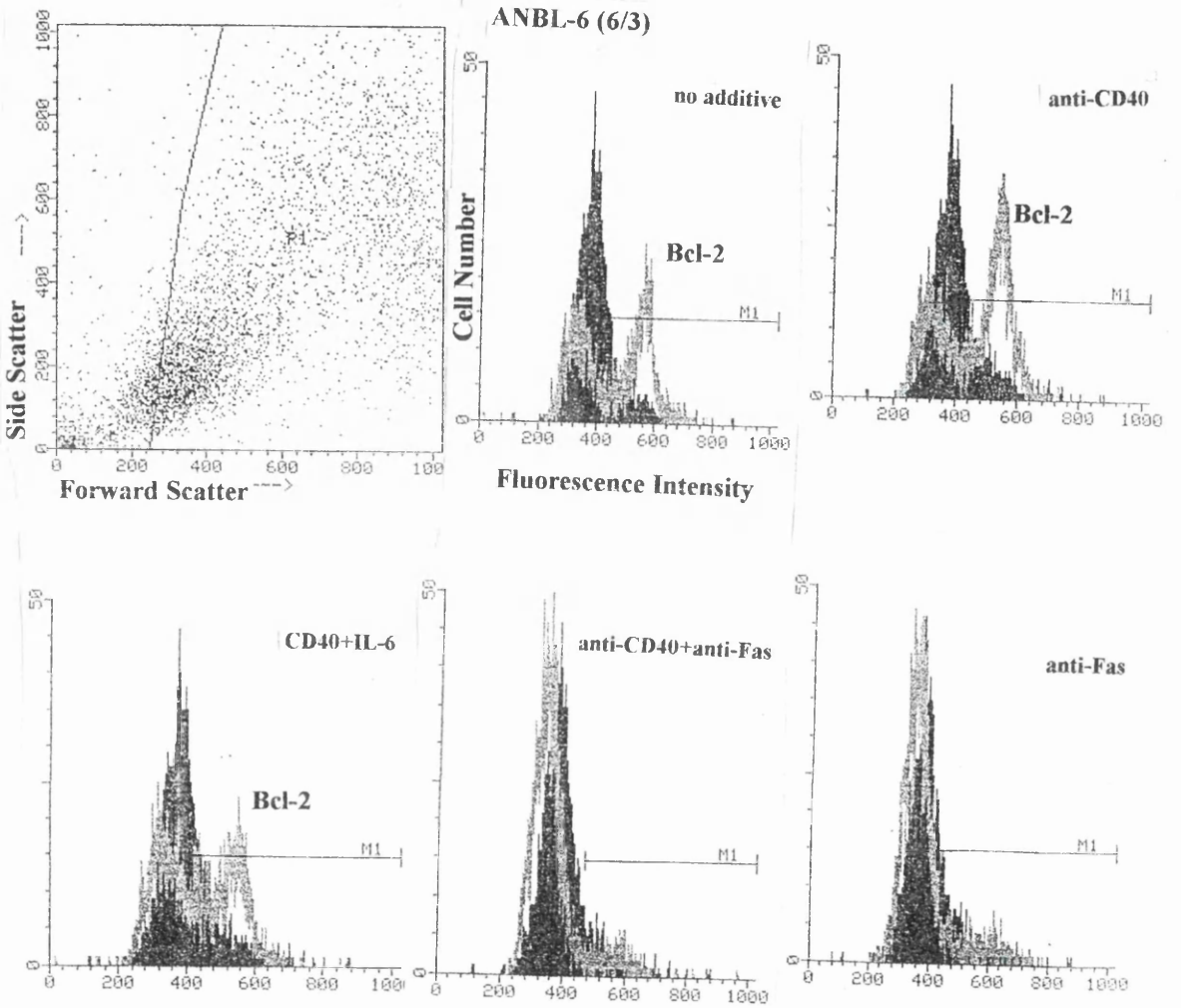
Bcl-2 expression in the myeloma cell line IM-9 following a three day incubation in the CD40 system with either no additive, anti-CD40+IL-6, antiCD40+anti-Fas or anti-Fas. Cells were permeabilised and labeled with an FITC-labeled mouse anti-human Bcl-2 MoAb. 10,000 cells were analysed per experiment. Bcl-2 expression is down-regulated following incubation with anti-Fas.



Negative control (mouse IgG) is peak with black background

Figure 6.6b

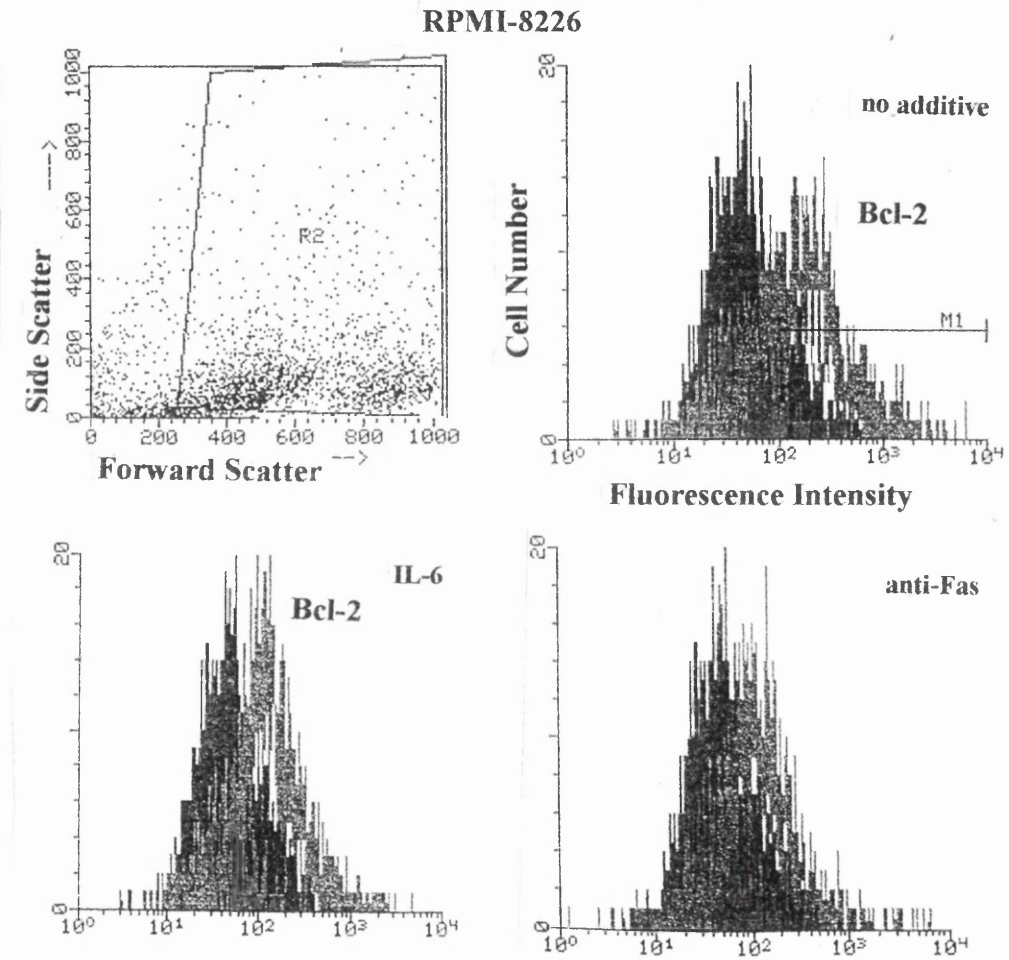
Bcl-2 expression in the myeloma cell line ANBL-6 (6/3) following a three day incubation in the CD40 system with either no additive, anti-CD40, anti-CD40+IL-6, antiCD40+anti-Fas or anti-Fas. Cells were permeabilised and labeled with an FITC-labeled mouse anti-human Bcl-2 MoAb. 10,000 cells were analysed per experiment. Bcl-2 expression is down-regulated following incubation with anti-Fas.



Negative control (mouse IgG) is peak with black background

Figure 6.6c

Bcl-2 expression in the myeloma cell line RPMI-8226 following a three day incubation in the CD40Lig-L system with either no additive, IL-6, or anti-Fas. Cells were permeabilised and labeled with an FITC-labeled mouse anti-human Bcl-2 MoAb. 10,000 cells were analysed per experiment. Bcl-2 expression remains unchanged following incubation with anti-Fas.



Negative control (mouse IgG) is peak with black background

CHAPTER 7

DISCUSSION

Discussion

This study into the mechanisms which control apoptosis in myeloma plasma cell lines has shown that Fas was almost universally expressed on all the myeloma cell lines tested. Pilot studies were then performed to determine whether any of these cell lines would be susceptible to Fas-mediated apoptosis. It was found that incubation with anti-Fas at a concentration of 200ng/ml for a period of up to 20 hours did not result in apoptosis in any of the cell lines tested (JIM-1, JIM-3, U266, IM-9 and IL-6 independent ANBL-6), see section 4.4. Increasing the concentration of anti-Fas to 500ng/ml had no effect on these results. However, it was found that prolonged incubation with anti-Fas (up to a period of 72 hours) resulted in comprehensive activation induced cell death in certain cell lines at a concentration of anti-Fas as low as 100ng/ml. This death initially became obvious 24 hours following Fas ligation with a 30-50% reduction in cell viability but 100% cell death in susceptible cell lines was only observed following 48-72 hours of Fas ligation.

It was also found that there was a varying intensity of expression of CD40 and Fas in all the myeloma cell lines tested (Table 5.1) and that not all of those lines which were positive for Fas expression were susceptible to Fas-mediated apoptosis (Sections 6.3, 6.3.1). This is consistent with previous studies (126,236). This variability in susceptibility to apoptosis implies that even monoclonal plasma cells may represent a variety of differentiation stages (276-279). The culture techniques adopted in this study were aimed at examining the effect of activation of Fas and CD40 on antigen expression, proliferation and induction of activation induced cell death. Comparisons were made by assaying the cells with either a) no accessory L cells, b) CD32L cells or c) CD40Lig-L cells. A summary of the results of these assays is tabulated in appendix 1. The results for the CD32L system assays are summarised in graphical form in appendix 2, Figures 7.1-7.3, with representative examples of flow cytometric light scatter analyses shown in appendix 2a. The two methods adopted to detect apoptosis amongst this panel of cell lines proved to be rapid, informative and cost effective. The effect of stimulation of proliferation or alternatively inhibition of proliferation was also corroborated by the tritiated thymidine uptake although this was not always as informative as the morphological analysis and flow cytometric results.

Both the CD40- and CD40Lig-L- culture systems were found to be effective in potentiating CD40 activation in myeloma cell lines with a synergistic increase in proliferation achieved by the addition of either IL-4 or IL-6. IL-6 had the greatest proliferative effect on the IL-6-dependent passages of ANBL-6 as well as the IL-6 dependent JT line (which is an IL-6 dependent myeloma line developed by myself from the peripheral blood of a patient with plasma cell leukaemia) as demonstrated by a marked increase in tritiated thymidine uptake. Addition of IL-6 to cultures of IL-6 independent myeloma cell lines also enhanced proliferation. This enhancement of proliferation was potentiated by CD40 ligation (in both the CD32L and CD40Lig-L culture systems). This indicates that there is a synergistic proliferative effect on myeloma cells induced by a combination of CD40 activation and the presence of IL-6 which agrees with previous studies indicating that there may be a link between CD40 and IL-6 signalling pathways both in B cells (90) and in myeloma cells (88).

The CD40Lig-L culture system proved to be the most effective method of testing CD40 stimulation since less additives were required than when using the CD32L system, the background tritiated thymidine uptake was much lower for the CD40Lig-L assays than with CD32L assays and an overall potentiation of the response was achieved by the use of L cells as opposed to no L cells. If, however, CD40 responses are to be compared with lack of CD40 activation, then the CD32L system would be preferable. Also, cross-linkage of CD40 on the surface of myeloma cells with it's natural ligand on the surface of the CD40Lig-L cells better ressembles the *in vivo* situation. These assays have proved that myeloma cell lines can be effectively stimulated without accessory L cells but the proliferation rate and cell viability following three days culture is optimised in the CD40Lig-L system. The reason for the high background uptake of tritiated thymidine in the CD32L system assays is a mystery since the same number of both the CD40Lig-L and CD32L cells in the same volume of medium were irradiated for the same length of time in both systems. A recent study examining the effect of proliferation of a variety of B cell malignancies using the CD40 system (280) has shown that the presence of irradiated CD32L cells themselves, when cultured with fresh myeloma plasma cells resulted in a substantial increase in tritiated thymidine uptake compared with the same cells cultured in the absence of a CD32L monolayer. This implies that these CD32L cells not only function as Fc-receptor presenting cells, but also produce growth-promoting factors, or induce the production of paracrine or autocrine B cell-derived growth factors such as IL-6. Indeed, endogenously produced

human IL-6 could be measured in supernantants of cultures consisting of CD32L cells and malignant B cells. Planken et al. (280) also showed that murine IL-6 was transcribed by the CD32L cells although the human IL-6 produced in this CD40 system assay must have been responsible for the observed proliferation since murine IL-6 has no proliferation enhancing effect on human B cells, and human IL-6 was measured in the supernatants. Moreover, anti-human-IL-6 antibodies (not cross-reactive with murine IL-6) considerably reduced the ^{3}H-thymidine incorporation in myeloma cells suggesting that the malignant B cells themselves, or accessory cells present in the system produced the IL-6 when co-cultured with the fibroblasts.

Not all the myeloma cell lines tested were found to be susceptible to Fas-mediated apoptosis. All of the IL-6 dependent lines tested - passages 26/12, 16/1 and the original clone of ANBL-6 (P32) as well as JT - were found to be non-susceptible to activation induced cell death. The results for P32 and JT are shown in appendices 1.12 and 1.13 respectively. The IL-6 independent myeloma cell lines U266 and JIM-1 were also resistant which is interesting since the subclone of JIM-1, JIM-3, is susceptible to Fas-mediated apoptosis. The cell lines JJN-3, IM-9, RPMI-8226 and all IL-6 independent subclones of ANBL-6 tested (passages 17/2, 27/2, 6/3 and 22/5) were susceptible to AICD. JJN-3 and RPMI-8226 exhibited the least marked apoptotic response as observed by flow cytometric light scatter analysis (Figure 6.3.1d). Activation of the Fas antigen on the myeloma cells tested in these culture systems was potentiated by co-activation of CD40. Effective cell death was also observed, however, when cells were stimulated via anti-Fas antibody alone.

In those lines non-susceptible to Fas-mediated apoptosis (JIM-1, U266 and all IL-6 dependent cell lines), the uptake of tritiated thymidine was similar when these cells were incubated in the presence of anti-Fas, with or without CD40 ligation (in both the CD32L and CD40Lig-L culture systems), to when they were incubated with no additive.

Total (100%) Fas-mediated apoptosis in the cell line JIM-3 was observed when these cells were cultured in the CD32L and CD40Lig-L systems, whereas only 40-50% cell death was observed when these cells were cultured with no L cells - as observed by the percentage viability assessed by morphology and trypan blue dye exclusion (appendix 1.2). Inhibition of proliferation however, as observed by tritiated thymidine uptake, was only 38% following

CD40- and Fas- ligation with only 24% inhibition following Fas activation alone in the CD32L culture system. This indicates that the uptake of tritiated thymidine is not always indicative of the morphological picture.

JJN-3 exhibited no increased proliferation when cultured in either the CD32L or CD40Lig-L systems compared with cultures containing no L cells (appendix 1.3). The best inhibition of proliferation, however, was seen when these cells were cultured in the CD32L system with anti-Fas, with or without anti-CD40 (48-64% inhibition by tritiated thymidine uptake and 100% inhibition by morphological and cell count analysis).

The cell line U266, which was the only line tested shown to transcribe IL-6 mRNA by RT-PCR (Table 7) indicating autocrine production of this cytokine, exhibited similar viability and proliferation by tritiated thymidine uptake in all three culture systems (appendix 1.4). This is interesting since it has been postulated that the transfected mouse fibroblasts used in these assays may stimulate autocrine human IL-6 production in myeloma cells (280). Also, addition of exogenous IL-6 to cultures of U266 cells in all three culture systems did not significantly increase proliferation compared to those cultured with no additive. As a result of this observation, it is not altogether surprising that these cells are non-susceptible to Fas-mediated apoptosis since all the IL-6 dependent cell lines tested were Fas-resistant.

The cell line IM-9 exhibited the fastest proliferative rate of all the myeloma cell lines tested as seen by the extent of tritiated thymidine uptake observed (appendix 1.5). This was only slightly potentiated by CD40 ligation and co-stimulation through IL-6 in all three culture systems (no L cells, CD32L and CD40Lig-L cells). It is interesting to note that this cell line had the highest constitutive mRNA and protein expression of Fas and CD40 as observed by *in situ* hybridisation and flow cytometric analysis. Comprehensive Fas-mediated apoptosis was observed when these cells were incubated with anti-Fas with or without CD40 activation. The optimum increase (2-fold) in cell count as observed by trypan blue dye exclusion was found when these cells were cultured in the CD40Lig-L system with either IL-6 or IL-4.

RPMI-8226 was only effectively killed by co-activation of Fas and CD40 since incubation of this cell line with anti-Fas alone in the CD32L culture system or with no L cells only

resulted in an 8% and 25% inhibition of proliferation respectively (appendix 1.6). Co-ligation of Fas and CD40 in the CD40 and CD40Lig-L culture systems only resulted in a 40-50% inhibition of proliferation as observed by uptake of tritiated thymidine indicating that this line was not completely susceptible to Fas-mediated apoptosis. This accords with a previous study by Westendorf et al. (236) which also estimated apoptosis by tritiated thymidine uptake. Extensive (70-80%) cell death was observed however by trypan blue dye exclusion and morphological analysis following CD40 and Fas stimulation in both of these culture systems.

ANBL-6 (passage 17/2, IL-6 independent) exhibited the best overall proliferation assessed by tritiated thymidine uptake when cultured with no L cells with an 80-90% increase in proliferation observed when cultured with exogenous IL-6, with or without CD40 co-ligation (appendix 1.7). Nearly 100% Fas-mediated cell death was observed in cultures of ANBL-6 (passage 27/2, IL-6 independent) using the CD32L and CD40Lig-L systems compared with cultures containing no L cells in which Fas ligation resulted in only a 60-68% inhibition of proliferation (appendix 1.8). Cultures of ANBL-6 (passage 22/5, IL-6 independent) indicated that the optimum cell counts (ie proliferation and viability) were observed in the CD40Lig-L system, the highest being with IL-6 co-stimulation (appendix 1.9). This being despite the fact that uptake of thymidine was lowest in this system (optimum uptake was observed in CD32L assays).

ANBL-6 (passage 16/1, IL-6 dependent) was only cultured using the CD32L and CD40Lig-L systems due to insufficient cell numbers. All the IL-6 dependent myeloma cells tested in this study have an extremely slow doubling time (48 hours). Cell counts and uptake of thymidine was low except when these cells were cultured with IL-6 in the CD40Lig-L system (appendix 1.10). Fas-activation, with or without CD40 co-ligation, resulted in only a 5-10% (background) inhibibtion of proliferation with 80-90% of the cells still viable by cell count and morphological analysis. Similarly ANBL-6 (passage 26/12, IL-6 dependent) cells, when tested in all three culture systems resulted in the optimum proliferation being observed in the CD40Lig-L system with IL-6 as an additive (appendix 1.11). Despite the low thymidine uptake observed when both passages 16/1 and 26/12 were cultured with IL-4, or anti-Fas with or without CD40 ligation in the absence of IL-6 cell

viability remained high and morphological analysis revealed the majority of the cells to be healthy.

The original passage of ANBL-6 (P32) was also studied using all three culture systems. Cultures using no L cells and CD40Lig-L cells exhibited a marked increase in proliferation caused by the presence of exogenous IL-6 with or without CD40 ligation (appendix 1.12). Despite background counts being high, culture using the CD32L system resulted in high thymidine counts with all additives, which were significantly enhanced by co-culture with IL-6 with or without anti-CD40. This effect could be due to the stimulation of autocrine IL-6 production by the ancillary CD32L cells (280). Activation of Fas in all three culture systems did not result in decreased viability amongst these cells. Co-incubation of ANBL-6 (P32) with anti-Fas and IL-6 in the CD40 and CD40Lig-L culture systems resulted in a marked proliferative effect with no induction of apoptotis (see below). As in the other IL-6 dependent myeloma cells tested viability in the absence of IL-6 remained high during a three day culture despite a low uptake of tritiated thymidine.

The myeloma cell line JT (IL-6 dependent) exhibited very slow growth which was only moderately enhanced by IL-6 co-stimulation as seen by cell counts and thymidine uptake in all three culture systems. As seen with the IL-6 dependent passages of ANBL-6, Fas-activation of these myeloma cells showed no effect on viability (appendix 1.13).

Mononuclear cells prepared from the peripheral blood of normal healthy donors were used as controls to test the efficacy of the culture systems (appendix 1.14). These were also used to compare the proliferative effect of trimeric soluble recombinant human CD40 ligand (consisting of the concentrated supernatant of cos cells which had been transfected with a leucine zipper CD40L construct) which was a generous gift of Dr. Richard Armitage of the Immunex Corporation in Seattle, USA. This form of CD40L has been shown to share all the activities of membrane-bound CD40 ligand. Although this trimer had a marked proliferative effect on both mononuclear cell controls and the myeloma cell lines tested, there was insufficient quantity to use in the replicate experiments required for this study.

ANBL-6 cells have previously been shown to die by apoptosis upon IL-6 withdrawal but this was seen to be a very slow process with no apoptotic effect observed until 4-5 days

culture in the absence of cytokine (281). Only 70% of these cells were killed following a two week incubation without IL-6. Similarly, in the present study, IL-6 dependent passages of ANBL-6 cultured for 3 days in the absence of IL-6 showed a background uptake of tritiated thymidine and the cells looked viable under light microscopy although there was no evidence of mitosis occuring. Only background (15%) signs of apoptotic cell death were observed signifying that these cells were in growth arrest. Significant apoptotic cell death became evident only after 5 days in culture (results not shown).

IL-6 added to the IL-6-dependent passages of ANBL-6 was shown to have no effect on their resistance to Fas-mediated apoptosis (appendix 1.12). Addition of IL-6 to cultures of (IL-6 independent) myeloma cells normally highly susceptible to Fas-mediated apoptosis however, in the presence of CD40 co-ligation, resulted in a protective effect (50% of IM-9 cells and 80% of ANBL-6 (22/5) cells were still viable following a three day incubation in the CD40Lig-L culture system with anti-Fas + IL-6). This effect was abrogated (completely in the case of IM-9 and partially in the case of ANBL-6) by the addition of anti-CD40 (clone G28-5) to the culture system implying that this monoclonal antibody had a blocking effect on CD40 ligation between CD40 ligand expressed on the L cells and CD40 on the surface of the myeloma cells (Appendix 3, Figures 7.4,7.5).

It is also of interest that, although the IL-6 dependent passages of the myeloma cell line ANBL-6 studied in this thesis were found to be strongly positive for CD40 expression, this expression was down-regulated once IL-6 independence was achieved (Table 5.1). An upregulation of CD40 expression was observed when these IL-6 independent passages were stimulated by anti-Fas monoclonal antibody and the cells were rendered susceptible to Fas-mediated apoptosis (Table 6.4b). It has been reported (120) that activation of CD40 in resting B cells, which normally do not express Fas and are non-susceptible to Fas-mediated apoptosis (102,137), induces the expression of high levels of Fas antigen and renders these cells susceptible to Fas-induced cell death. This upregulation of Fas expression following CD40 activation was not observed in the myeloma cell lines tested in this study although all of them except JIM-1 expressed moderate to high levels of Fas without prior stimulation (5.5.1). It was interesting to note however, that Fas-ligation over a three day period resulted in the upregulation of CD40 expression in the majority of the myeloma cell lines which were susceptible to Fas-mediated apoptosis (except for IM-9 which expressed 100%

positivity for CD40 throughout stimulation). This was manifested after 43 hours of Fas stimulation (5.5).

Jelinek et al (236) have also reported that ANBL-6 (IL-6 dependent) and U266 were resistant to Fas-mediated apoptosis (even after a three day incubation with anti-Fas at a concentration as high as 5μg/ml) and that RPMI-8226 was sensitive. They also found that not all the RPMI-8226 cells were killed by incubation with anti-Fas (only about 50% of the cells were killed following a 21 hour incubation with anti-Fas @250ng/ml). This group tried pre-treating U266 cells with interferon-γ, which has been reported to enhance the cytocidal action of anti-Fas (126), but this had no effect on its resistance to Fas-mediated apoptosis. They also verified that the sequence of the cDNA encoding Fas in this cell line was wild type. Fas-positive myeloma cell lines which are resistant to Fas-mediated cell death could therefore be useful in studying intracellular signalling events initiated after cross-linkage with Fas. The authors found no correlation between Bcl-2 levels in the myeloma cell lines tested and their susceptibility to Fas mediated apoptosis. These results agree with previous findings by others both in myeloma cells (126) and other cell lineages (120, 175, 179, 181, 193).

The same authors also looked at Fas expression in fresh myeloma, MGUS and primary amyloidosis samples. They found that 15 out of 28 myeloma samples expressed Fas (including 3 out of 4 patients with extramedullary involvement) compared to 3/6 and 2/7 Fas positive cases of MGUS and primary amyloidosis respectively. They found that ANBL-6 was negative for Fas expression whereas U266 and RPMI-8226 were strongly positive. In the present study however, the IL-6 dependent passages of ANBL-6 were found to be weakly positive (40%) for Fas expression by flow cytometry (Table 5.1). Interestingly, the IL-6 independent passages of ANBL-6, derived from the original clone P32, manifested an upregulated expression of Fas (50-90%). The presence of IL-6 in the IL-6 dependent cell lines tested by Jelinek et al. (including ANBL-6) during anti-Fas treatment had no effect on their apoptotic response, nor did it have any effect on Fas expression. These results were confirmed by this study when the original passage (P32, IL-6 dependent) of ANBL-6 was incubated in both the CD40- and CD40Lig-L -culture systems in the presence of CD40 stimulation, anti-Fas and IL-6. Tritiated thymidine uptake was as high as, or higher than that observed when the cells were incubated with IL-6 ± CD40 activation (appendix 1.12). A

similar picture was seen when these cells were examined under light microscopy. No difference in viability was observed between ANBL-6 (P32) cells incubated a) without anti-Fas, b) with anti-Fas or c) with (anti-Fas + IL-6 ± CD40 activation). The majority of the cells (85-95%) were still viable.

Shima et al. (126) have also investigated Fas-mediated apoptosis amongst fresh myeloma samples and myeloma cell lines and found that, although all the cell lines and the majority of patient samples tested were positive for Fas expression (at different intensities), they were not all susceptible to Fas-mediated apoptosis. RPMI-8226 was susceptible to Fas mediated cell death whilst U266 was not which is in keeping with both this study and previous studies (236).

The results shown in section 6.4 indicate that a high level of expression of both cell surface CD40 and Fas is required for a myeloma cell line at least, to become susceptible to the onset of activation induced cell death with a high level of Fas alone being insufficient to initiate the process.

The results of experiments performed in section 6.6 indicate that expression of the proto-oncogene *bcl-2* is down-regulated in those myeloma cell lines which are susceptible to Fas-mediated apoptosis, or, in the case of RPMI-8226, Bcl-2 expression is negative and remains so following Fas activation. There are conflicting reports about whether Fas-mediated apoptosis is regulated by Bcl-2 (175,180). Strasser et al (175) state that the activation induced lymphocyte cell death induced by Fas activation is not regulated through Bcl-2 expression and Lee et al. (180) state that Bcl-2 protects against Fas-based T cell-mediated cytolysis. It has recently been proposed that *bcl-2* plays a critical role in the growth and in spontaneous or Dexamethasone-induced apoptosis in myeloma cell lines (161) but it remains to be seen whether or not this proto-oncogene protects myeloma cells from Fas-mediated AICD. Two previous studies (126,236) have indicated that the level of Bcl-2 expression in myeloma cells does not correlate with their susceptibility to undergo Fas-mediated apoptosis. Similarly, Bcl-2 expression has been reported not to correlate with the susceptibility of activated B cells to Fas-mediated apoptosis (181). This obviously requires further clarification since Fas-mediated apoptosis may be a Bcl-2 independent mechanism of AICD.

None of these studies on myeloma cells have investigated the possibility that one of the other members of the *bcl-2* family, such as *bcl-x* or Bax (195,196) may be responsible for regulating Fas-induced cell death in myeloma cells. Reports have shown that expression of Fas on the surface of lymphocytes does not necessarily correlate with sensitivity to Fas-mediated apoptosis (282,283). Klas et al. (283) have shown that Fas-induced cell death does not occur until late in the time course of activation of T cells (72 hours). Analysis of the time course of Bcl-x_L expression following activation with anti-CD3 and anti-CD28 antibodies has revealed that Bcl-x_L expression peaks at 48 hours and then declines. That is, cells only become sensitive to Fas-induced cell death when Bcl-x_L levels are declining (176).

Consistent with these findings in T cells, Fas activation has also been demonstrated to inhibit the later stages of CD40-dependent B cell proliferation (120), with maximal inhibition of CD40-mediated proliferation observed after 72 hours incubation in the CD40Lig-L culture system. In the present study, myeloma cell lines were not found to be completely susceptible to Fas-induced cell death until 72 hours post activation of Fas (4.4, 4.4.1, 4.4.2) which would support the concept that perhaps there is a down-regulation of *bcl-x_L* in Fas activated myeloma cells also. CD40 stimulation of B cells which renders these cells susceptible to Fas-mediated apoptosis has been reported not to result in Bcl-x_L expression (284). Fas-induced cell death could be invoked when B cells were stimulated with CD40 ligand alone, but not when cells were stimulated with anti-μ and CD40 ligand (121). It is only when cells were stimulated under conditions similar to the latter (anti-μ + anti-CD40) that expression of Bcl-x_L was induced in B cells (284). These results suggest that antigen binding (mimicked by anti-μ or anti-Ig) results in a protective effect saving B cells from the onset of activation induced cell death and thus allowing an immune response to develop (285). There have been no comprehensive studies of *bcl-2* homologue expression with relation to Fas activation induced cell death in myeloma cells to date and this is of great interest in future studies. Indeed, preliminary experiments are already underway by the author (MML).

The regulation and function of *bcl-x* has been studied during B cell development (286) and the protein product Bcl-x has been found to be expressed in pre-B cells but downregulated at the immature and mature stages of B cell development. Bcl-x_L, but not Bcl-2 was rapidly induced in peripheral B cells upon surface immunoglobulin cross-linking and CD40

signalling (284,286). Transgenic mice which over-expressed Bcl-x_L within the B cell lineage exhibited marked accumulation of peripheral B cells in lymphoid organs with enhanced survival of developing and mature B cells. These studies demonstrate that Bcl-x_L is developmentally regulated in the B cell lineage and functions to protect developing and mature B cells from apoptosis. The pattern of Bcl-x_L expression is strikingly different from that of Bcl-2. Pre-B cells, a developmental stage in which Bcl-2 is down-regulated (135,136), expressed the highest levels of Bcl-x_L. This indicates that these two proteins play different roles in B cell selection and homeostasis. It may be that Bcl-2 and Bcl-x_L differ in their ability to counter death signals generated at specific stages during development. Both proteins share remarkable structural homology and localise to identical intracellular sites, suggesting that they inhibit cell death by a similar biochemical mechanism (287,288). The anti-apoptotic functions of Bcl-2 and Bcl-x_L are controlled in part by several interacting proteins (196, 200,201), one of which (Bad), exhibits a differential ability to heterodimerise with Bcl-2 and Bcl-x_L (200). It could be then, that the expression of the interacting partners of Bcl-2 and Bcl-x_L is differentially regulated during B cell development.

Studies of murine myeloma cell lines have shown that those resisitant to cyclohexamide-induced apoptosis expressed increased levels of *bcl-x_L* mRNA than those which were susceptible to apoptotic cell death (289). These results suggest a predominant role of *bcl-x_L* in preventing, at least drug-induced, apoptosis in murine and possibly human myeloma cells and suggest that the expression of *bcl-2* or *bcl-x_L* genes in B cell tumours may depend on the differentiation stage of the precursor normal cell. No significant differences in the expression of *bax*, which was expressed in both, between cyclohexamide-susceptible and -resistant murine myeloma cells was observed. The protective role of bcl-x_L may be related to its capacity to inactivate Bax and possibly other apoptosis-inducing proteins by forming heterodimers (196,200,204).

Schwarze and Hawley (290) have recently reported the up-regulation by IL-6 of *bcl-x_L* but not *bcl-2* gene expression in the mouse B9 hybridoma cell line. Myeloma cell lines have been cultured *in vitro* for many years and may have acquired, during this period, additional characterisitcs, such as rapid proliferation, which may compensate for their incapacity to resist apoptosis induction *in vitro.* Primary plasmacytoma cells initially grow very slowly in culture and are dependent on IL-6 for their propogation. It would be very interesting to

examine *bcl-x_L* and *bcl-x_S* expression in these tumours and to follow their expression through progression of the newly established cell line into long-term passage and the development of IL-6 independence. The finding that all the murine primary plasmacytomas tested expressed bcl-x_L indicates that the inhibition of apoptosis is probably an important step in the early development of myelomas as well as other tumours. The progression from IL-6 -dependence to -independence in human multiple myeloma cells cultured *in vitro* may involve activation of the *bcl-x_L* gene.

Tian et al. (165) have studied the role of p53, *bcl-2* and *bax* in Dexamethasone-induced apoptosis in a panel of multiple myeloma cell lines. They found that Bax transcripts were abundant in all the lines tested and therefore did not correlate with sensitivity to dexamethosone. Levels of p53 and *bcl-2* varied and did not in every case correlate with dexamethosone-sensitivity. The myeloma cell lines IM-9, U266 and RPMI-8226 were all found to express p53, *bax* and *bcl-2* by RT-PCR using sequence-specific primers. Loss of function of this tumour suppressor gene occurs in about 50% of all human cancers. p53 is a DNA-binding protein which can both induce cell cycle arrest and promote apoptosis, and functions, at least in part, as a transcriptional regulator. p53 appears, either directly or indirectly, to suppress *bcl-2* gene expression leading to the speculation that p53 loss in human tumours may contribute to the high levels of abnormal patterns of Bcl-2 protein production observed in many types of cancer (291,292). In addition to inhibition of Bcl-2 gene expression, p53 can also induce an increase in Bax gene expression (291). These effects of p53 on *bcl-2* and *bax* gene expression can result in a marked decrease in the ratio of Bcl-2 to Bax protein, and therefore render these cells more susceptible to apoptotic stimuli. Bax is the first pro-apoptotic gene to be identified which is a direct transcriptional target of p53. This group have found that there is a marked downregulation of Bax protein levels in drug-resistant breast and ovarian tumours as well as leukaemias. They also found significant increases in Bcl-x_L protein expression in association with drug-resistant leukaemias and solid tumours confirming that alterations in the expression of Bcl-x_L may also be relevant to mechanisms of drug-resistance in some types of cancer.

Reed et al. (293) have proposed a model for Bcl-2 family protein interactions based on the current available evidence. They postulate that Bax promotes apoptosis, probably through the formation of homodimers. This is opposed when Bcl-2, Bcl-x_L, Mcl-1, or possibly other

homologues of Bcl-2 that have anti-death activity (eg A1), heterodimerise with Bax thus neutralising its function. A second class of cell death promotors, which include Bcl-x_S and Bad, indirectly induce apoptosis by binding to Bcl-2, Bcl-x_L and probably other anti-apoptotic members of the Bcl-2 protein family, thus sequestering them and preventing them from heterodimerising with Bax. This leaves Bax homodimers unopposed. A recently described member of the Bcl-2 protein family, Bak (197), may function similarly to Bax.

In situ hybridisation using CD40 and Fas riboprobes of myeloma cell lines following a three day culture in the CD40 system revealed that all the myeloma lines tested in this study were positive for both transcripts when unstimulated. The strongest expression of CD40 mRNA was observed in IM-9, RPMI-8226 and IL-6 dependent myeloma lines in unstimulated cells. The intensity of CD40 expression increased following incubation with anti-CD40 with or without IL-4 or IL-6. The intensity of CD40 RNA expression did not alter following a three day incubation with anti-Fas (with or without anti-CD40). The weakest intensity of Fas mRNA expression was observed in JIM-1 and the IL-6 dependent passages of ANBL-6 in unstimulated cells. No change in this expression was observed following incubation with anti-CD40 with or without cytokines but a stronger intensity of Fas RNA expression was observed in those cell lines following incubation with anti-Fas with or without CD40 ligation (6.5).

A summary of the results determining transcription and translation of the genes of interest in this study are shown in Table 7. Since there is now a convenient method of estimating whether cells are producing endogenous IL-6 by flow cytometric analysis, it will be interesting to examine IL-6 protein expression in these myeloma cell lines in relation to their activation status. Previous methods of IL-6 detection by bioassay or enzyme linked immunosorbent tests were laborious and time-consuming.

In the majority of cases, the myeloma cell lines examined showed evidence of both transcription and translation of the genes encoding *bcl-2,* Fas and CD40. Exceptions to this were found in the IL-6 dependent passages of ANBL-6 in which translation of Bcl-2 was not always evident despite positive transcription. JIM-1 was also found not to translate the Fas and CD40 protein products despite transcription being detected by both ISH and RT-PCR. The U266 cell line was similarly found to express extremely low levels of CD40

protein despite positive transcription. Translation of the IL-6 receptor was not detected by flow cytometry in JIM-1 or JIM-3 despite evidence of transcription occuring. These results are consistent with previous reports that, at least the *bcl-2* gene, is consistently expressed in germinal centre B cells and myeloma cell lines but is not always translated (155,156). It could be that the regulation of these genes in this disease is post-transcriptional.

Fas ligand is normally only expressed in immunologically privileged tissues (testis, central nervous system and eye). These areas are known to accept grafts without rejection and grafts of these tissues placed at heterotopic sites are also not rejected. Histologic analysis suggests that normal testis grafts survive by inducing apoptosis in infiltrating leukocytes (294). These studies show that the lack of severe inflammation or immune responses in the testes and eye is at least partly because the Fas ligand present in these tissues kills lymphocytes that infiltrate the tissues and are activated to express Fas. Thus, Fas-FasL interactions are the principal mediators of immune privilege in the testis and eye. These results also suggest that tissues engineered to express FasL (for example as a transgene) may be protected from rejection, since they will destroy invading lymphocytes.

Shima et al. (126) have discussed the question of how myeloma cells sensitive to anti-Fas may survive *in vivo*. They hypothesised several possibilities:

1. Apoptosis may occur *in vivo* but the cells may proliferate more rapidly than they undergo apoptosis.
2. Fibroblast conditioned medium has been shown to decrease the apoptosis of human immunodeficiency virus (HIV) infected mononuclear cells suggesting the possible existence of a factor that inhibited the apoptosis (295). In support of this theory, Westendorf et al. (236) observed that plasma cells in freshly isolated bone marrow (MNC) cultures were resistant to Fas-mediated apoptosis, but that isolated plasma cells were sensitive. This suggests that a bone marrow MNC-derived protective factor(s) may be secreted, or a cell-cell mediated signal delivered which negates Fas-induced cell death. It has been postulated that there may be a soluble form of Fas antigen in vivo which could interfere with the interaction between Fas and its ligand (296).
3. Most myeloma cells express CD38 antigen and it has also been postulated that a signal through this molecule may protect cells from apoptosis (297).

4. Impaired T cell cytotoxicity, including abnormal Fas ligand expression, may be involved in the pathogenisis of this disease.

Activation via the death domains of TNFR1 and Fas triggers apoptosis and activation of the transcription factor NF-κB (298,299). Since the adhesion of myeloma cells to BMSC has been shown to result in the induction of IL6 transcription in BMSC which is conferred through the NF-kB binding motif in the IL6 promotor (317) it is possible that IL-6 interferes with the onset of Fas-mediated apoptosis in myeloma cells by inhibition at some point during its signaling pathway. The protective effect exerted by IL-6 in those IL-6-independent cell lines, normally susceptible to Fas-mediated AICD (Appendix 3) would support this theory.

Activation of CD95 recruits the Fas-associated death domain-containing molecule FADD which in turn binds and presumably activates the FADD-like ICE (FLICE), a member of the ICE family of proapoptotic proteases (303,304,315). FLICE has homology to both FADD and the ICE family of cysteine proteases. It binds to the death effector domain of FADD and upon overexpression induces apoptosis. A dominant negative derivative of FADD (FADD-DN) has been shown to disrupt the assembly of the Fas signalling complex (316). It is hoped that expression vectors containing *bcl-2*, *bcl-x_L*, *crmA* and a dominant negative inhibitor of FADD (FADD-DN) will soon become available to this laboratory and that these tools will enable further study of the Fas signaling pathway in these cell lines. The FADD-DN construct inserted into myeloma cells will allow the Fas pathway to be blocked and the effect of IL-6 on this will be interesting to observe

All these possibilities remain to be fully explored and either proven or discounted. It is exciting to imagine that the aetiology of this disease, which has been relatively little researched compared to other forms of leukaemia, may be within reach. Multiple myeloma may eventually be treatable due to the rapid advances in the study of cell death mediated by a final common pathway of protease cascade activation which could potentially be blocked or triggered at various stages in its execution.

Table 7
Comparison of results

Cell line	ISH (Gene transcription detected)						RT-PCR (Transcription)					ICC (Gene translation detected)	FACS (Translation)			
	bcl-2	IL-6R	CD40L	FasL	Fas	CD40	bcl-2	IL-6	IL-6R	Fas	CD40	Bcl-2	Bcl-2	IL-6R	Fas	CD40
JIM-1	+	-	-	-	+weak	+	+	-	+	+	+	+	+	-	-	-
JIM-3	+weak	-	-	-	+	+	+	-	+	+	+	+	+	-	+	+
JJN-3	+	-	-	-	+	+	+	-	-	+	+	+	+	-	+	+weak
U266	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	-
IM-9	+	-	-	-	+	++	+	-	-	+	+	+	+		+	+
RPMI-8226	+weak	-	-	-	+	++	+	-	+	+	+	+	+/-	+	+	+
ANBL-6(IL-6 dep.)	+	-	-	-	+weak	++	+					+	-	+	+	+
ANBL-6(IL-6 indep.)	+weak	-	-	-	+	+	+	-	+	+	+	+	+/-	+	+	+weak
JT						++										
RK						++	+					+				

	ISH	RT-PCR	ICC	FACS	ISH	RT-PCR	FACS	ISH	RT-PCR	FACS	ISH	RT-PCR	FACS
	bcl-2	bcl-2	Bcl-2	Bcl-2	IL-6R	IL-6R	IL-6R	Fas	Fas	Fas	CD40	CD40	CD40
JIM-1	+	+	+	+	-	+	-	+	+	-	+	+	-
JIM-3	+	+	+	+	-	+	-	+	+	+	+	+	+
JJN-3	+	+	+	+	-	-	-	+	+	+	+	+	+
U266	+	+	+	+	-	+	+	+	+	+	+	+	-
IM-9	+	+	+	+	-	-		+	+	+	+	+	+
RPMI-8226	+	+	+	+	-	+	+	+	+	+	+	+	+
ANBL-6(IL-6 dep.)	+	+	+	-	-		+	+		+	+		+
ANBL-6(IL-6 indep.)	+	+	+	+	-	+	+	+	+	+	+	+	+
JT											+		
RK		+	+								+		

ISH in situ hybridisation, RT-PCR reverse transcription PCR, ICC immunocytochemistry, FACS fluorescent antibody cell sorting, dep.=dependent, indep.=independent

LIST OF APPENDICES

APPENDIX 1

Effect on proliferation or inhibition of such by CD40 and Fas-ligation of myeloma cell lines with or without accessory cytokines. Comparison between the a) CD40, b) CD40Lig-L culture systems and c) culture with no accessory L-cells.

The results of replicate experiments are summarised using these three culture systems as follows:

1. By tritiated thymidine uptake with the percentage variation in counts between triplicate wells in brackets. The mean of replicate experiments is tabulated and the percentage proliferation or inhibition of proliferation calculated as follows:

* % proliferation = - [1-^{3}H-TdR incorporation with anti-CD40 MoAb/(-)]x100

  % inhibition = [1-^{3}H-TdR incorporation with anti-Fas MoAb/(-)]x100

(-) = counts per minute for myeloma cells cultured with no additive

* Proliferation due to cytokine co-stimulation ± CD40 ligation also calculated using this formula.

In all experiments the background uptake of tritiated thymidine has been subtracted except where otherwise stated. Background counts were those obtained when a row of twelve wells of a microtitre plate were incubated for three days in the presence of medium alone (no L cells), or medium in wells containing irradiated CD32L (CD40 system assays) or CD40Lig-L cells only.

2. Cell count (mean) by trypan blue dye exclusion (x10^{6})

3. Average percentage viability calculated by cell counts and morphological analysis.

Appendix 1.1

JIM-1 (CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas
Thymidine uptake in cpm	203	18829 (16)	17071 (9)	16701 (17)	14062 (25)
(% variation in cpm of triplicate wells)	265	11487 (13)	14220 (10)	17087 (4)	12217 (4)
	546	12959 (15)	12209 (7)	11574 (12)	9243 (12)
	213	11691 (24)	14664 (12)	13577 (7)	11929 (9)
	179	11086 (23)	11939 (12)	10095 (13)	8145 (11)
mean (cpm)		13210	14020	13806	11119
% proliferation			6	5	
% inhibition of proliferation					16
Cell count (mean of above) x10-6		2.9	4.6	7	2.8
% viability (mean of above)		90	91	88	86

JIM-1(blanks not subtracted, CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	889	12162 (15)	14169 (5)	14595 (2)	12883 (18)	15508 (23)	11193 (14)	10882 (9)
(% variation in triplicate wells)	10138	8084 (6)	8972 (19)	7726 (15)	7363 (24)	7676 (14)	6678 (23)	5163 (4)
	16092	13887 (19)	18529 (29)	16259 (7)	21097 (4)	16631 (12)	10346 (13)	13355 (4)
mean (cpm)	9040	11378	13890	12860	13781	13272	9406	9800
%proliferation			22	13	21	17		
% inhibition of proliferation							32	14
Cell count (mean) x10-6		1.6	2.6	4.4	3.9	2.6	1.8	2.1
% viability		77	83	89	85	78	79	76

JIM-1(no L cells)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	62	8432 (25)	8098 (6)	7635 (21)	7751 (4)	10324 (12)	9091 (25)	8960 (11)
(% variation in triplicate wells)	400	6185 (10)	6464 (10)	8364 (17)	9076 (13)	6353 (21)	6119 (20)	7752 (16)
	122	7861 (22)	7155 (5)	8675 (11)	6634 (12)	7499 (25)	6513 (14)	8297 (17)
	54	6823 (4)	10204 (10)	10299 (20)	10404 (18)	8962 (24)	6840 (2)	7853 (11)
mean (cpm)		7325	7980	8743	8466	8285	7141	8216
% proliferation			9	19	16	13		12
% inhibition							3	

Appendix 1.2

JIM-3 (CD40LigL)

Additive	blank	no addiitve	IL-4	IL-6	anti-Fas
Thymidine uptake (cpm)	203	9096 (17)	7314 (14)	10349 (13)	4573 (10)
(% variation in cpm of triplicate wells)	213	6856 (7)	6141 (12)	5745 (20)	4284 (10)
	265	3991 (16)	4265 (4)	6349 (18)	3057 (20)
	546	4438 (15)	4094 (14)	5006 (10)	3184 (13)
mean (cpm)		6095	5454	6862	3775
% proliferation			0	13	
% inhibition of proliferation					38
Cell count (mean of above) x10-6		2.3	1.1	1.4	0
% viability (mean of above)		79	81	86	0

JIM-3 (blanks not subtracted, CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	889	8789 (24)	8132 (15)	11289 (8)	14879 (9)	9650 (11)	5188 (5)	6984 (9)
(% variation in triplicate wells)	4110	5409 (12)	5783 (12)	6868 (14)	9837 (12)	6019 (12)	4234 (3)	4574 (4)
	16092	5905 (17)	5744 (22)	6527 (8)	5932 (15)	5200 (10)	3215 (12)	3810 (19)
mean (cpm)	7030	6701	6553	8228	10216	6956	4212	5123
% proliferation			0	23	52	4		
% inhibition of proliferation							37	24
Cell count (mean) x10-6		1.3	1	0.9	0.8	1.9	0	0
% viability		75	69	69	73	73	0	0

JIM-3 (no L cells)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	586	4459 (20)	5908 (7)	5314 (23)	6215 (23)	4638 (3)	1553 (23)	2539 (26)
(% variation in triplicate wells)	400	5032 (16)	5384 (21)	5224 (6)	10193 (24)	6369 (14)	3322 (6)	3253 (9)
	122	6263 (9)	7485 (20)	6645 (15)	7588 (10)	10068 (6)	2425 (20)	
	54	6498 (13)	4701 (19)	6976 (6)	9819 (25)	6545 (14)	3714 (21)	3723 (4)
	57	4027 (15)	4625 (20)	3429 (23)	5935 (15)	3549 (3)	2772 (9)	2700 (6)
mean (cpm)		5256	5621	5518	7950	6234	2757	3054
% proliferation			7	5	51	19		
% inhibition of proliferation							48	42

Appendix 1.3

JJN-3 (CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas
Thymidine uptake (cpm)	183	8517 (15)	8280 (12)	9428 (2)	7308 (21)
(% variation in cpm of triplicate wells)	207	8469 (23)	8126 (20)	7685 (8)	5985 (20)
mean (cpm)		8493	8203	8557	6647
% proliferation			0	0	
% inhibition of proliferation					22
Cell count (mean of above) x10-6		0.4	0.5	0.9	0.06
% viability (mean of above)		82	84	92	30

JJN-3 (CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	634	5336 (2)	6748 (20)	6196 (20)	8469 (4)	6237 (20)	0 (11)	4191 (12)
(% variation in triplicate wells)	267	6901 (20)	4539 (10)	7174 (16)	5670 (18)	6451 (15)	6180 (25)	4818 (21)
	4318	5006 (24)	5375 (15)	6534 (8)	7947 (11)	3075 (13)	0 (14)	0
mean (cpm)		5748	5554	6635	7362	5254	2060	3003
% proliferation			0	15	28	0		
% inhibition of proliferation							64	48
Cell count (mean) x10-6		0.6	0.7	2.5	1	1.3	0	0
% viability		90	86	91	74	82	0	0

JJN-3 (no L cells)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	197	6088 (16)	7125 (20)	8664 (6)	6758 (20)	7546 (17)		5279 (15)
(% variation in triplicate wells)	83	6649 (20)	9323 (15)	8258 (21)	10803 (15)	5913 (19)	3074 (8)	4703 (17)
	84	12373 (17)	14460 (21)	13039 (18)	12116 (12)	12630 (6)	7204 (8)	6737 (6)
mean (cpm)		8370	10303	9987	9892	8696	5139	5573
% proliferation			23	19	18	4		
% inhibition of proliferation							39	33

Appendix 1.4

U266 (CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas
Thymidine uptake (cpm)	213	9291 (24)	10174 (10)	10195 (17)	9503 (5)
(% variation in cpm of triplicate wells)	287	6165 (14)	6844 (12)	8109 (20)	8106 (11)
	179	7310 (7)	8824 (14)	8131 (18)	11164 (25)
mean (cpm)		7589	8614	8812	9591
% proliferation			14	16	26
% inhibition of proliferation					
Cell count (mean of above) x10-6		0.5	1.4	1.4	0.6
% viability (mean of above)		62	83	83	57

U266 (Blanks not subtracted, CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	889	11035 (6)	9026 (11)	13115 (4)	10413 (22)	10659 (5)	8355 (7)	10638 (20)
(% variation in triplicate wells)	8,167	6853 (9)	10124 (14)	7593 (6)	12122 (22)	9005 (16)	5590 (20)	5798 (14)
mean (cpm)		8944	9575	10354	11268	9832	6973	8218
% proliferation			7	16	26	10		
% inhibition of proliferation							22	8
Cell count (mean) x10-6		1.3	2	5	5	1.3	0.7	1
% viability		60	71	72	80	71	56	61

U266 (no L cells)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	400	5267 (12)	5050 (11)	5557 (4)	6178 (20)	5488 (11)	3930 (15)	6520 (16)
(% variation in triplicate wells)	122	6376 (7)	8463 (9)	9341 (7)	8840 (19)	8383 (18)	5624 (22)	7485 (7)
	84	8450 (14)	7642 (2)	7914 (19)	10434 (12)	7471 (18)	8948 (7)	9771 (11)
mean (cpm)		6698	7052	7604	8484	7114	6167	7925
% proliferation			5	14	27	6		18
% inhibition of proliferation							8	

Appendix 1.5

IM-9 (CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas
Thymidine uptake (cpm)	331	197000 (9)	189000 (10)	189000 (12)	4811 (20)
(% variation in cpm of triplicate wells)	213	76354 (19)	61369 (23)	63378 (5)	4558 (24)
	287	93530 (5)	97121 (7)	112000 (7)	15262 (11)
	179	94638 (12)	98188 (18)	105000 (11)	10247 (25)
mean (cpm)		115381	111420	117345	8720
% proliferation			0	2	
% inhibition					92
Cell count (mean of above) x10-6		4.8	7.4	7.6	0.04
% viability (mean of above)		95	94	94	6

IM-9 (CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	2617	203000 (3)	213000 (15)	196000 (11)	299000 (13)	161000 (6)	16121 (20)	21126 (15)
(% variation in triplicate wells)	3288	317000 (18)	328000 (3)	307000 (9)	309000 (15)	279000 (13)	8899 (12)	5214 (5)
mean (cpm)		260000	271000	252000	304000	220000	12510	13170
% proliferation			4	0	17	0		
% inhibition of proliferation							95	95
Cell count (mean) x10-6		2.8	3.9	4	4	5	0.06	0.07
% viability		87	92	90	92	96	7	12

IM-9 (no L cells)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	83	347000 (21)	412000 (3)	386000 (15)	406000 (17)	269000 (14)	14859 (20)	14147 (8)
(% variation in triplicate wells)	58	382000 (12)	412000 (13)	379000 (6)	504000 (14)	327000 (6)	7743 (8)	13847 (18)
	148	329000 (6)	272000 (11)	248000 (22)	254000 (16)	305000 (12)	4874 (14)	6896 (12)
mean (cpm)		353000	365000	338000	388000	300000	9159	11630
% proliferation			3	0	10	0		
% inhibition of proliferation							97	97

Appendix 1.6

RPMI-8226 (CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas
Thymidine uptake (cpm)	203	9840 (23)	11436 (15)	15493 (16)	5545 (20)
(% variation in cpm of triplicate wells)	265	8472 (12)	9262 (23)	12932 (12)	3617 (16)
	546	4266 (29)	3404 (9)	4417 (21)	2863 (11)
mean (cpm)		7526	8034	10947	4008
% proliferation			7	45	
% inhibition of proliferation					47
Cell count (mean of above) x10-6		0.9	1	1.3	0.06
% viability (mean of above)		92	95	91	20

RPMI-8226 (CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	117	8197 (18)	8805 (14)	9891 (4)	10962 (10)	10466 (11)	3787 (16)	7425 (19)
(% variation in triplicate wells)	93	4303 (1)	5389 (6)	7098 (0.3)	7571 (15)	5107 (21)		5000 (1)
	162	4579 (6)	5380 (5)	5379 (13)	6836 (14)	5686 (9)	2929 (6)	3221 (4)
mean (cpm)		5693	6525	7456	8456	7086	3358	5215
% proliferation			15	31	49	24		
% inhibition of proliferation							41	8
Cell count (mean) x10-6		0.5	0.7	1.1	5.2	0.5	0.04	0.06
% viability		92	78	95	90	90	33	29

RPMI-8226 (no L cells)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	400	3721 (24)	5306 (17)	4560 (20)	7899 (17)	5089 (14)	3784 (15)	3784 (17)
(% variation in triplicate wells)	122	6345 (18)	8727 (13)	8706 (4)	8724 (5)	8828 (14)	2940 (20)	3809 (24)
	54	5775 (18)	6773 (15)	5784 (12)	8051 (14)	5219 (6)	4066 (7)	4269 (18)
mean (cpm)		5280	6935	6350	8225	6379	3597	3954
% proliferation			31	20	56	21		
% inhibition of proliferation							32	25

Appendix 1.7

ANBL-6 (17/2), (CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas
Thymidine uptake (cpm)	819	4696 (21)	3338 (16)	4652 (15)	1221 (17)
(% variation in cpm of triplicate wells)	265	7833 (7)	8109 (11)	9381 (21)	2672 (23)
	546	5120 (8)	4968 (12)	4468 (4)	2084 (15)
mean (cpm)		5883	5472	6167	1992
% proliferation			7	5	
% inhibition of proliferation					66
Cell count (mean of above) x10-6		1.7	1.5	1.8	0.04
% viability (mean of above)		93	92	93	2

ANBL-6 (17/2), (Blank not subtracted, CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	10138	4991 (19)	6466 (3)	7198 (14)	8971 (14)	5644 (5)	2353 (11)	2147 (15)
(% variation of triplicate wells)	16092	6400 (6)	7336 (20)	6003 (11)	7195 (6)	6998 (2)	2823 (16)	3504 (21)
	8944	9742 (19)	6485 (20)	9911 (5)	10579 (20)	9601 (13)	4311 (25)	3710 (25)
mean (cpm)		7044	6762	7704	8915	7414	3162	3120
% proliferation			0	9	26	5		
% inhibition of proliferation							55	56
Cell count (mean) x10-6		1.7	2.6	1.8	1.7	1.5	0.02	0
% viability		87	79	81	78	81	7	0

ANBL-6 (17/2), (no L cells)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	58	7518 (11)	10294 (25)	14931 (20)	13362 (18)	16752 (22)	3244 (24)	3106 (13)
(% variation in triplicate wells)	54	7868 (25)	11602 (11)	9759 (24)	14749 (9)	11795 (18)	3949 (12)	4294 (19)
	57	2853 (21)	5071 (22)	3808 (19)	5307 (17)	6081 (17)	1396 (11)	2889 (25)
mean (cpm)		6080	8989	9499	11139	11543	2863	3430
% proliferation			4	56	83	90		
% inhibition of proliferation							53	44

Appendix 1.8

ANBL-6 (27/2), (CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas
Thymidine uptake (cpm)	331	88594 (13)	79187 (18)	72799 (20)	9233 (9)
(% variation in cpm of triplicate wells)					
% proliferation			0	0	
% inhibition of proliferation					90
Cell count (mean of above) x10-6		1.7	1.7	1.3	0
% viability (mean of above)		89	90	93	0

ANBL-6 (27/2), (CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	2,671	9139 (14)	7993 (7)	8462 (8)	15434 (24)	8111 (10)	720 (17)	1452 (20)
(% variation in triplicate wells)	3,288	7924 (21)	10482 (20)	7573 (5)	11979 (19)	6867 (20)	0 (15)	0 (13)
mean (cpm)		8532	9238	8018	13707	7489	360	726
% proliferation			8	0	61	0		
% inhibition							96	91
Cell count (mean) x10-6		1	1	1	1	1	0	0
% viability		86	83	87	83	86	0	0

ANBL-6 (27/2), (no L cells)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	125	6013 (16)	7406 (14)	6296 (19)	8226 (23)	6829 (23)		3129 (25)
(% variation in triplicate wells)	586	6122 (17)	7811 (20)	6435 (14)	9556 (10)	6588 (11)		1942 (8)
	58	9279 (6)	12022 (15)	9474 (5)	11434 (14)	10896 (14)	2331 (20)	2916 (5)
	83	8787 (10)	12180 (21)	15226 (9)	19374 (6)	13197 (8)	2126 (15)	4538 (14)
	148	8704 (18)	7805 (17)	7250 (8)	11205 (15)	8901 (21)	3018 (16)	3213 (14)
mean (cpm)		7781	9445	8936	11959	9282	2492	3148
% proliferation			21	15	54	19		
% inhibition of proliferation							68	60

Appendix 1.9

ANBL-6 (22/5), (CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas
Thymidine uptake (cpm)	287	5974 (19)	7079 (5)	7528 (6)	4168 (7)
(% variation in cpm of triplicate wells)	183	6292 (21)	7562 (9)	7025 (21)	5663 (6)
	207	7273 (10)	6066 (5)	5293 (18)	4507 (6)
mean (cpm)		6513	6902	6615	4779
% proliferation			6	2	
% inhibition of proliferation					27
Cell count (mean of above) x10-6		3.3	3.4	4.2	0.01
% viability (mean of above)		94	95	96	7

ANBL-6 (22/5), (CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	117	11612 (13)	11153 (5)	15318 (16)	15631 (2)	14368 (11)	3074 (9)	3323 (15)
(% variation of triplicate wells)	93	9367 (22)	13918 (22)	12229 (14)	12323 (11)	8132 (15)		4799 (7)
	162	9435 (15)	12428 (6)	10259 (7)	14614 (5)	11245 (8)	3244 (19)	4466 (11)
	1,086	8453 (18)	9953 (11)	10478 (15)	8468 (7)	9511 (14)	3400 (5)	2863 (14)
mean (cpm)		9717	11863	12071	12759	10814	3239	3863
% proliferation			22	24	31	11		
% inhibition of proliferation							67	60
Cell count (mean) x10-6		2.2	1.6	0.7	3.4	1.2	0	0
% viability		85	88	87	83	84	0	0

ANBL-6 (22/5), (no L cells)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	62	9650 (14)	12739 (6)	11216 (7)	12148 (5)	11078 (8)	3401 (19)	3912 (11)
(% variation of triplicate wells)	125	5499 (24)	7272 (16)	11486 (19)	11979 (25)	6860 (25)	3001 (26)	2902 (22)
mean (cpm)		7575	10006	11351	12064	8969	3201	3407
% proliferation			32	50	59	18		
% inhibition of proliferation							58	55

Appendix 1.10

ANBL-6 (16/1) (CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas
Thymidine uptake (cpm)	331	3979 (22)	6404 (20)	44729 (21)	3743 (22)
(% variation in triplicate wells)	287	5718 (15)	5831 (14)	6503 (3)	5538 (11)
	179	4474 (7)	4059 (3)	3357 (8)	3688 (25)
mean (cpm)		4724	5431	18196	4323
% proliferation			15	285	
% inhibition of proliferation					8
Cell count (mean of above) x10-6		0.3	0.4	0.5	0.5
% viability (mean of above)		82	79	86	78

ANBL-6 (16/1), (Blanks not subtracted, CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	3288	5761 (10)	5516 (4)	4912 (18)	9930 (6)	7062 (3)	4461 (13)	4936 (15)
(% variation in triplicate wells)	4318	7122 (25)	8159 (12)	8227 (13)	6670 (10)	8526 (20)	5740 (10)	7815 (17)
	7037	5878 (4)	5975 (7)	5008 (12)	5395 (18)	5045 (4)	6742 (13)	5143 (1)
mean (cpm)		6254	6550	6049	7332	6878	5648	5965
% proliferation			5	3	17	10		
% inhibition of proliferation							10	5
Cell count (mean) x10-6		0.3	0.5	1	0.6	1	1.4	0.2
% viability		100	90	94	91	93	93	92

Appendix 1.11

ANBL-6 (26/12) (CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas
Thymidine uptake (cpm)	331	4145 (25)	7839 (25)	28396 (12)	3097 (17)
(% variation in triplicate wells)	287	4745 (12)	5084 (15)	5300 (17)	3513 (10)
	183	4595 (16)	5163 (15)	5376 (14)	6651 (7)
mean (cpm)		4495	6029	13024	4420
% proliferation			34	190	
% inhibition of proliferation					0
Cell count (mean of above) x10-6		0.6	1.5	1	0.6
% viability (mean of above)		88	95	88	100

ANBL-6 (26/12), (Blanks not subtracted, CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	3288	3802 (13)	4145 (2)	4530 (15)	9798 (20)	7014 (14)	4667 (11)	4589 (15)
(% variation in triplicate wells)	4318	6058 (14)	6203 (25)	6498 (4)	8600 (20)	9006 (21)	5257 (10)	6482 (20)
	7037	7560 (15)	7887 (4)	8847 4)	9456 (7)	9287 (7)	8478 (6)	9753 (8)
mean (cpm)		5807	6078	6625	9285	8436	6134	6941
% proliferation			5	14	60	45		19
% inhibition of proliferation							0	
Cell count (mean) x10-6		1	1	0.8	1	0.9	0.7	0.4
% viability		95	92	100	97	94	97	87

ANBL-6 (26/12) (no L cells)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	148	2897 (7)	2816 (21)	2918 (13)	4212 (12)	4277 (25)	3044 (15)	2996 (10)
(% variation in triplicate wells)	70	4702 (6)	4544 (13)	4544 11)	9326 (20)	6691 (1)	4381 (14)	4632 (7)
mean (cpm)		3800	3680	3731	6769	5484	3713	3814
% proliferation			3	2	78	44		
% inhibition of proliferation							0	0

Appendix 1.12

ANBL-6 (P32) (CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas	anti-Fas+IL-6
Thymidine uptake (cpm)	183	3097 (26)	8742 (23)	126000 (9)	4007 (20)	
(% variation in triplicate wells)	207	458 (8)	3874 (13)	52818 (22)	608 (10)	52103 (15)
mean (cpm)		1778	6308	89409	2308	52103
% proliferation			250	490	30	280
% inhibition of proliferation						
Cell count (mean of above) x10-6		0.3	0.6	0.5	0.5	0.9
% viability (mean of above)		85	82	87	73	73

ANBL-6 (P32), (Blanks not subtracted, CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas	Anti-CD40+anti-Fas+IL-6
Thymidine uptake (cpm)	4318	111000 (13)	130000 (10)	152000 (10)	383000 (15)	393000 (18)	131000 (7)	144000 (11)	
(% variation in triplicate wells)	8167	42784 (26)	72263 (2)	65091 (6)	114000 (2)	121000 (5)	69205 (16)	64898 (5)	365000 (12)
mean (cpm)		76892	101132	108546	248500	257000	100103	104449	365000
% proliferation			32	41	220	230	30	36	370
% inhibition of proliferation									
Cell count (mean) x10-6		0.4	0.4	0.9	0.9	0.6	0.1	0.4	0.3
% viability		100	95	90	94	87	64	81	90

ANBL-6 (P32) (no L cells)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	70	1292 (11)	1142 (13)	1354 (24)	123000 (8)	130000 (8)	1245 (12)	1050 (22)
(% variation in triplicate wells)								
% proliferation			0	5	940	996	0	
% inhibition of proliferation								19

Appendix 1.13

JT (CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas
Thymidine uptake (cpm)	183	2865 (15)	2472 (15)	2572 (16)	2257 (14)
(% variation in triplicate wells)	207	1591 (24)	1893 (13)	1775 (9)	2223 (25)
mean (cpm)		2228	2183	2174	2240
% proliferation			0	0	0
% inhibition of proliferation					
Cell count (mean of above) x10-6		0.2	0.3	0.2	0.2
% viability (mean of above)		84	80	80	77

JT (Blanks not subtracted, CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	4110	5138 (18)	6352 (25)	5611 (20)	11007 (2)	7224 (19)	5361 (12)	6426 (22)
(% variation in triplicate wells)	7037	3349 (24)	4075 (21)	4925 (10)	5072 (17)	3638 (4)	3461 (3)	3360 (11)
mean (cpm)		4244	5214	5268	8040	5431	4411	4893
% proliferation			23	24				15
% inhibition of proliferation					89	28	4	
Cell count (mean) x10-6			0.08	0.1	0.3	0.3	0.3	0.2
% viability			80	66	100	94	82	75

JT (no L cells)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40+IL-6	IL-6	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	70	4442 (8)	4932 (22)	6664 (12)	6323 (22)	4530 (25)	3344 (3)	4849 (23)
(% variation in triplicate wells)								
% proliferation			11	50	42	2		
% inhibition of proliferation							25	9

Appendix 1.14

MNC (Control, CD40LigL)

Additive	blank	no additive	IL-4	IL-6	anti-Fas
Thymidine uptake (cpm)	207	47227 (22)	60927 (5)	66908 (11)	45270 (15)
(% variation in triplicate wells)					
% proliferation			29	42	
% inhibition					4

MNC (CDw32L)

Additive	blank	no additive	anti-CD40	anti-CD40+IL-4	anti-CD40 +anti-Fas	anti-Fas
Thymidine uptake (cpm)	1633	2729 (25)	14524 (10)	14068 (18)	11955 (22)	5928 (24)
(% variation in triplicate wells)	455	7777 (10)	10373 (12)	24373 (11)	6502 (24)	6648 (15)
mean (cpm)		5253	12449	19221	9229	6288
% proliferation			137	266	76	20
% inhibition of proliferation						

MNC (CD40L soluble trimer)

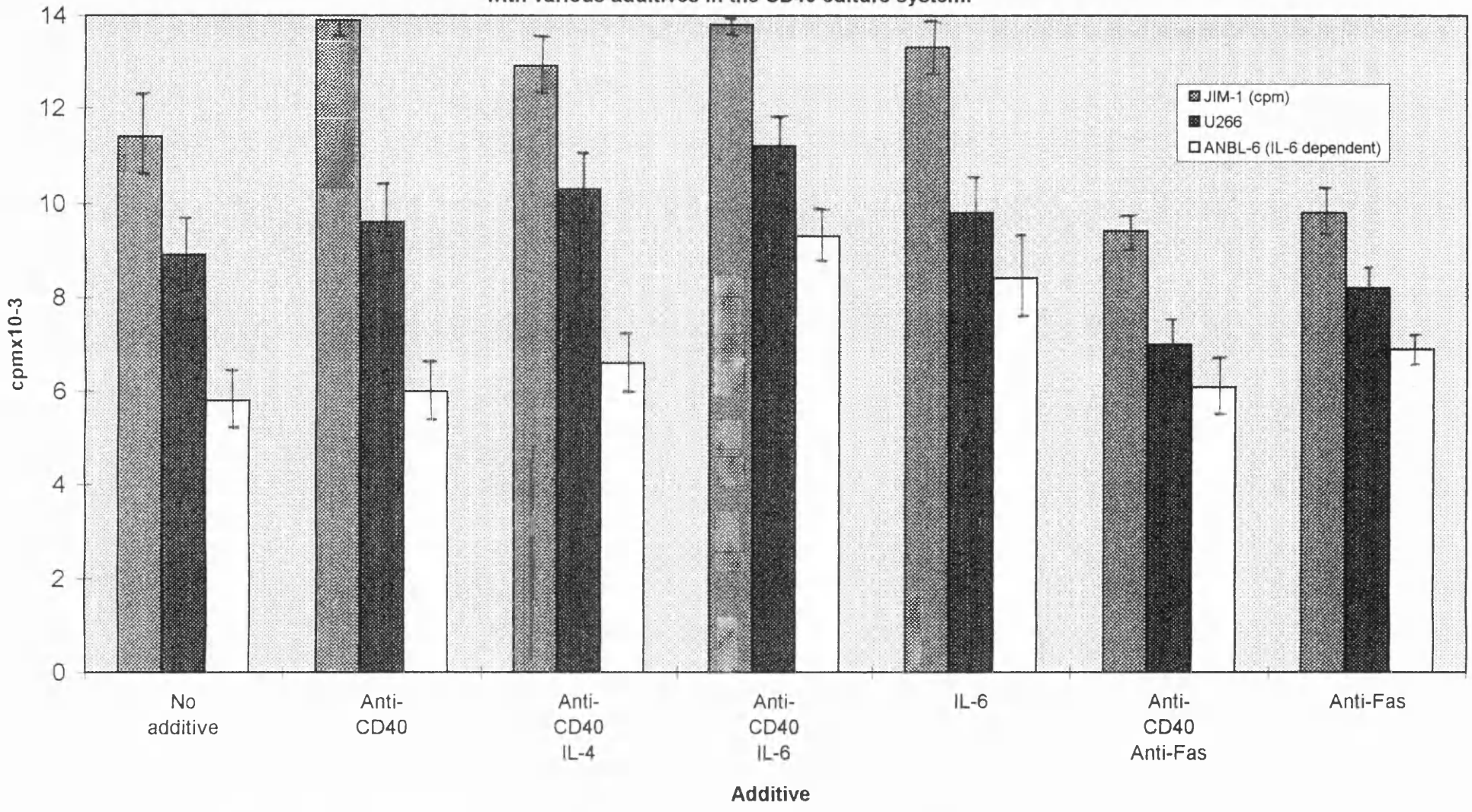
	blank	no additive	control sup.	CD40L (1/10)	CD40L+IL-4	CD40L+IL-6	CD40L+Fas	anti-Fas
Thymidine uptake (cpm)	252	4155 (20)	4008 (20)	81160 (10)	83557 (14)	39086 (6)	25672 (20)	2014 (13)
(5 variation in triplicate wells)								
% proliferation			0	1853	1911	841	518	
% inhibition of proliferation								52

Abbreviations:

Control Sup. = control supernatant
CD40L (1/10) = soluble trimer of human CD40 ligand (a 1:10 dilution)
MNC = mononuclear cells

Figure 7.1

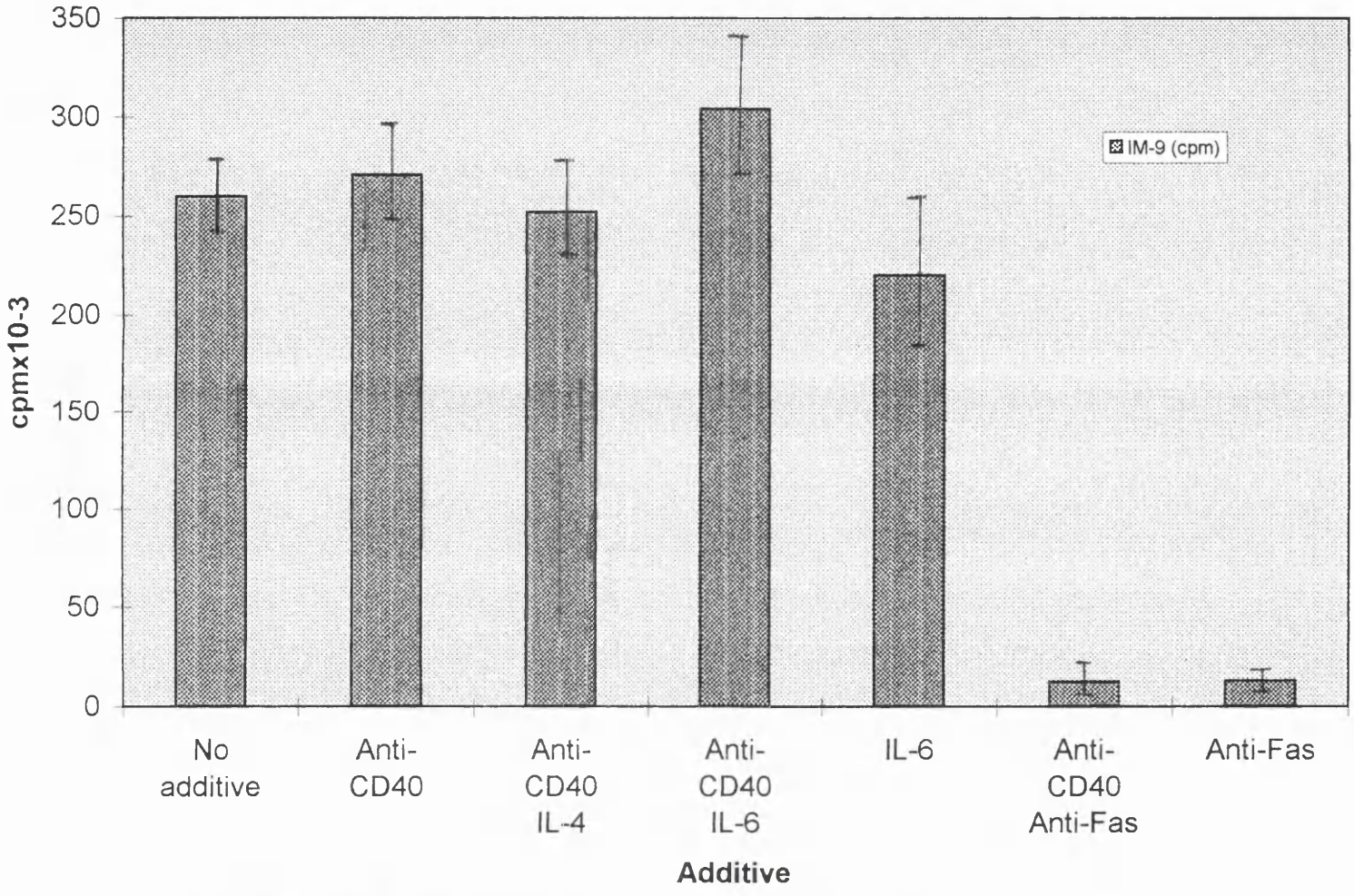
Tritiated thymidine uptake in myeloma cell lines non-susceptible to Fas-mediated apoptosis after a 3 day incubation with various additives in the CD40 culture system.



Error bars depict the standard error of the mean (S.E.M.) of replicate experiments

Figure7.2

Tritiated thymidine uptake in the myeloma cell line IM-9, susceptible to Fas-mediated apoptosis after 3 days culture with various additives in the CD40 culture system.

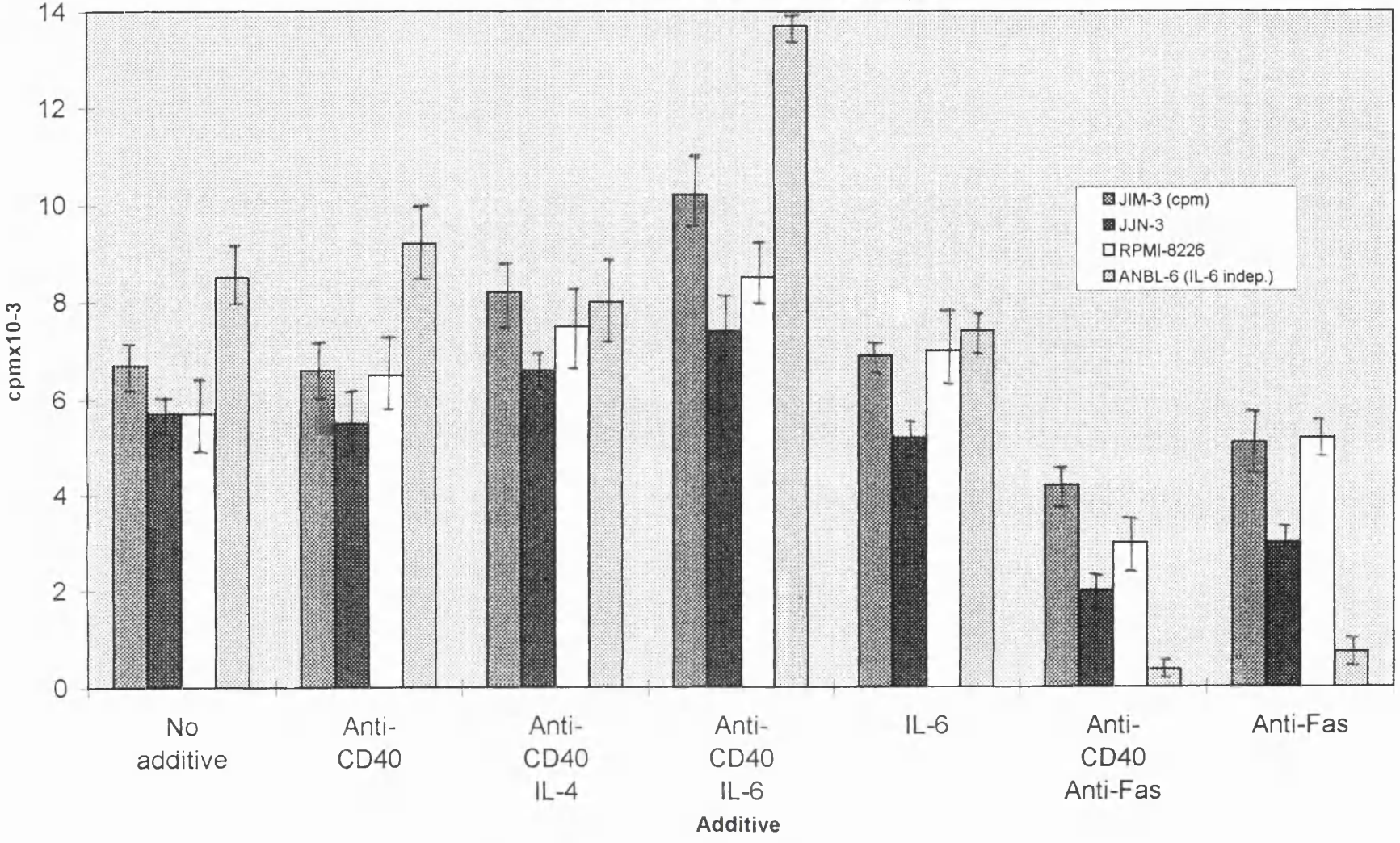


Error bars depict the standard error of the mean (S.E.M.) of replicate experiments

Appendix 2

Figure 7.3

Tritiated thymidine uptake in myeloma cell lines susceptible to Fas-mediated apoptosis after a 3 day incubation with various additives in the CD40 culture system.



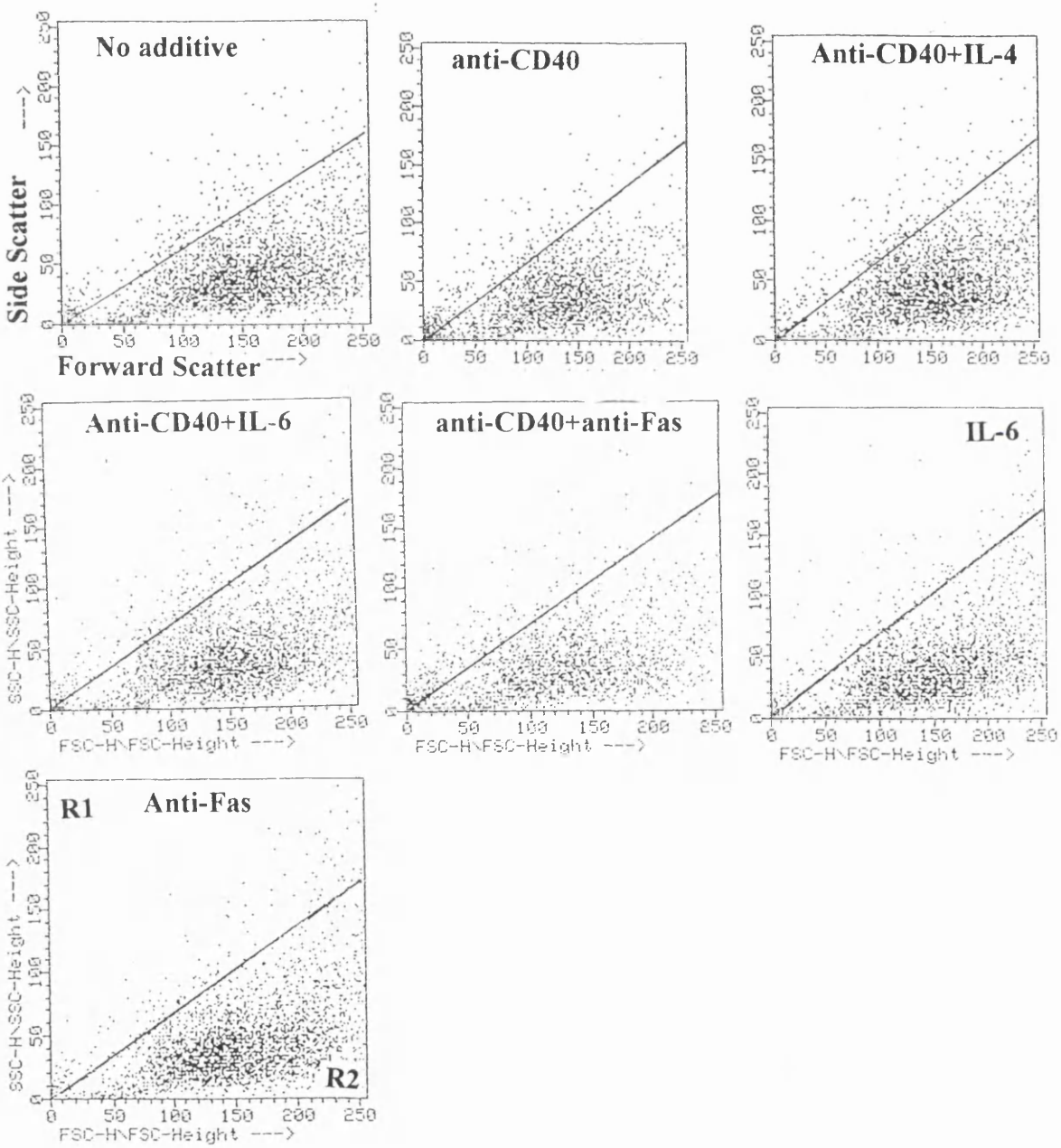
Error bars depict the standard error of the mean (S.E.M.) of replicate experiments

Appendix 2a

Figure 7.1a

Flow cytometric light scatter analysis of the myeloma cell line JIM-1 cultured in the CD40 system following a 3 day incubation with various additives. An arbritary axis was drawn to distinguish between live (R2) and apoptotic (R1) cells. 4000 cells per experiment were analysed. No shift in scatter was observed with any of the additives indicating that Fas activation had no effect on their viability.

JIM-1

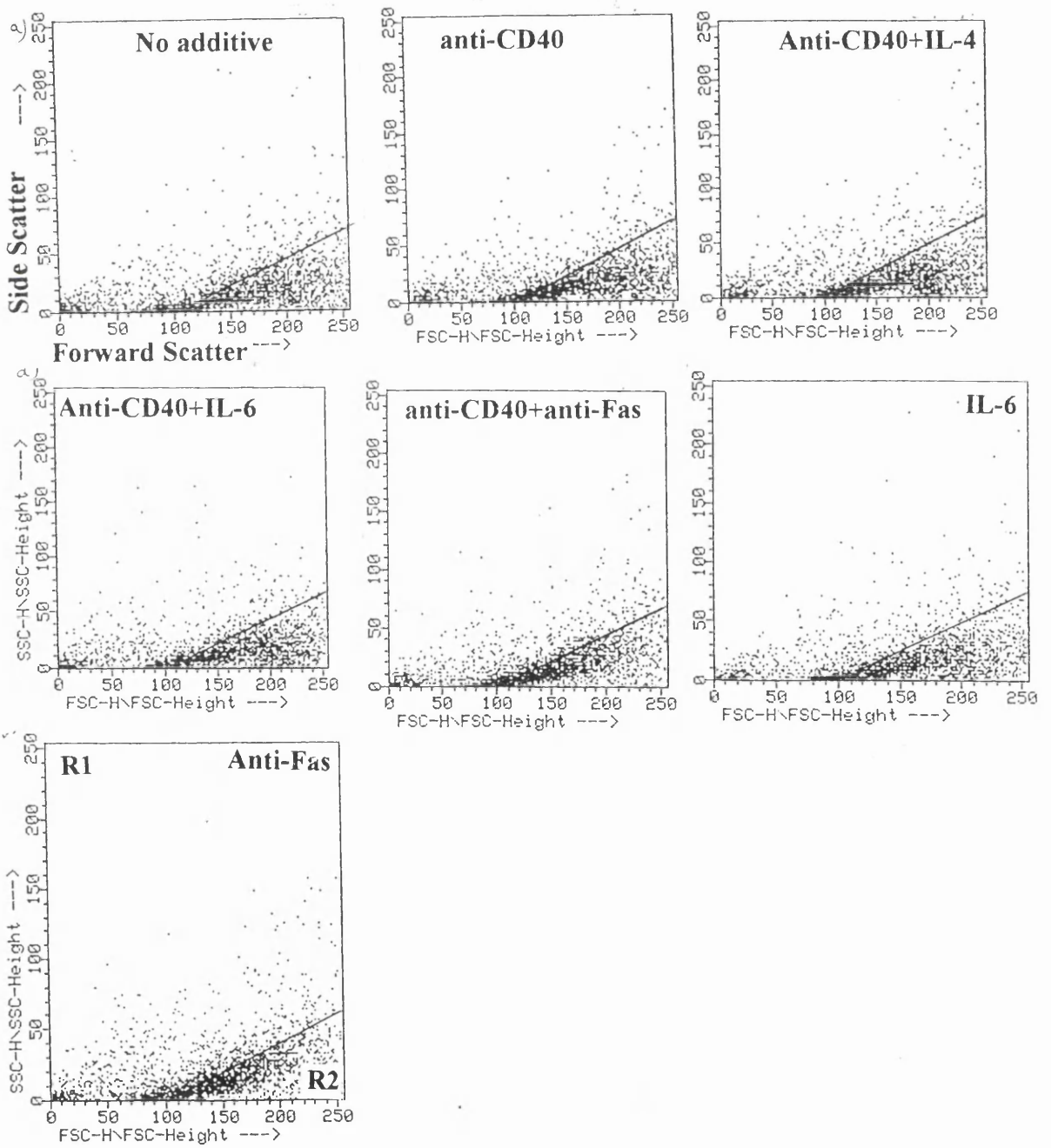


Appendix 2a

Figure 7.1b

Flow cytometric light scatter analysis of the myeloma cell line U266 cultured in the CD40 system following a 3 day incubation with various additives. An arbritary axis was drawn to distinguish between live (R2) and apoptotic (R1) cells. 4000 cells per experiment were analysed. No shift in scatter was observed with any of the additives indicating that Fas activation had no effect on their viability.

U266

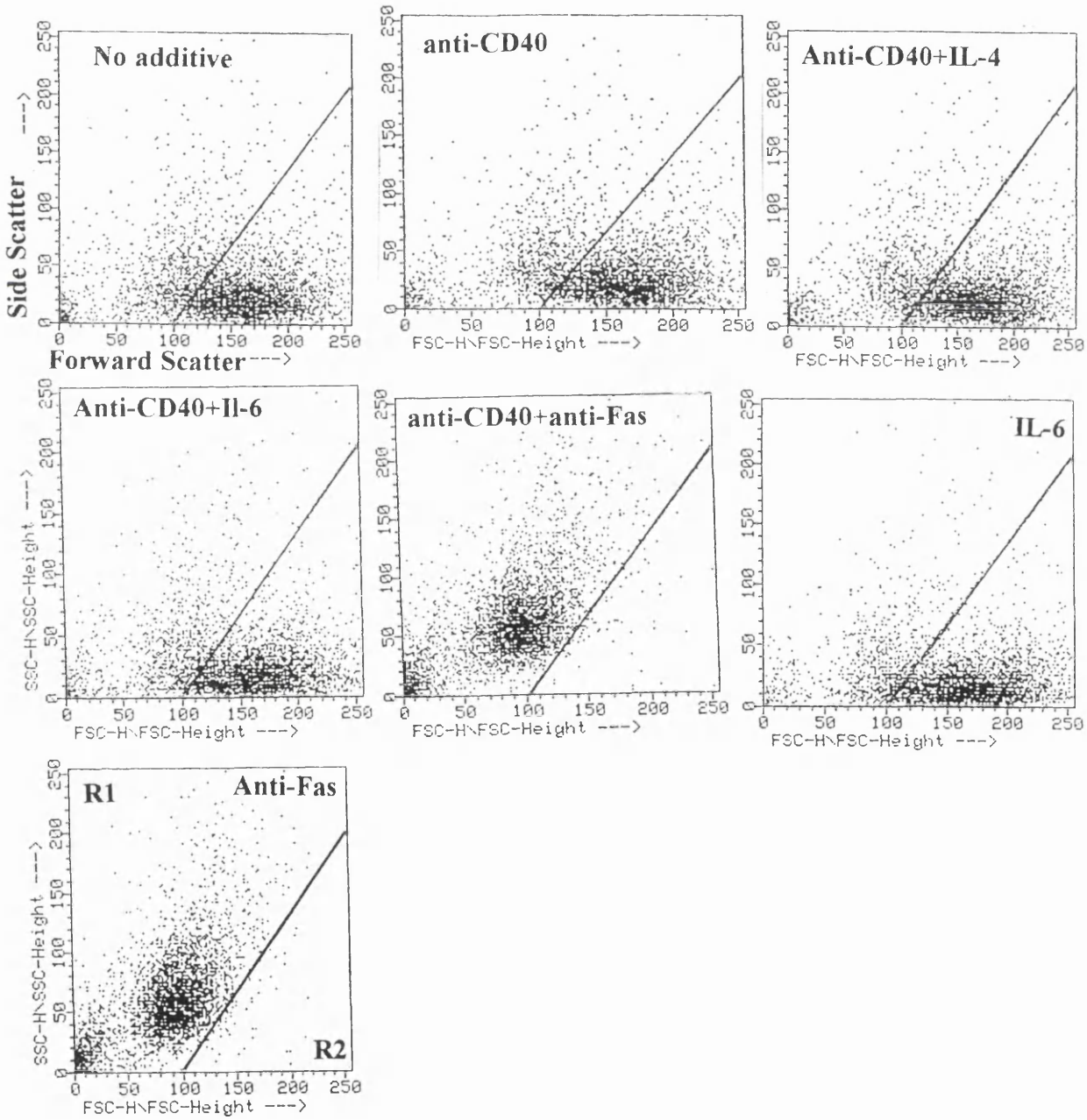


Appendix 2a

Figure 7.3a

Flow cytometric light scatter analysis of the myeloma cell line JIM-3 cultured in the CD40 system following a 3 day incubation with various additives. An arbritary axis was drawn to distinguish between live (R2) and apoptotic (R1) cells. 4000 cells per experiment were analysed. A major shift in scatter, indicative of an increase in cell granularity and a decrease in cell size, was observed in those cells incubated with anti-Fas ± anti-CD40 indicating that Fas activation had a potent apoptotic effect with or without CD40 co-ligation.

JIM-3

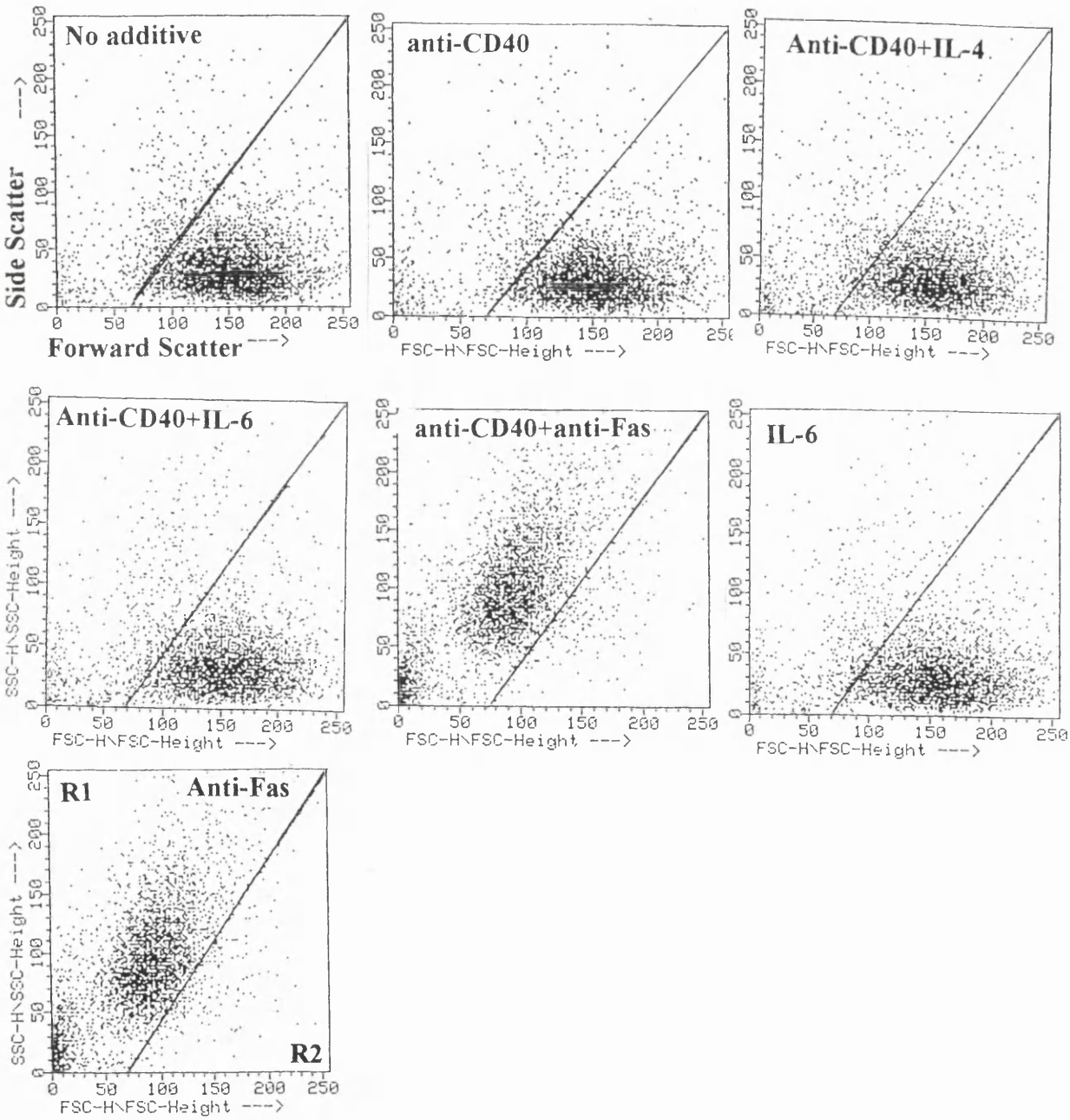


Appendix 2a

Figure 7.3b

Flow cytometric light scatter analysis of the myeloma cell line ANBL-6 (passage 17/2) cultured in the CD40 system following a 3 day incubation with various additives. An arbritary axis was drawn to distinguish between live (R2) and apoptotic (R1) cells. 4000 cells per experiment were analysed. A major shift in scatter, indicative of an increase in cell granularity and a decrease in cell size, was observed in those cells incubated with anti-Fas ± anti-CD40 indicating that Fas activation had a potent apoptotic effect with or without CD40 co-ligation.

ANBL-6 (17/2)



Appendix 3

Figure 7.4

Protective effect of IL-6 on IL-6 independent myeloma cell lines susceptible to Fas-mediated apoptosis. IM-9 and ANBL-6 (22/5) were incubated for three days in the CD40Lig-L culture system either without additives, or with IL-6 (1ng/ml); IL-6 + anti-Fas (100ng/ml); IL-6+anti-Fas+anti-CD40 (0.5μg/ml) or anti-Fas alone.

Cell line

IM-9

Method of detection:

Additive	Flow cytometry	% viability (Trypan Blue)	% inhibition (^{3}H-TdR incorp.)	Morphology (Wright's stain)
no additive	apo^-	100		92%viable
IL-6	apo^-	73	0	90% viable
IL-6+anti-Fas	$*apo^- apo^+$	44	40	50% viable
IL-6+anti-Fas+anti-CD40	apo^{++}	0	100	0% viable
anti-Fas	apo^{++}	0	100	0% viable

Cell line

ANBL-6 (22/5)

Method of detection:

Additive	Flow cytometry	% viability (Trypan Blue)	% inhibition (^{3}H-TdR incorp.)	Morphology (Wright's stain)
no additive	apo^-	98		90%viable
IL-6	apo^-	100	0	92% viable
IL-6+anti-Fas	$*apo^- apo^+$	96	0	80% viable
IL-6+anti-Fas+anti-CD40	$*apo^- apo^+$	44	42	50% viable
anti-Fas	apo^{++}	30	83	35% viable

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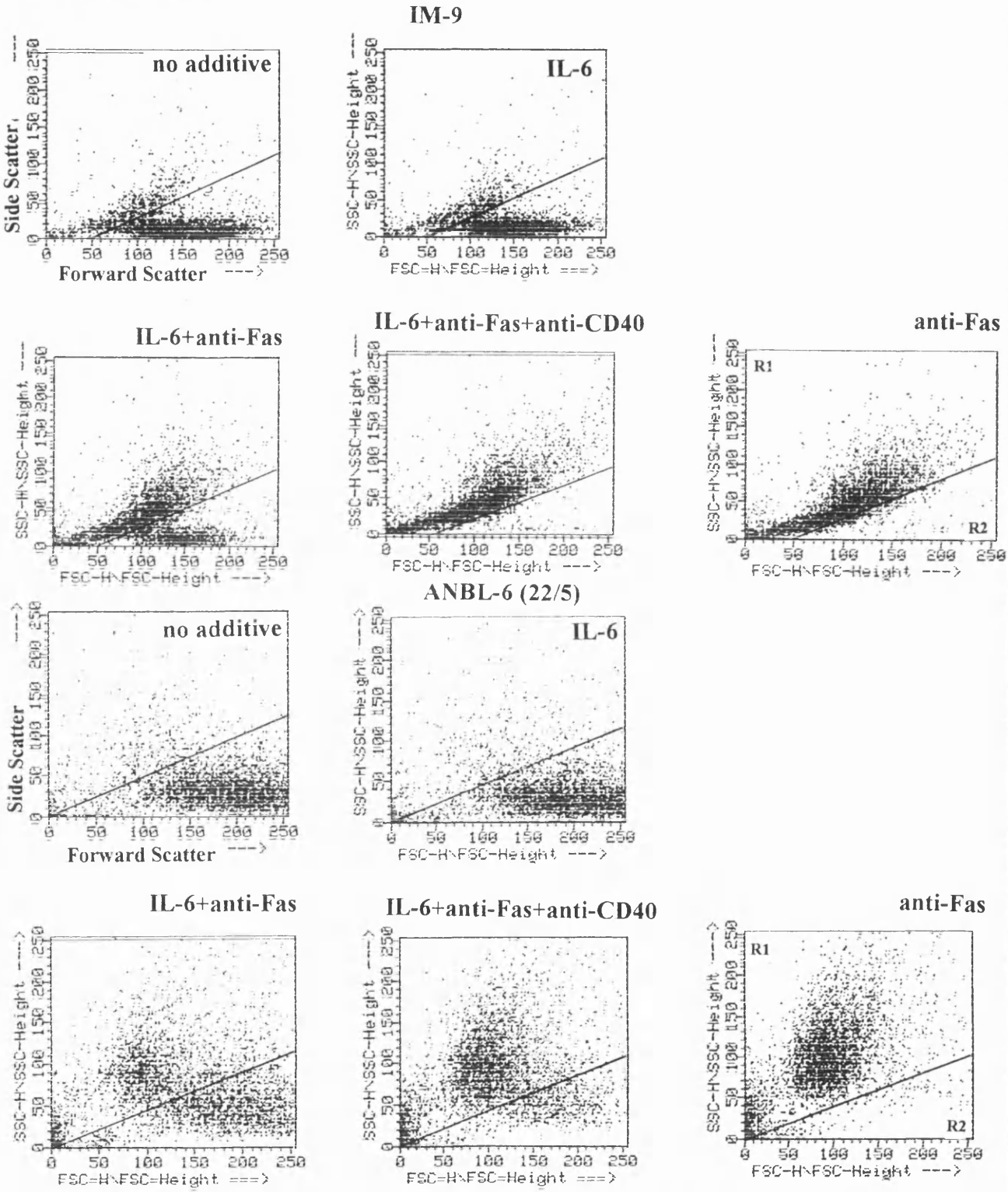
* Two populations observed
apo^- = no apoptosis observed, apo^+ = apoptosis observed,
apo^{++} = virtually 100% apoptosis observed

Detection of apoptosis was using a combination of flow cytometric light scatter analysis, trypan blue dye exclusion, morphology under light microscopy after staining the cells with Wright's stain and inhibition of proliferation assessed by tritiated thymidine uptake (^{3}H-TdR incorporation).

Appendix 3
Figure 7.5

Protective effect of IL-6 on (IL-6 independent) myeloma cell lines susceptible to Fas-mediated apoptosis as demonstrated by Flow cytometric light scatter analysis. IM-9 and ANBL-6 (22/5) are protected from Fas-induced AICD by co-incubation of IL-6 and anti-Fas, together with CD40 stimulation.



Three day culture of the myeloma cell lines ANBL-6 (passage 22/5, IL-6 independent) and IM-9 in the CD40Lig-L system. $5x10^4$ myeloma cells/well were added to an irradiated monolayer ($5x10^3$ cells/well) of mouse fibroblasts transfected with the human CD40 ligand gene in triplicate wells of a microtitre plate in a total volume of 200µl/well. Additives IL-6 (1ng/ml); anti-Fas (100ng/ml) or anti-CD40 (0.5µg/ml) were added prior to incubation. An arbritary axis was drawn to distinguish between live (R2) and apoptotic (R1) cells. 4000 cells per experiment were analysed.

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