APOPTOSIS, CELL PROLIFERATION AND ROLE OF ONCOGENES IN LYMPHORETICULAR MALIGNANCIES

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by

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PUBLICATIONS

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PREFACE

This Ph.D. research project was initiated because of the current interest in the role played by various DNA damaging agents in inducing cell death by apoptosis, and the role played by cell proliferation studies in predicting a response to chemotherapeutic agents. As regards cell proliferation, it is quite well established that tumour size bears a close relationship between its aggressiveness and proliferation rate.[1] However measuring the proliferation rate does not necessarily help in the management of a particular patient.[2] Gaining an insight into factors that influence proliferation rates by looking at oncogenes like c-myc or cell death like Bcl-2 or p53 expression, could help in planing more effective therapeutic strategy.

The induction of cell death by apoptosis as a chemotherapeutic strategy has already gained widespread credibility.[3] Kramer isolated an antibody called anti APO-I which has been shown to cause B lymphoblastoid tumour regression by inducing apoptosis.[4,5] Analogues of somatostatin and leutinising hormone have caused tumour regression in breast or pancreas via apoptotic mechanisms. Other chemotherapeutic agents like 5-fluorouracil and Topo isomerase II inhibitors or DNA damaging agents like ionising radiation also cause apoptosis.[6] For the non Hodgkin's lymphomas there are many different classifications.[7] For instance Rapport (1966) Lukes and Colin (Bennet 1974) British national lymphoma investigation (1974) Kiel classification (Gerard Merchant 1974). This creates a problem for the clinicians. Of all the classifications the Kiel classification is sounder because it combines morphology with the immunobiology of tumours and separates B from T cell types

However even with this classification tumours within the same diagnostic category can have widely differing clinical outcomes.[8] Therefore the need for studying other parameters like apoptotic rates, Bcl-2 expression in addition to proliferation rates becomes imperative if a better therapeutic strategy or improved prognostic information are to be attainable. The first chapter of this thesis gives the background to the study. It also gives information on apoptosis, cell proliferation, and role of oncogenes such Bcl-2, p53 and c-myc, in those two processes.

Chapter 2 sections 1-4 gives information about assessment and correlation of cell proliferation, Bcl-2 expression and apoptotic activity, in paraffin embedded tonsilar tissue and lymphoma cases. The rest of the sections of Chapter 2 consists of a flowcytometric study of apoptosis, cell proliferation and the role of oncogenes, in those two processes using two murine leukaemia cell lines (DP16-1 and C88), as well as two human leukaemia cell lines (Raji and HL-60). The response to irradiation in those cell lines is also investigated and the relationship between cellular differentiation and apoptosis is also explored. Chapter 3 contains the results from all the experiments in Chapter 2.
Chapter 4 is the discussion and Chapter 5 ends with recommendations for further studies.

HYPOTHESIS:

It has been described in the literature that cell death by apoptosis acts as a modulator of cell proliferation in order to maintain homeostasis. This has been shown for instance in the haemopoietic system, in the skin, and gastrointestinal system. Published scientific work carried out in cell cultures and animal studies has demonstrated the role of oncogenes such as Bcl-2 and c-myc as well as tumour suppresser genes like p53 in regulating cell proliferation. The research I undertook was to test the hypothesis that cell death by apoptosis modulates cell proliferation, and to test the role of oncogenes in the two processes.

ABSTRACT:

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TITLE: APOPTOSIS, CELL PROLIFERATION AND THE ROLE OF ONCOGENES IN LYMPHORETICULAR MALIGNANCIES.

This study was conducted using paraffin embedded material of randomly selected lymphoma cases from the archives of Leicester Royal Infirmary.

The cases had been confirmed by immunophenotyping and immunocytogenetic analysis of B and T cell gene rearrangements using PCR (Polymerase chain reaction). These included 20 each of low grade and high grade non Hodgkin's lymphomas of both sexes and age groups 4-84 yrs. Prognostic information was obtained from the case notes. An *in situ* End labelling technique was used to study apoptotic indices. Cell proliferation rates were studied by *in situ* hybridisation method to detect histone using mRNA digoxigenin-11-dUTP probe. Bcl-2 expression was assessed using antigen retrieval and an immunoperoxidase technique. Apoptotic indices, proliferation rates, Bcl-2 expression and tumour grade were correlated to each other. Flowcytometric study of four cell lines (HL-60, Raji, C88and DP16-1) was also undertaken as regards apoptosis, cell proliferation and oncogene expression. Apoptosis was assessed using existing techniques on thymocytes Cell proliferation was assessed using proliferation markers like Ki-67, PCNA and BrdUrd. Apoptosis was confirmed by Electron microscopy and DNA ladders on Gel electrophoresis. Cell differentiation was demonstrated by photomicrographs of cytospin preparations.

The study showed that the high grade lymphomas had the highest apoptotic indices and proliferation rates. More of the low grade tumours were Bcl-2 positive. Most mortalities occurred in tumours which had high proliferation rates and were Bcl-2 negative suggesting that those two parameters were bad prognostic indicators. A larger and extended study would be required to confirm my observations. For the cell lines apoptosis showed an inverse correlation with cell proliferation. There appeared to be a link between apoptosis and cell differentiation and the role of oncogenes was established. The information derived from cell lines could be exploited in designing drugs which produce apoptosis, cell differentiation and counteracting cell proliferation thus reducing tumour load.

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GLOSSARY

ACT D	Actinomycin D
BCIP	5-Bromo4 Chloro Indolyl phosphate
bp	Base pairs
BSA	Bovine Serum Albumen
CD	Cluster differentiation antigen
DEPC	Diethyl Pyrocarbonate Carbonate
DMSO	Dimethyl sulphoxide
EM	Electron microscope
FITC	Fluorescein Isothiocyanate
HP	High power field
IGH	Immunoglobulin heavy chain
J	Journal
K	Kappa
Kb	Kilobase
LB	Loading buffer
MEL	Murine leukaemia
mRNA	Messenger RNA
NaCl	Sodium chloride
nM	Nanomolar
NBT	Nitroblue tetrazolium
PK	Protein kinase
PI	Propidium iodide
PBS	Phosphate buffered saline

pM	Picamolar
SDS	Sodium dodecyl sulphate
SSD	Salmon sperm DNA
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TdT	Terminal Deoxy nucleotidyl Transferase
TE	Tris EDTA
Thaps	Thapsigargin
TNP	Trinitrophenol
TCR	T-cell receptor
UV	Ultraviolet light
UP	Ultrapure water

CHAPTER 1

INTRODUCTION

Lymphoma classification has been a source of frustration for clinicians and pathologists for many years.[8] Different entities in one classification mean different things to other people using another classification. To date there is no single classification that can predict how a particular tumour in an individual patient might behave. The need to look at other prognostic indicators has become increasingly relevant. Characteristically follicular NHL lymphomas are low-grade tumours of B lymphocytes which are widely disseminated at presentation and may undergo transformation to high grade.[9]

Since the study of Pekham and Cooper, it has been shown consistently that low-grade NHL exhibit low proliferative activity while high grades do the opposite. Studies using various markers of cellular proliferation like, tritiated Thymidine, Ki-67, and S-phase fraction, have indicated that assessment of this parameter is of prognostic value within the low grade.[9] A high Ki-67 is associated with reduced survival in the low grade NHL (Hall-1988).[10] A high S-phase fraction was associated with reduced survival (Susan Rehn -1990) and an S-phase fraction greater than 5% was associated with high grade transformation.[11]

CELL PROLIFERATION

Tumour growth is based on changes in the regulation of cell proliferation. In order to understand the molecular pathology of tumours, one should understand the molecular basis of cell proliferation.[1] The aggressiveness of a tumour is best characterised by its doubling time as defined by the time it takes the tumour to double its volume. Tumour size is usually related to cell number and in tumour growth these can be affected by three mechanisms:

1. Shortening of the cell cycle, which results in more cells being produced per unit time.
2. A decrease in the rate of cell death.
3. An increase in the growth fraction.

Cell proliferation is defined as an increase in cell number as a result of completion of the cell cycle.[12] Individual cells enter the cell cycle at different frequencies.[13] For example in a reactive lymph node, follicular centroblasts have a high rate of cycle entry while others like mantle zone lymphocytes have a low rate. The eukaryotic cell cycle is divided into G_1, S, G_2 and M phases. The G_1 phase is subdivided into competence, entry, progression and assembly.[12] There is also a quiescent phase known as the G_0 phase.

The events in the cell cycle are tightly controlled.[14,15] Transition between G_1/S and G_2M boundary is under the control of proteins called Cyclins.[15] Cyclin D and E control G_1/S transition, while A and B control G_2/M transition.[15,16] Cyclins act by binding activating cyclin dependent kinases (Cdk).[12,15]

Cyclin A binds to Cdk2 and Cdc2, cyclin B binds to Cdc2, cyclin E binds Cdk2 while cyclin D binds to Cdk2, 4 and 5. The configuration of cyclins with the Cdk results in phosphorylation of other proteins which are needed for the progression of the cell cycle.

Cyclin deregulation could lead to neoplastic development. The genes for cyclin A, D1, D2 and E are amplified in breast and colonic carcinomas.[15] The transition between various phases of the cell cycle is under control by certain check points. These check points ensure that M-phase follows S-phase and the S-phase does not start before the G_1 when growth factors and nutrients are available. G_2/M check point is controlled through inhibition of cyclin A and B. In yeast *Sacromyces cervercia* the rad9 gene is required.[16]

In man the G_2/M DNA damage check is not well known but it is thought to involve inhibition of Cdc2 kinase activity by inhibition of activation of Cd25 phosphatase. A p53 tumour suppresser gene (wild type) acts at G_1/S boundary following DNA damage and arrests G_1 progression to S-phase. A protein P21 which is regulated by wild p53 can bind to Cdk2 and Cdk4 and inhibits activation of cyclins A, D1, D2 and E following radiation damage. Levels of P21 rise, causing G_1/S arrest.

The mechanisms that control the cell cycle are complex. These include genetic, extra and intracellular influences.[1,17] Published information on factors that control cell cycle comes

mainly from yeast and murine fibroblasts but such information is also applicable to man.[13] In yeast for instance, a cell makes commitment to enter a cycle at a point called START.[17] In yeast *S. Pombe*, the gene controlling this process is called Cdc2. A human equivalent is a 34Kd protein designated $P34^{Cdc2}$. This protein complexes with cyclins during the cell cycle. Both *in vivo* and *in vitro*, the extent of cell proliferation is regulated by signals in the environment which are of two types, spatial restriction (contact inhibition) chemical factors and growth factors. The growth factors are by far the most important.[1]

A variety of growth factors have been identified and the most important ones from the clinical point of view are the cytokinases which are involved in the growth and differentiation of haemopoietic cells.[1,17] Other growth factors include insulin growth factor (IGFI), platelet derived growth factor (PGF), oligopeptides (Bombesin, Bradykinin, Serotonin) and steroid based compounds like oestrogens and retinoids. Autocrine, paracrine and endocrine pathways could operate to control cell proliferation, for instance autocrine and paracrine stimulation may follow production of gastrin and bombesin releasing peptides by small cell lung carcinoma. Some factors act as inhibitors of cell proliferation. These include tumour necrotic factor, TGFβ and interferon. TGFβ has also growth stimulatory effects i.e. in Schwann cells and osteoclasts in culture.

Following a growth stimulus, a series of steps involving growth factors with their receptors leads to the transmission of growth signal via cytoplasmic transduction pathways to the nucleus where DNA replication occurs.[12,18]

Step 1 Following a growth signal, extra cellular factors bind to their receptors on the cells forming a transduction particle on the plasma membrane.

Step 2 The union of receptor and growth factor leads to phosphorylation of cytoplasmic proteins with tyrosine, serine and threonine residues, further transmitting the growth signal. Three cytoplasmic pathways have been identified in this process, the phosphotidylinositol pathway, the cyclic-AMP and the phosphocholine pathway.

Step3 Activation of the phosphotydylinositol pathway follows phosphorylation of Plcγ (Phospholipase-C) which cleaves lipid Phosphatidylinositol 4,5-biphosphate (PIP2) resulting in production of 1,2 diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). The presence of DAG and calcium activates calcium dependent growth regulatory enzymes mainly PKC (Protein kinase C). The Phosphocholine pathway also uses PK via DAG. A third pathway uses Cyclic AMP to access PK (Protein kinase)

Activation of the cyclic-AMP cascade is mediated by membrane associated proteins with inihibitory subunits (G1) and stimulatory subunit (GS) which control adenyl cyclase activity and production of cyclic-AMP dependent kinases (PK) resulting in phosphorylation of target protein substrates.

Step 4 This eventually leads to induction of target genes. In case of cyclic-AMP these contain promoter elements which possess a family of related transcription factors known as CREB- binding proteins.

There are other genes like the Retinoblastoma gene which act in the opposite way i.e. suppression of cell proliferation. The gene codes for a 110-114Kd nuclear protein ($P110^{RB}$). It suppresses cell proliferation. The binding of $P110^{RB}$ by DNA viral proteins i.e. SV40 large T antigen, adenovirus E1A and human papilloma virus tends to abrogate growth inhibitory properties of 110^{RB}, thus facilitating tumour production.

Step5 After activation of the transcription genes, several enzymes for DNA replication like Transin and Ornithine decarboxylase are synthesized in early G_1 and in late G_1, dihydrofolate reductase, polymerase, thymidilate synthetase and thymine kinase are synthesised. Other proteins needed for replication like histones and native RNA are made.

Step 6 Finally the enzymes and proteins move from ribosomal site to the nucleus where DNA replication takes place. The assembly of enzymes in the nucleus ends the G_1

phase. The S-phase follows in which DNA is replicated in both directions at both sites each called a replication fork.

Step7 The cell cycle ends in the M-phase or mitotic phase when a cell divides into 2 daughter cells.

Another prognostic indicator that is currently being looked at in ever increasing detail is Apoptotic index.

APOPTOSIS

In 1972 Kerr, et al proposed apoptosis as a mode of cell death with widespread implications in tissue kinetics.[19] In 1980, Wyllie in Edinburgh defined apoptosis as a mode of cell death in which the cell participates in its own death. In some systems this is preceded by protein synthesis. He clearly defined for the first time both morphologically and biochemically, apoptosis as a mode of cell death characterised by cell shrinkage and condensation into small fragments.[20,21,22,23] Eventually the cell breaks up into small fragments surrounded by membranes (apoptotic bodies).

Biochemically it is caused by an endonuclease which cleaves internucleosomal linker DNA into DNA fragments that are 200 base pairs or multiples thereof.[20,23,24,] This is seen on gel electrophoresis as DNA ladders. This mode of cell death is to be distinguished from necrosis, in which cell death is a passive process brought about by hostile environmental conditions like viral infections, hypothermia or hypoxia.[20]

This later form of cell death is followed by disruption of the cell membrane and cytoplasmic organelles, and is followed by an inflammatory reaction. In contrast, apoptosis is an energy requiring, gene directed process which in some systems involves RNA and protein synthesis[20,21].

Apoptosis was already recognised in 1972 but its full biological significance has only recently been fully appreciated.[19] For instance, cell death in tumours occurs by apoptosis and this would have a bearing on tumour regression. In the immune system there is a

selection for those cells bearing useful high affinity antigen receptors while other cells die by apoptosis. [25]

Incidence [26]

1) **Normal Embryonic and Foetal Development.**

Apoptosis is seen during palatal fusion or inter digital web deletion. Mullerian duct regression in foetal testis. Apoptosis is seen in intestinal villus crypt remodelling.

2) **Metamorphosis**

(Amphibian tail regression).

3) **Hormone Dependent Atrophy**

Endometrium cyclic proliferation and deletion. Breast cyclic proliferation and atrophy, prostatic atrophy after castration.

4) **Growth factor Dependent Survival**

IL-2 Withdrawal from thymocytes

IL-3 Withdrawal from haemopoietic precursors

Serum deprivation from thymocytes.

5) **Immune Cell Outogeny**

Auto reactive T-cells in the immune system are eliminated by apoptotic cell death.

6) **Immune Killing**

Cytoxic T cell killing, natural killer cell killing and K-cell killing use the mechanism of apoptosis.

7) **Toxin Exposure**

i.e. gliotoxic induced macrophage death. Cytotoxic drugs like Actinomycin D induce apoptosis in cell lines such as HL-60. Cycloheximide causes cell death for instance in Raji cells by apoptosis. Topoisomerase inhibitors such as Camphotecin cause cell death by apoptosis.

8) **Irradiation**

Intestinal mucosal cells, thymocytes and leukaemic cell lines exposed to irradiation die by apoptosis.

9) **Resolution of Inflammation**

Senescent neutrophils are eliminated by apoptosis.

10) Tumour cell Deletion.

Tumour regression with or without treatment often occurs by the process of apoptosis. Using anti APO-I antibody in T-cell leukaemia can cause apoptotic cell death. Apoptosis occurs during thymic T-cell maturation and in the B-cells in germinal centers during affinity maturation. Only those cells with high affinity receptors survive while others die by apoptosis.[26,27]

Functions of cell Death[24,28]

1) Elimination of cells that appear to have no function

A nematode C. elegans has been studied extensively in this respect. It consists at birth of 1090 cells. 131 of these cells die by programmed cell death. Many of these die within 1 hour before they can differentiate. Another example is seen in the thymocytes in which T cells die before they function. In the development of the chick nervous system some cells die before they can differentiate.

2) Cells that are produced in excess

For example during the development of the chick nervous system half of the neurons are eliminated by this process and it ensures that the number of neurons closely matches the target sites. This also occurs in C. elegans.

3) Cells that develop improperly

This occurs in vertebrate neuronal development, i.e. neurons that have improper connections die by apoptosis.

4) Cells that have completed their functions

This is seen for instance in metamorphosis. In humans - elimination of senescent neutrophils.

Morphological Characteristics of Apoptosis[20,24]

There are three phases in the process according to Wyllie. Phase 1 is the reduction in nuclear size, condensation of chromatin into crescenteric caps at the periphery of the nucleus followed by nucleolar disintegration with dissolution of transcriptional complexes from the fibrillar centre. Cells undergoing apoptosis become rounded and detached from their neighbours. The cell shrinks in volume, the cytoplasmic organelles become compacted. The smooth endoplasmic reticulum dilates, the dilated cysternae fuse with

the cell membrane giving a blebbing appearance to the surface. The cytoplasmic organelles remain intact.

Phase 2 which overlaps phase 1 is heralded by blebbing of the cell surface, and crenation of nuclear outline. The nucleus splits into fragments which become bound by membranes. Apoptotic bodies are phagocytosed by neighbouring cells or macrophages. In the third and final stage there is a progressive degeneration of residual nuclear and cytoplasmic organelles. In this stage the cell membrane becomes permeable to vital dyes. Apoptotic bodies remain recognisable for very short periods of 4-6 hours and this is the time it takes for them to be completely phagocytosed by macrophages. It is this short time span and the fact that the process occurs asynchronously in tissues which have made the study of apoptosis so difficult.

Mechanisms[24,28]

Activation of endogenous endonuclease leading to cleavage of linker DNA between nucleosomes is a pivotal event in apoptosis. The enzyme is activated by Ca^{2+} and Mg^{2+} ions but inhibited by Zinc ions (Zn^{2+}).

Table 1 Triggering factors for apoptosis under different model systems

Circumstances	**Example**
Fas/Apo-1 activation	Thymocytes
TNF (Tumour necrotic factor)	Various cell lines
Beta transforming growth factor	Hepatocytes
Androgen withdrawal	Prostate
Irradiation	DP16-1 and C88 cell lines
Drugs like Actinomycin-D, Cycloheximide, Camphotecin	HL-60 cell line
Serum withdrawal	Fibroblasts

Cells grown in Zn^{2+} ion free culture medium undergo apoptosis. Support for Ca^{2+} requirement for the process comes from the evidence that HL-60 cells grown in calcium free environment do not undergo apoptosis The Ca^{2+} ionophore A23187 is a potent inducer of apoptosis. The requirement for RNA and protein synthesis during apoptosis in some biological systems like thymocytes is supported by the fact that Actinomycin D, inhibitor of RNA synthesis, and cycloheximide, an inhibitor of protein synthesis, inhibit the process of apoptosis in these cells. In other models where protein or RNA synthesis are not required such as HL-60 cell culture, these drugs cause apoptosis.

Role of Oncogenes[20,25,26,29,]

Oncogenes that have been well documented in this process include Bcl-2, p53 and c-myc in vertebrates and ced_3 and ced_4 in a nemetode - C.elegans. Bcl-2 oncogene has a protective role against apoptosis. For instance it promotes survival of B-lymphoid cells in the affinity maturation of the immune system.

1) **Bcl-2**[30,31,32,33]

Bcl-2 has been shown to exert a protective role against apoptosis. Cortical thymocytes into which Bcl-2 is engineered to be constitutively expressed fail to undergo apoptosis under different stimuli. Transgenic mice experiments show that those carrying Bcl-2 gene show prolonged B-cell survival and B-clonal expansion. Other Bcl-2 related genes are Bax. $Bclx_L$ and $Bclx_S$. $Bclx_L$ like Bcl-2 inhibits apoptosis. Bax and $Bclx_s$ promote apoptosis. In the nematode model, (C. elegans) the genes ced3 and ced4 promote apoptosis while ced9 protects cells against apoptosis, like Bcl-2 in humans.

2) **p53**[34,35,36]

Normal (wild type) p53 protects cells against DNA damaging agents like radiation and chemical agents by initiating a delay in G_1 and S phases. This gives the cell time to repair. In those which are severely damaged, p53 initiates an apoptotic program. Transgenic mice experiments prove the role of p53 in this respect. Those lacking p53 when exposed to radiation do not show any cell death by apoptosis, but their damaged cells survive and go on to develop tumours. A

tumour suppresser gene like Rb(Retinoblastoma), cell cycle progression products like Cdc2, Cyclin A and E2F-1 have also been linked with apoptosis. Overexpression of p53 and E2F-1 in cells which do not have Retinoblastoma (Rb gene) causes apoptosis.

3) **c-myc Oncogene**[37,38]

This usually participates in cell proliferation but its role in apoptosis has been shown by experiments on fibroblast cells grown in culture. In the presence of growth factors, fibroblasts respond to c-myc by proliferation. In absence of growth factors they respond by going into apoptosis. c-myc co-operates with Bcl-2 to overcome apoptosis and promote tumour development in transgenic mice.

Since the preliminary paper by Wyllie and his associates in 1980 on apoptosis, there has been a flood of papers in literature on the subject using leukaemic cell lines.[38] It has become clearly evident that there are multiple pathways to the execution of an apoptotic program. It has been demonstrated by Wojecieh Gorczyca et al. in 1992 that protease inhibitors like Disopropyl fluorophosphate (DFP) can inhibit formation of double stranded DNA breaks hence apoptosis in HL-60 cell treated with Camphotecin.[39] It became evident that proteolysis is an early and necessary event in the execution of an apoptotic program.

There is an ever growing list of protein molecules involved in the execution of the apoptotic pathway since I did my work. It does appear now that all these pathways are triggered by ICE or one of the related proteases like PARP(poly ADP-ribose polymerase), Cmk (Chloromethyl ketone), TNF (Tumour necrosis factor) or PrICE (protease resembling ICE).[40] ICE is an IL-β converting enzyme which has 29% homology with ced3 gene. Ced3 in a nematode Cernorhabitidis elegans is responsible for execution of apoptosis. ICE proteases play a central role in apoptosis and could be targets for new drugs to induce apoptosis.

Cell Death versus Cell Proliferation

There various examples in nature as well as experimental models to show that apoptosis counteracts cell proliferation. Apoptosis has been proposed as a system of cell deletion that complements mitosis in the regulation of animal cell population. It is seen for instance in the shaping of the organism during embryogenesis.[26] It is also seen in the development of the immune and haemopoietic systems.[25] Despite the appreciation of apoptosis as a basic biological phenomenon, there continues to be a lack of appreciation of its role in tissue kinetics. Cell size in a population is jointly determined by the cell birth rate (K_B) and cell loss rate (K_L). While K_B is well defined and can be assessed by metaphase blocking method, cell loss (K_L) is more difficult to measure since it includes exfoliation, migration and cell death. Experimental models on the study between cell proliferation and cell death include skin, liver and endometrium.

1) **Skin model**[41]

In 1974 Potten proposed a murine epidermis model. It consists of a vertical columns of cells with stacked cells with each stack overlying 10-11 basal cells ranging from 3-4 in a central zone and 6-7 in a peripheral zone. In the centre of a basal zone is a stem cell (S). This stem cell gives rise to a daughter cell which is capable of amplification divisions in the basal layer. This gives rise to cells which are differentiated and forms layers above the basal layers. These basal cells with a stem cell at the centre form an epidermal proliferating unit (EUP). The differentiated cells give rise to keratinised cells which are finally lost by desquamation.

Valid models of growth control that can be tested experimentally require precise identification of kinetically different types of cells present in the system, compartments and establishment of rate of transition between them. The complexity of mammalian epidermis makes such measurements difficult. The amphibian model (Toad) with no dermal papilla has been preferred as it is simple and easier to get to the epidermal pool size. The stratum corneum is single layered and it is lost as a whole (molts) every 6-10 days at 20°C. Studies in the toad epidermis have surprisingly shown that there is not a delicate balance between K_B

(cell birth) and K_D (cell death). There is a much larger K_B than K_D with a ratio varying from 1:2 to 4:1 (Buitz 1985).[32] The surplus from these cells are lost from the skin by apoptosis. By pituitary ablation (pars distalis) and or hydrocortisone implants, K_B:K_D can rise to 7-10 compared to 2-3 in normal toads, without any big change in the epidermal pool size.

In these experiments there was an increased apoptotic rate resulting only in a slight increase in the epidermal pool size for at least 14 days. It was followed by decreased mitotic and apoptotic rates. This resulted in only a slight increase in the epidermal pool size. These results indicate that apoptosis is complementary to mitosis in the toad epidermis.

2) Rat Liver model[42]

A rat liver model used by Patricia Servais further illustrated the link between cell proliferation and cell death. Female Sprague Dawley rats were fed for 5 days with the sex steroid analogue, cryopterone acetate (CPA) and killed at 2 day intervals for 11 days. For the control they were not fed with cryopterone. The drug was shown to induce liver hyperplasia as determined by an increase in the liver weight of the treated controls. The liver cell proliferation rate as assessed by PCNA was also increased. This was paralleled by an increase in the apoptotic rate which reached a maximum in the first 2 days and declined to a pre-treatment level upon cessation of treatment.

3) Rabbit Endometrium model[43]

In the endometrium, oestrogen tends to increase the migration and proliferation of stem cells. Progesterone on the other hand can promote division of daughter cells in the lumen with differentiation to form glands as well as non-proliferating cells which produce uteroglobin. Progesterone has been shown to inhibit oestrogenic proliferative effect in culture by production of a growth inhibitory factor. A similar factor has been observed in high density cultures. By growing endometrial cells on collagen substrates in culture, it has been possible to reproduce 3 dimensional structures similar to endometrial glands and to study the regulatory

mechanism of endometrial growth. These mechanisms are cell proliferation, migration, and cell death. These changes take place through time in culture and with the same periodicity as changes in DNA.

It has also been shown that there is a negative feedback between cell proliferation and cell death in order to maintain total culture mass. The homeostasis is maintained by cell proliferation factor (CPF) and cell death factor (CDF). Endometrium has been shown to have morphological features of apoptosis.

Current research interest has centred on the role of oncogenes like p53 and Bcl-2 in apoptosis. The role of p53 has been underlined by the fact that cell lines without p53 or with mutated p53 have needed large doses of drugs to induce apoptosis.[44] The same phenomenon has been seen in radiation sensitivity. An oncogene which has been studied extensively because of its protective role against apoptosis is Bcl-2.[,30,31,32] Since I did this research, two papers have appeared in the literature, one by Anjou Thomas et al (1995)[45] and one by David McConkey et al (1997)[46] regarding the role of Bcl2 and the related protein Bax in the chemosensitivity of chronic lymphocytic leukaemia to drugs. It has been clearly demonstrated in these studies that cells with none to low levels of Bcl2\Bax ratio were chemosenstive and vice versa for those which had high Bcl2\Bax ratios. Also those which had no detectable endonuclease were drug resistant. I therefore undertook a study with the following aims:

Aims

1) To study cell proliferation and apoptosis in lymphomas, using histological paraffin embedded material and leukaemia cell lines grown in culture.
2) To assess any correlation between cell proliferation and apoptosis and to what extent the two processes influence each other.
3) To assess the role played by oncogenes in apoptosis.
4) To study the link between cell differentiation and apoptosis.

CHAPTER 2

METHODS

2.1 A Study of Correlations Between Apoptosis Proliferation and BCL-2 Expression in non-Hodgkin Lymphomas.

2.1.1 Introduction

The incentive for oncologists and radiotherapists to study the biological relationships of apoptosis and cell proliferation is that it could result in improved treatment.[47] As part of an effort to improve prognostic criteria this retrospective study assessed 20 low grade and 20 high grade non-Hodgkin's lymphomas as to their apoptotic index, proliferation index and Bcl-2 expression and these were related to histological grade, and where appropriate to each other.

The study of apoptosis by morphological criteria is hampered by the fact that the process is short-lived,[20] and after a few minutes, the apoptotic bodies are phagocytosed. In any given tissue the process occurs asynchronously and it can therefore, be very difficult measure.[2] Apoptotic rate in this study was assessed by an *in situ* end labelling technique (ISEL) to label DNA breaks in apoptotic cells using Digoxigenin-11-dUTP oligonucleotide analogue introduced by a terminal deoxynucleotidyl transferase (TdT). Compared to a morphological count of apoptotic bodies in haematoxylin/eosin stained histological slides the ISEL technique is more sensitive and detects the early stages of apoptosis.[48]

Proliferation rate was assessed by *in situ* hybridisation for histone mRNA using Digoxigenin-11-dUTP labelled oligonucleotide probes. Histone mRNA is stabilised during the S-phase of the cell cycle and the levels increase 20-50 fold thus acting as a marker of DNA replication and the S-phase. Digoxigenin labels were detected for both methods by anti-digoxigenin alkaline phosphatase staining. Bcl-2 expression was demonstrated by immunoperoxidase technique after antigen retrieval. Assessment of Bcl-2 status has prognostic implications as previous studies have shown that the lymphomas which are Bcl-2 negative fair badly.[49]

Case selection and diagnosis

Twenty cases each of low and high grade NHL along with tonsil controls were selected randomly from the Pathology archives of Leicester University and the Leicester Royal Infirmary. Cases included 16 females and 24 males with age ranges of 4-84 years. These cases were well defined by histology, immunohistochemistry, light chain Ig restriction mRNA *in situ* hybridisation, and heavy chain Ig rearrangements by PCR and Southern Blot gene rearrangements (see table 2).

Table 2 Diagnostic categories, age ranges, and sex ratios of lymphoma cases

Low Grade NHL Diagnosis	No. of Cases	Age range	Sex ratio m/f
ML lymphocytic	6	61-78	4:2
ML follicular centrocytic/centroblastic	9	20-80	5:4
ML follicular/diffuse centrocytic/centroblastic	2	48-64	1:1
ML PTCL Lennert's lymphoma	1	55	0:1
ML T cell rich B cell lymphoma	2	79-83	1:1
High Grade NHL			
ML B centroblastic	13	45-84	9:4
ML B centroblastic secondary	2	63-68	0:2
ML B immunoblastic	2	39-74	0:2
ML T cell lymphoma Ki-1 positive	1	81	1:0
ML T cell peripheral	1	80	0:1
ML T lymphoblastic	1	4	0:1

2.1.2 *In situ* End Labelling using Digoxigenin-11 dUTP [48,50]

Formalin fixed and paraffin embedded sections 4μM thick were cut and mounted on glass slides which had been coated with 2% 3 aminopropyl triethoxysilene (Sigma, Poole, Dorset, UK). Sections were dewaxed through xylene, then were passed through 99% ethanol and 95% ethanol for 10 minutes each time. They were rehydrated with ultrapure water.

Permeability of the sections was achieved by application of Proteinase K (Sigma, UK), 5μg/ml dissolved in 0.05 M Tris/Hcl at pH of 7.6. Incubation was carried out for 1 hour at 37°C. The sections were washed in ultrapure water and fixed in 0.4% paraformaldehyde for 20 min at 4°C. They were washed in ultrapure water and put in TdT (Terminal Deoxynucleotidyl Transferase) buffer pH 7.2 for 5 minutes. The TdT buffer consisted of (30 mM Trizma base pH 7.2, 140 mM sodium cacodylate and cobalt chloride).

End labelling was carried out using Digoxigenin-11-dUTP (Boehringer Mannheim, Lewes, Sussex, UK) dissolved in TdT buffer at 50 picamol concentration. To some sections 50μl of similar solution with 50 units of Terminal Transferase enzyme (Boehringer Mannheim, Sussex, UK) was applied. The sections were left at 37°C for 2 hrs. For the negative controls, the enzyme was omitted. Tonsilar tissue served as positive control. Incorporated Digoxigenin-11-dUTP was detected by incubating sections with 100μl of alkaline phosphatase Fab fragments of polyclonal sheep anti-digoxigenin diluted 1:600 in 3% BSA/TBS (Boehringer Mannheim, UK). for 30min at 37°C and then washed in ultrapure water.

Alkaline phosphatase activity was detected by applying a substrate solution consisting of Nitroblue Tetrazolium (Sigma, UK), BCIP (5-Bromo-4-chloro-3 Indolyphosphate) dissolved in 1 molar Tris pH 9.5. 100μl volumes were applied to each section and left at room temperature in the dark overnight. Positivity was indicated by dark brown staining of the apoptotic nuclei. Counting was done using a Leitz microscope with a graticule and at x400 magnification in equivalent areas which had been selected to do cellular proliferation Apoptosis was measured by counting apoptotic nuclei, tingible bodies and macrophage staining. An average apoptotic count per 1000 cells done on two different occasions was taken. For the negative controls to which no enzyme was added there was no staining.

Tonsilar tissue served as a good positive control as apoptotic activity was easily demonstrated in germinal centres. It is necessary to avoid tissue with necrotic areas

where such areas would stain brown. It was also important to avoid exposing sections to proteinase K for longer periods than 1hr otherwise considerable over digestion of tissue and non specific staining resulted.

2.1.3 BCL-2 Antigen Detection by Antigen Retrieval and ImmunoPeroxidase Method [51,52]

Formalin fixed paraffin embedded tonsil sections 3μ thick were cut and mounted on silane-coated slides. They were dewaxed through xylene - 10 minutes, 99% alcohol - 10 minutes and 95% alcohol - 5 minutes followed by rehydration with ultra pure water.
Antigen retrieval was done by immersing slides in 6 molar citrate buffer solution bath inside a microwave, temperature set at 70°C and heating for a total of 15 minutes. The buffer was changed every 5 minutes. The sections at the end of 15 minutes were put in a moist chamber to cool for 20 minutes.

Immunoperoxidase detection of Bcl-2 was carried out using a standard immunoperoxidase technique. Permeabalised sections were immersed in 6% hydrogen peroxide bath at room temperature for 2 minutes to abolish endogenous peroxidase activity and 100μl of normal rabbit serum (Dako UK) diluted 1/20 in TBS (Tris buffered saline) was applied. The sections were left in a moist chamber for 10 minutes at room temperature. The rabbit serum was decanted off and Bcl-2 (mouse anti-human) antibody diluted 1:20 in Tris buffered saline was applied to the sections in 100μl volumes and left for 60 min. at room temp. The sections were washed in PBS for 20min. Secondary biotinylated rabbit anti-mouse antibody diluted in TBS 1:400 was applied for 30 minutes at room temperature.

Sections were washed in TBS and 100μl of horse radish peroxidase (BDH, UK) was applied. Brown colour development was achieved by addition of Diamino Benzidine (BDH UK) to the slides for 10 minutes. Sections were then stained with Haematoxylin and Eosin and mounted on slides.

Cases were graded: 0 - if no staining 1 - only light staining
2 - moderate staining 3 - maximum staining

Normal lymph node tissue acted as a good negative control where the germinal centres did not stain and known lymphoma cases as positive controls with the follicular centres staining. A grading of 0 to 3 appeared sufficient to place the cases into categories according to the amount of brown staining observed.

2.1.4 mRNA Histone Hybridisation Using Digoxigenin-11 dUTP Labelled DNA Probe[53]

Paraffin sections 4μm thick were mounted on slides which had been coated with 2% silane. (Sigma, Poole, Dorset, UK). The sections were then dewaxed through xylene, taken through decreasing grades of ethyl alcohol and rehydrated to water. Permeabilisation of the sections was achieved by application of proteinase K (Sigma, UK) at 5 ug/ml concentration and dissolved in 0.05 molar Tris buffer pH 8.6 and sections were left for 1 hour at 37°C. They were washed in DEPC water and fixed in 0.4% paraformaldehyde followed by a wash in DEPC water.

Hybridisation

This was carried out as previously described.[53] 50μl of prehybridisation cocktail was applied to sections for 1 hour at 37°C (see Table 4). 50μl of hybridisation probe cocktail containing DNA probes complementary to H_2, H_3 and H_4 histone genes and labelled with digoxigenin, was applied and sections left in the dark overnight at 37°C. Sections were washed in 2x SSC/30% formamide-10min, followed by a wash in 3%BSA/DEPC water-5minutes. The *in situ* signals were detected by applying100μl of anti-digoxigenin alkaline phosphatase 1:600 in 3%BSA followed by a wash in TBS and visualisation using -200μl of substrate (NBT, BCIP) till brown colour developed.

Counting was done on two separate occasions in the areas which were actively proliferating, as revealed by the Haematoxylin and Eosin stained sections from the same paraffin embedded block. The proliferation index was derived by taking the average from two counts of 1000 lymphoma cells using a Leitz microscope at x400 magnification and a graticule. For the results see tables 4 and 5.

For the photomicrographs (illustrations) see appendix-page 152.

TABLE 3 Table listing the components of hybridisation and substrate solutions

	Prehybridisation Cocktails
1	300µl Paraformaldehyde
2	120µl of 5M NaCl
3	100µl of 10 x P.E. Pyrophosphate/EDTA buffer pH. 7.4
4	15µl of SSDNA (Heat denatured salmon sperm DNA)
5	265µl of ultrapure water
6	200µl of 50% Dextran sulphate
	Substrate
1	NitroBlue Tetrazolium (Sigma, UK)
2	BCIP-5 bromo, 4 chloro, indolyl phosphate (Sigma, UK)
	Substrate Buffer
1	1M $MgCl_2$
2	5M NaCl
3	1M Tris pH 9.5
4	Ultrapure water

2.2 Flowcytometric Demonstration of Apoptosis in a Human Leukaemia Cell line (HL-60) and Raji Cell Line.

2.2.1 Introduction

Apoptosis is a mode of cell death triggered by various stimuli and in which the cell participates in its own suicide, and is biochemically characterised by fragmentation of DNA into oligonucleosomal fragments with 200 base pairs or multiples thereof.[19,20,21] These stimuli could be drugs, irradiation, extremes of temperature i.e. 44°C or microbiological agents. In some cells like thymocytes protein and RNA synthesis is required and drugs like Actinomycin D which inhibit RNA transcription, or

Cycloheximide which inhibit protein synthesis, can induce apoptosis.[54] Other DNA damaging agents like Camphotecin which is a Topoisomerase-1 inhibitor, can induce apoptosis.[55]

Demonstration of apoptosis using DNA histograms, relies on demonstration of sub G_1 peaks on DNA histograms, as apoptotic cells with their low DNA content tend to appear in that position.[23]

2.2.2 Material and Methods

All chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, England) unless otherwise stated. Cell lines HL-60 and Raji were grown in RPMI1640 medium (Gibco, UK), supplemented with 10% Foetal calf serum, 100μg of Streptomycin and 100IU of Penicillin. The cells were grown at 37°C in a humidified culture cabinet with 5% CO_2. They were used when growing at an exponential phase, and at a concentration of 1×10^6/ml.

Induction of apoptosis was undertaken by incubating the cells in culture with Actinomycin D at increasing concentrations of 0.25μg/ml, 0.5μg/ml, 1μg/ml and 2μg/ml for 5 hours. Also Camphotecin was used to induce apoptosis at a concentration of 0.3μmolar for 1hr, 2hrs and 3hrs. For the control samples no drugs were used. For the Raji cell line, apoptosis was induced by incubating the cells in culture for 72 hrs, with Cycloheximide at concentrations of 100μg, 250μg, 500μg and 1gm.

The cells were harvested at the end of the incubation period and washed twice in ×1 PBS and fixed with ice cold 70% Ethanol for 1hr.
The fixed cells were spun down at 400g and washed twice in ×1PBS.
The cells were stained with Propidium iodide-final concentration 10μg/ml and RNase 100units/ml, left for 1hr. They were filtered through a nylon mesh to remove debris before analysing with FAC-STAR flowcytometer.
Induction of apoptosis in these cell lines was confirmed in a separate experiment (see chapters 2.5 and 3.5), by doing DNA ladders.
For the results-DNA Histograms, see chapter 3.2

2.3 Demonstration of Apoptotic Activity in Murine Leukaemia Cell Lines-DP16-1 and MEL-C88

2.3.1 Introduction.

Apoptosis is a process which occurs in response to various stimuli and is mediated by an endogenous endonuclease which cleaves DNA into oligonucleosomal fragments of 200 base pairs or multiples thereof.[56,57] In some cells, like thymocytes, incubation of their nuclei with (Ca^{++}) leads to spontaneous apoptosis and it is thought that Ca^{++} is important for the enzyme activity.[1] In the early phase of apoptosis an influx of Ca^{++} is seen in many cell systems.[24,57]

Using calcium ionophore A23187 induces apoptosis in the HL-60 cell line, and using calcium chelating agents inhibits apoptosis in this cell line. In Murine leukaemia cell lines DP16-1 and MEL-C88, an increase in cytosol calcium can trigger apoptosis.[57] A compound called Thapsigargin, which is a sequenterpene lactone derived from Thapsia garganica plant, is an irreversible inhibitor of calcium-ATPase enzyme. This enzyme tends to pump calcium from cytosol into the endoplasmic reticulum. Blockage of such a pump leads to an accumulation of cytosol calcium triggering apoptosis.

2.3.2 Material and methods

The Murine leukaemia cell line DP16-1 was a donated by Dr S Benchimol, University of Toronto, Canada. The Murine leukaemia cell line C88 was donated by Dr E Conley, Leicester University, UK. The cell lines were grown in Eagles medium (Gibco, UK.) supplemented with 10% Foetal calf serum (Sigma, UK.), 100IU Penicillin and 100μg Streptomycin (Sigma, UK). The cell lines were grown in 5% CO_2 and at 37°C in a moist culture cabinet.

While growing at an exponential rate, and at a concentration of 1×10^6/ml for the DP16-1 induction of apoptosis was achieved by using in culture various concentrations of Thapsigargin for 6hrs Concentrations of 5μg, 10μg, 20μg were tried. For the C88 cell line Thapsigargin was used at concentrations of 10μg, 20μg, 30μg and 40μg for 24hrs. For the controls Thapsigargin was omitted. The cells were harvested at the end of the incubation period, washed twice in ×1PBS and fixed in 70% Ethanol on ice for 1hr. They

were washed twice in ×1PBS and stained with Propidium iodide final concentration 10μg/ml. RNAse (100units) was added and samples left for 1hr. The cells were filtered in a nylon mesh and analysed with a FAC-STAR flowcytometer. Induction of apoptosis in these cell lines was confirmed in a separate experiment (see chapters 2.5 and 3.5) by doing DNA ladders and EM on sorted and unsorted samples.

For the results -DNA Histograms see chapter 3.3

2.4 Flowcytometric Demonstration of Apoptotic Activity in a Human Leukaemia Cell Line HL-60 and a Murine Leukaemia Cell Line C88 Using an End Labeling Technique with Digoxigenin-11-dUTP

2.4.1 Introduction

During apoptosis, activation of an endogenous endonuclease which attacks DNA at internucleosomal linker regions marks an early event[22]. The morphological features of apoptosis occur later. A flowcytometric technique pioneered by Yael Gavriel enables labelling of DNA breaks at the 3′ OH ends, with a nucleotide analogue like Digoxigenin-11-dUTP or biotinylated dUTP introduced by a Terminal deoxynucleotidyl transferase (End labelling technique), or a polymerase enzyme (Nick translation method).[28,48] These are then detected by anti-Digoxigenin antibody or in case of Biotinylated nucleotide an anti avidin antibody. This method allows simultaneous staining of DNA, giving the position of the cell cycle, where apoptosis occurs.

2.4.2 Material and methods

All chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise stated. HL-60 cell line was grown in RPMI1640 medium (Gibco, UK) supplemented with 10% Foetal calf serum, 100IUpenicillin and 100μg of Streptomycin. The Murine leukaemia cell line C88 was a donation Dr. E. Conley, Leicester University, UK. The C88 cell line was grown in Eagles Medium (Gibco, UK) supplemented with 10% Foetal calf serum, 100IU Penicillin, 100μg of Streptomycin and L-glutamine. Both cell lines were grown in a humidified culture cabinet with 5% CO_2 at 37°C. The cells were used when growing at an exponential rate and at a concentration of 2×10^6/ml.

For the HL-60 apoptosis was induced by incubating cells with Camphotecin 0.3μM in culture for 3hrs. For the C88 cell line apoptosis was induced by incubating the cells with Thapsigargin 30μg (Cal Biochem-novo Biochem, UK) for 24hrs. Confirmation that apoptosis was induced was done using DNA ladders (see chapters 2.5 and 3.5). Cells from both cell lines were harvested at the end of the incubation periods, and washed twice in ×1PBS. They were fixed in 1% buffered formalin on ice for 15 minutes. The cells were washed twice in ×1PBS and fixed for 45 min. in 70% Ethanol on ice. The cells were spun down at 800g for 6 min. and washed ×2 in ×1PBS. The cells were washed in 1ml of TdT buffer (30mM Trizma base, 140 mM Sodium cacodylate and 1nM Cobalt chloride with a pH of 7.4). They were then adjusted to a concentration of 1×10^6cells/ml.

End labelling solution for the HL-60 cell line was constituted as follows:

1) TdT buffer - 100μl + 100pmol Dig-11-dUTP but no enzyme control.

2) TdT buffer - 100μl + 100pmol Dig-11-dUTP and 20units of TdT enzyme (Terminal deoxynucleotidyl transferase)

3) TdT buffer - 100μl + 0.2 nM Dig-11-dUTP + 20units of TdT enzyme

4) TdT buffer - 100μl + 0.2nM Dig-11-dUTP but with no enzyme

For the C88 cell line labelling solution was used as follows:

1) TdT buffer - 100μl + pmol Dig-11-dUTP +10 units TdT enzyme (APO1).

2) TdT buffer - 100μl + 100pmol Dig-11-dUTP but no enzyme control. (APO1).

3) TdT buffer - 100μl + 100pmol Dig-11-dUTP +20 units of T.D.T enzyme (APO2).

4) TdT buffer - 100μl + 100pmol Dig-11-dUTP no enzyme control (APO2).

5) TdT buffer - 100μl + 100pmol Dig-11 dUTP + 5 units of T.D.T enzyme (APO3).

6) TdT buffer - 100μl + 100pmol Dig-11-dUTP but no enzyme control (APO3)

Control samples like the blank were included in the experiment for each line to zero the flowcytometer and also FITC only stained samples were included. The cells from both cell lines were washed in 5% dry fat milk dissolved in x4 SSC (Saline trisodium Citrate)/0.1% Triton-X-100.

They were resuspended in 100μl volumes of labelling solutions with enzymes or no enzyme for 30 minutes at 37°C in a water bath. The cells were washed in the buffer (5% dry fat milk dissolved in ×4 SSC/0.1% Triton-X-100).

Except for the blank control, the cells were stained with FITC anti Digoxigenin 0.1μg dissolved in 250μl of buffer (5% dry fat milk dissolved in × 4 SSC/0.1% Triton-X-100) and left at room temperature for 30 minutes.

The cells, except for the blank and FITC only sample, were counterstained with Propidium iodide at a final concentration of 10μg/ml. The samples from both cell lines were analysed by the FAC-STAR flowcytometer, with Argon laser tuned in at 488nM and FITC as well as PI (Propidium iodide) fluorescence were recorded.
For the results -DNA Histograms see chapter 3.4

2.5 DNA Extraction and Gel Electrophoresis on the Sorted and non Sorted Samples of HL-60, Raji, C88 and DP16-1

2.5.1 Introduction

In 1980, Wyllie demonstrated that the death of thymocytes *in vitro* when exposed to glucocorticoids had morphological features of apoptosis.[20] He further demonstrated that the cells chromatin was cleaved into a series of fragments with an average size of 200bp and integral multiples thereof. These are seen on gel electrophoresis as DNA ladders. This is due to activation of calcium and magnesium dependent endonuclease.[21,55] This could be inhibited by Zinc ions.[4] Apoptosis could occur in absence of DNA ladders. Agarose gel electrophoresis offers a way of demonstration of DNA fragmentation. The purpose of the experiment was to confirm by demonstration of DNA ladders that apoptotic activity represented by the sub G_1 peaks on histograms of the sorted and non sorted samples of HL-60, Raji, C88 and DP16-1 seen in Chapter 3.1, 3.2, 3.3, 3.4 and 3.5 was genuine.

2.5.2 Materials and methods

Materials

Phenol

Chloroform

x1TE (10mM Tris, 1mM EDTA pH 8.0)

SDS 10% (in H_20)

RNase (50mg/ml in 10mM Tris pH 7.5, 0.15mM NaCl - heated at 95°C for 15 min.)

Extraction Buffer-

10mM Tris pH 8.0, 0.1mM EDTA, 20μg/ml RNase, 0.5% SDS, Proteinase K25μg/ml.

Methods

For culture conditions see (Chapter 2.2.2 and 2.3.2)

As previously described apoptosis was induced in HL-60 cell line by incubating the cells with Camphotecin 0.15μM for 1, 2 or 3hrs. and 0.3μM for 3hrs. For the sorted samples 0.15μM for 3hrs. was used. Alternatively Actinomycin D 0.25μg, 0.5μg, 1μgm or 2μg was used for a 6hr. incubation period.

For the Raji cell line Cycloheximide was used at concentrations of 100μg, 250μg,500μg and 1g for 72hrs. For the sorted sample Cycloheximide was used at 250μg for 72hrs.

For the C88 cell line non sorted sample apoptosis was induced by incubating the cells with Thapsigargin (CalBiochem-NovBiochem, UK) at concentrations of 10μg, 20μg, 30μg and 40μg in culture 24 hrs. For the sorted sample 40μg Thapsigargin for 24hrs. were used. For the DP16-1 cell line Thapsigargin was used for 6hrs. incubation period at concentrations of 5μg, 10μg, 20μg and the sorted sample Thapsigargin was used at 30μg for the 6hr. incubation period.

DNA extraction and Agarose gel electrophoresis

DNA extraction:

$2x10^6$ cells/ml were washed twice in x1PBS. Centrifugation was performed in a small eppendorfs leaving a small pellet to which 0.5ml of extraction buffer was added. The

pellets were incubated in a water bath at 37°C for 1hr. 10µl of Proteinase-K solution was added to each tube and incubation was carried out at 50°C for 3hrs.

Phenol Extraction

0.5ml of Phenol was added to each tube and the tubes were inverted several times in order to mix the organic and aqueous phases.

The sample was centrifuged at 400g for 2min. in a microcentrifuge.

The top aqueous phase containing DNA was carefully separated off avoiding the bottom phase containing proteins. The Phenol extraction was repeated again until a clear top phase was seen.

Chloroform extraction.

This stage ensured that the extracted DNA was free of Phenol. The procedure was carried out by adding the same volume of chloroform to the DNA solution. This was followed by mixing the sample and spinning it down in a microcentrifuge at 400g for 2min.

DNA precipitation

This was achieved by taking the top phase, measuring its volume and adding twice the volume of cold 100%. Ethanol as well as adding 20µl of 5M NaCl/ml to DNA alcohol mixture. The samples were left on ice for 30 minutes at -20°C to allow DNA precipitation. DNA appeared in solution as tiny threads of gelatinous material. Centrifugation was carried out in eppendorfs at 400g for 5 minutes. The pellets were resuspended in 1ml volumes of 70% Ethanol/PBS to rehydrate the DNA. The DNA samples were spun down further in a microcentrifuges for 5 minutes and the pellets of DNA were resuspended in x1TE, volume as determined by the concentration of DNA required.

Gel electrophoresis

Gel moulds consisting of 2% agarose dissolved in x1Tris acetate/EDTA buffer were made. The samples of DNA were mixed with the desired loading buffer (LB)

Bromophenol blue in an appropriate ratio (e.g., 4:1 for 5x LB, 9:1 for 10x LB). The samples were heated for 10 minutes at 65°C before loading into the wells of the gels.

A constant voltage of 6V/cm (distance measured between the electrodes) was applied.

A check was done to see that gels were running satisfactorily by looking for the generation of bubbles at the electrodes, temporarily switching the power pack to amps and observing the current reading and finally observing the migration of the dye fronts. The gels were allowed to run until the dye in the loading buffer had migrated an appropriate distance. The power pack was turned off and the power leads removed.

The gels were stained by putting them into trays containing ethidium bromide (1-2μg/ml) in x1TE. They were left for 20-60 minutes. The gels were rinsed with ultrapure water and examined under ultra violet light. A UV protective visor was won to protect the eyes and gloves were worn to protect the skin.

In the lanes from the samples treated with drugs to induce apoptosis bands of DNA (DNA ladders) could be seen clearly. Photographs of the gels were then taken.
For results see chapter 3.5.

2.6 Assessment of Cell Proliferation and with Apoptosis in a Human Promyelocytic Cell Line (HL-60) Using PCNA (Proliferating Cell Nuclear Antigen).

2.6.1 Introduction

PCNA (Proliferating Cell Nuclear Antigen) is an evolutionary conserved 36Kd acidic protein that is essential for DNA synthesis.[11] It can be detected in proliferating cells by serum raised in patients with systemic lupus erythematosus.[62,63] PCNA functions as an auxiliary protein for DNA polymerase δ in DNA synthesis (Bravo et al 1987, Favrman 1990).[11] Immunofluorescent studies on cultured cells have shown PCNA to be a cell cycle related antigen (Celis in 1985).[63]

There are 2 types of PCNA. During the S-phase, type I is nuclear PCNA which is present at low levels in cells which are quiescent but capable of cell division.[11,62] This is not resistant to extraction by organic solvents. Type II is associated with replication sites and is resistant to extraction by solvents. During cell proliferation it is this second type of replication associated PCNA which is raised.[11] It has been shown that some growth factors like platelet derived growth factors (PDGF) increase the stability of PCNA messenger RNA and facilitates its transcription. Immunoreactivity of monoclonal antibody against PCNA-PC10 has been shown to correlate with Ki-67 in nodal lymphomas (Hall 1990).[62] In gastrointestinal lymphomas, the proportion of cells staining with PCNA has also been shown to correlate with grade.[62]

2.6.2 Material and Methods

HL-60 cell lines grown in culture RPMI-1640 medium supplemented with 10% Foetal calf serum (Gibco, UK) 100IU Penicillin and 100μg of Streptomycin. The cells were grown in a moist culture cabinet containing 5% CO_2 and at 37°C. They were treated with varying concentrations of Camphotecin at 0.15nM and 0.3nM for 2 and 3hrs. For control samples, no drug was administered. The cells were harvested at the end of the incubation periods, washed twice in ×1PBS and fixed for 1hr on ice in 70% Ethanol/x1PBS. They were washed twice in x1PBS and adjusted to a concentration of 1×10^6 cells/ml. Permeablisation of cells was achieved by suspension of cells in 0.5% Triton-X-100/×1PBS for 10min.

10μl of a commercial monoclonal FITC conjugated PC10 antibody (DAKO F-863) was added to (1×10^6) cells and incubation was carried out at 37°C in a water bath for 30 minutes. An isotype control antibody (DAKO-X-933) was also included and added in equivalent proportions to the control cells. A blank and FITC (DAKO F0313-104) sample diluted 1/20 in 0.25% Triton X-100/ x1PBS was also included. At the end of the incubation period, the cells were washed twice in ×1PBS and except for the blank and FITC only control, were counterstained with Propidium Iodide - final concentration of 10μg/ml. The samples were analysed by means of a flowcytometer with Argon laser tuned in at 488nm and both PI and FITC fluorescence were recorded.

For results see chapter 3.6.

2.7 Flowcytometric Measurement of Cell Proliferation and Correlating it with Death by Apoptosis in HL-60 (A Human Promyelocytic cell line) and DP16-1 (A Murine Proerythroblastic cell line) Using Bromodeoxyuridine Incorporation Technique.

2.7.1 Introduction

Traditional tumour kinetics have used radioactive precursors of DNA synthesis like tritiated thymidine (3H-thymidine) and utoradiography. The general approach has been to identify a window in the cell cycle and measure the movement of a cohort of cells through that window.[64] The percentage of labelled mitosis (PLM) autoradiographic method where cells undergoing DNA synthesis are labelled with tritiated thymidine and uses mitosis as an identifiable window but the disadvantage of using this approach is that for tumours which are slow growing this could take many weeks. Also tritiated thymidine is a radioactive compound and not entirely safe for patients. Further interests in tumour cell kinetics arose by development of a monoclonal antibody against BrdUrd (Bromodeoxyuridine) by Gratliner in 1982[65]. This antibody used in combination with Propidium Iodide allows simultaneous measurement of DNA and Bromodeoxyuridine.

Using flowcytometric technique developed by Dolbeare,[64] one is able to assess cell proliferation, as a window can be set in any part of the cell cycle. The advantage of Bromodeoxyuridine is that it is not toxic to humans and can be administered safely both *in vivo* and *in vitro*[65].

2.7.2 Material and Methods

HL-60 cells grown in RPMI-1640 (Gibco, UK) medium and supplemented with 10% Foetal calf serum (Gibco, UK) 100IU of Penicillin, 100μg Streptomycin (Sigma, UK) were grown in a humidified culture cabinet containing 5% CO_2 at 37°C. The DP16-1 cells were grown in similar conditions using Eagles medium (Gibco, UK) supplemented with 10% Foetal calf serum, 100μg of Streptomycin, 100IU Penicillin and L-Glutamine. For the HL60 while growing in an exponential phase and at a concentration of 2×10^6 cells/ml, cells were incubated with 0.15μM of Camphotecin (Sigma, UK) for either 1hr, 2hrs. or 3hrs. For the DP16-1, $2x10^6$cells/ml were incubated with 30μg of Thapsigargin for 1,6,8 or 24hrs. For the controls samples, no drugs ware administered. The cells were washed free of the drug twice at the end of incubation periods. Bromodeoxyuridine

(Sigma, UK) 10nM/ml of culture final concentration was administered and cells left in culture for 1hr. No Bromodeoxyuridine was administered for the controls. The controls in this experiment included a blank, an FITC only sample, no Camphotecin or Thapsigargin controls, and no BrdUrd but anti BrdUrd control.

The cells were harvested and washed twice in ×1PBS. The cells were fixed in 70% Ethanol/×1PBS on ice for 45 minutes. The cells were washed ×2 in ×1PBS and adjusted to a final concentration of 1×10^6 cells/ml. A denaturation step was carried out by incubating the cells with 2N HCl 1ml for 1×10^6 cells over a 15 minute period. Cells were spun down at 400g for 5 minutes and the pellets were resuspended and washed in 1ml of 0.1M Sodium tetraborate pH 8.5 to neutralise the acid. The cells were permeabilised by washing them twice in 0.5% Tween 20 in ×1PBS. The cells were washed once in x1PBS. 100µl of anti-BrdUrd (DAKO M744) antibody diluted 2:7 in 0.5% Tween 20/x1PBS was added to the test samples. The samples were left at room temperature in the dark for 30 minutes. The cells were washed once in ×1PBS and except for the blank, 40µl of FITC Rabbit anti mouse (F313-104 DAKO) diluted 1/20 in 1% BSA/x1PBS, was added to the test samples and left on ice in the dark for 15 min. The cells were washed once in x1PBS and except for the blank, counterstained with PI (Propidium Iodide) final concentration 10µg/ml. PI and FITC fluorescence were analysed by means of a FAC-STAR flowcytometer with a laser beam tuned in at 488nm.
For results see chapter 3.7.

2.8 The Effects Of X-ray Irradiation on The Cell Cycle and Cell Proliferation of Human (Raji) and Murine leukaemia Cell Lines DP16-1 and C88

2.8.1 Introduction

A sound knowledge of the molecular events controlling radiation effects on the cell cycle is very important if effective treatment of cancers is to be achieved. Radiation in various forms i.e. X-rays or Gamma irradiation induce cell damage by inhibiting DNA synthesis and or replication.[70] Ionising radiation damage can cause single or double DNA strand breaks. It also leads to generation of free radicals that can cause DNA strand breaks.[71] Low doses of radiation cause death by apoptosis whereas high doses cause necrosis.

Radiation damage can cause cell cycle arrest in the G_1, S, or G_2-M phase depending on the cell line and dose of radiation given.[72] Kastan (et al.)[72] for instance, showed that in the M1 myeloblastic leukaemia cell line, giving 0.3-4Gy of radiation caused accumulation of cells in the G_1 and G_2-M phases of the cell cycle and G_1 phase arrest only occurred at the lower end of the radiation doses.[72] S-phase delay only occurs with high doses of radiation greater that 5 Grays. Also different cell lines have individual sensitivity to radiation damage.[73]

Some chemicals like Caffeine can sensitise cells to DNA damage by abolishing G_1, S or G_2 arrest. It also impairs DNA repair.[70] Fibroblasts from cells in patients with Ataxia telengiectasia are highly sensitive to ionising irradiation. Ataxia telengiectasia patients show various forms of defects in recombination including miss repair of DNA double strand breaks and high rates of immunoglobulin gene rearrangement.[70] Fibroblast cell lines from such patients have a reduced or absent G_1 arrest, S-phase or G_2-M delay. Some lymphoid cell lines like ST4 are also very sensitive to irradiation and respond by undergoing apoptosis whereas those cells not having the p53 protein like (M1 and W265) are radiation resistant. The same is true for the one having mutated p53 like the 9LY-R cell line.[74]

Patients with L-Fraumeni syndrome have mutated p53 and show resistance to irradiation damage. Wild type p53 has been shown to be involved in radiation induced G_1 arrest. Transgenic mice experiments have shown that those lacking p53 have reduced apoptosis in response to DNA damage. The DP16-1 cell line transfected with wild type of p53 showed death by apoptosis but this was not seen in those lacking p53.[75]

Those cells which co expressed Bcl2 and wild p53 were protected from radiation induced apoptosis but died via a mechanism of necrosis. Morphological features of apoptosis could be demonstrated after 8hrs whereas necrosis was seen after 48hrs. Cyclins play a role, for instance suppression of cyclin B1 mRNA is required in some cell lines for the G_2 arrest, in others tyrosine phosphorylation of P34 Cdc2- is required for the G_1 arrest. In

budding yeast the Rad 9 gene is required and in fission yeast the chKI/rad 27 gene is required.[72]

Some other investigators have studied the GADD 45 gene (growth arrest and DNA damage inducible genes.) GADD 45 gene has been found in humans and is strongly induced in humans following irradiation. Kastan found an excellent correlation between GADD 45 mRNA induction and p53 status. Cell lines containing both intact p53 genes showed a 2-10 fold rise in GADD 45 mRNA 4hrs after irradiation.[72]

2.8.2 Material and Methods

Two murine leukaemia cell lines DP16-1 and C88, and RAJI, a human lymphoblastoid cell line, were grown in a humidified culture cabinet containing 5% CO_2 at 37°C. The Raji cell line was grown in RPMI-1640 (Gibco, UK) supplemented with 10% Foetal calf serum 100IU, Penicillin and 100μgms of Streptomycin. The C88 and DP16-1 cell lines were grown in Eagles medium (Gibco, UK) supplemented with 10% Foetal calf serum, 100IU of Penicillin, 100μg of Streptomycin and L-Glutamine. For three consecutive days, the cells were spun down and resuspended in fresh culture medium. While growing in an exponential phase and at a concentration of $2x10^6$ cell/ml, they were irradiated with and X-ray radiation source (Faxitron-Hewlett Packard, UK) delivering 70 rads/min. For RAJI, 300, 600, 900, 1200, 1800, and 2000 rads were administered. For the DP16-1 - 100, 300, 500 and 1000 rads were given. For the C88 100, 250, 500 and 1000 rads were given. All the cell lines were put in a culture cabinet for 8hrs before DNA analysis. For C88 another set of samples, control and irradiated, were incubated with Bromodeoxyuridine-final concentration of 10nM/ml for 24hrs. For the C88, some of the 24 hour irradiated samples were prepared for DNA extraction and gel electrophoresis as previously described (see chapter 2.5and 3.5).

Bromodeoxyuridine incorporation in the C88 was detected as follows:

After 24 hour incubation with BrdUrd, cells were washed twice with x1PBS and $1x10^6$ cells were suspended in 1ml of 2N HCl for 15 minutes. They were spun at 400g and acid the was decanted off. The acid was neutralised by washing the cells in 0.1 tetra Borate

(pH 8.5) spun down at 400g followed by a wash in x1PBS. They were washed in 0·5% Tween 20/x1PBS. The cells were labelled with 20µl of anti-Bromodeoxyuridine dissolved in 0.5% Tween 20/x1PBS in a ratio 2:7. Incubation was carried out at 37°C in a water bath - 30 minutes. They were washed once in 0·5% Tween 20/x1PBS. Except for the blank sample, they were labelled with 20µl of FITC (DAKO) diluted in 0·5 % Tween 20/x1PBS. Incubation was carried out at room temperature for 30 minutes. The cells were washed once in x1PBS .Except for the blank and FITC only samples, cells were counterstained with Propidium iodide, final concentration of 10µg/ml. They were analysed with a FAC - STAR flowcytometer with a laser beam tuned in at 488nm.
For the results - Histograms, see chapter 3.8.

2.9 Experiment to Demonstrate Bcl-2 Oncogene Expression in two Murine Leukaemia Cell Lines DP16-1 and C88 Using a Flowcytometric Technique.

2.9.1 Introduction

Interest in Bcl-2 expression in the two cell lines stems mainly from the fact that these two cell lines have demonstrated a marked difference in susceptibility to apoptosis. Bcl-2 oncogene is known to protect cells from apoptosis.[58,69,70,71] Previous reports indicate that DP16-1 cell line has low Bcl-2 activity.[68] I used a flowcytometric approach to address the same problem.

2.9.2 Material and Methods

DP16-1 and C88 cell lines were grown in Eagles medium with L-Glutamine -(Gibco, UK) with 10% Foetal calf serum and 100IU of Penicillin and 100µg Streptomycin. They were grown in a humidified culture cabinet containing 5% CO_2 and at 37°C. The cells were used when growing at an exponential phase and at a concentration of $2x10^6$ cells/ml. The cells were harvested and washed twice in x1PBS. They were fixed in 70% Ethanol/x1PBS on ice for 1hr. They were washed twice in x1PBS.Cells for both cell lines DP16-1 and C88 were resuspended in 1ml of 20% normal rabbit serum/TBS for 20 minutes to reduce non specific binding of antibodies.

They were spun down at 400g and resuspended in 200µl of 0.25% Triton-X-100. 5µl of purified hamster anti mouse Bcl-2 monoclonal antibody (150 21A - Pharmigen, USA)

was added to $1x10^6$ cells. An isotype antibody purified hamster Ig standard anti-TNP (11101 D-Pharmigen) was used in equivalent proportions for the isotype control sample. This was included to assess non specific binding. P.E. (Phycoerythrin) only control at 1:250 dilution in 0.25% Triton-X-100/x1PBS and a blank sample were included. Incubation was carried out for 1hr at room temperature. The cells were washed twice in x1PBS.

250µl of phycoerythrin (P.E) antibody was added at 1:250 and 2:250 dilution in 0.25% Triton-X-100/x1PBS to the test samples. Incubation was carried out for 30 minutes at room temperature. For the blank no phycoerythrin antibody was used. The cells were washed once and resuspended in 1ml of x1PBS and analysed on FAC-STAR flowcytometer with laser beam tuned in at 488nm and P.E. (phycoerythrin) fluorescence recorded.

For the results-Histograms, see chapter 3.9.

2.10 The Role of C-myc in Apoptosis in HL-60 and DP16-1 Cell Lines As Investigated by Flowcytometric Technique.

2.10.1 Introduction

Previous reports have shown that c-myc oncogene plays an important role in apoptosis, i.e. RAT-1 fibroblasts with deregulated c-myc deprived of serum die via an apoptotic mechanism.[75] Interleukin-3 dependent cells expressing c-myc undergo apoptosis on the withdrawing of the cytokine.[75,76] Cells with higher levels of c-myc like HL-60 are more prone to apoptosis.[4]

The regions for c-myc protein required for apoptosis overlap with those required for co-transformation, auto regulation and inhibition of differentiation, suggesting that apoptotic functions of c-myc is related to its other functions.[72] Also the c-myc oncogene is involved in cell proliferation. Levels of c-myc are elevated when fibroblasts (Kelly et al. in 1988) and hepatocytes (Makino et al.)[72] are stimulated to proliferate. In 1988 G.A. Dent et al. published a flowcytometric method of simultaneous measurement of c-myc oncoprotein and DNA content,[74] thus enabling correlating c-myc levels with position of the cells in the cell cycle.

2.10.2 Material and Methods

HL-60 (a human promyelocytic leukaemia cell line) and DP16-1 (murine erythroleukaemia cell line) were grown in a humidified culture cabinet with 5% CO_2 at 37°C. For the HL-60 - RPMI 1640 medium (Gibco, UK) and 10% Foetal calf serum supplemented with 100 units Penicillin and 100µg Streptomycin was used. For the DP16-1 Eagles medium (Gibco, UK) supplemented with 10% Foetal calf serum, 100 units Penicillin, 100µg Streptomycin and L-Glutamine were used. The cells were sub cultured every alternate day and when they had reached a density of $1x10^6$ cells/ml, apoptosis was induced. For the DP16-1, 30µg of Thapsigargin (Cal Biochem-Novo Biochem, UK) was administered to 10mls of cells in culture and incubation period was 8hrs. For the control samples no drug was administered.

For the HL-60 Camphotecin 0.3µg was administered to 10mls of cells in culture and an incubation period of 3hrs was undertaken. For the control sample no drug was administered. The cells for both cell lines were harvested at the end of the incubation periods. They were washed twice in x1PBS and fixed in 70% Ethanol/x1PBS on ice for 1hr. The cells were washed twice in x1PBS and adjusted to a concentration of $1x10^6$ cells/ml in x1PBS. The cells were spun down in a microcentrifuge at 400g and supernatent removed and the cells were resuspended in 1ml 0.25% Triton-X-100/x1PBS for 15 minutes to facilitate permeabilisation.

The cells were spun down at 400g in a microcentrifuge, supernatant discarded and resuspended in 200µl of 0.25% Triton-X-100/x1PBS. For the murine leukaemia cell line (DP16-1)5µl of mouse monoclonal antibody to c-myc family proteins (ohm- 11- 904A Cambridge Research Biochemicals) was added to $1x10^6$ cells.

For the HL-60 cell line 10µl of monoclonal mouse anti human c-myc antibody (Cambridge research Biochemicals) was added to $1x10^6$ cells. Controls included in the experiment were the (Blank) and FITC-only samples.

For the murine cell line DP16-1 cell line an isotype, purified hamster IgG monoclonal immunoglobulin isotype standard-anti Trinitro phenol (Pharmigen, USA) was used in

equivalent proportions. The need for this control was to detect the effects of non specific adherence of antibody to the cells. Incubation was carried out for 1hr. in a 37°C water bath. The cells were washed twice in x1PBS and resuspended in 200µl of 0.25% Triton-X-100/x1PBS.

40µl of FITC (conjugated F(ab')2 fragments of rabbit anti-mouse immunoglobulins- (F 0313 -104) was added in 1/20 dilution in 0.25% Triton-X-100/x1PBS to cells except for the blank only samples. Incubation was carried out for 30 minutes at room temperature. The cells were washed twice with x1PBS and apart from the blank and FITC only sample, were counterstained with Propidium iodide a final concentration of 10µg/ml and analysed with FAC-STAR flowcytometer with argon laser beam tuned in at 488nm.
For results - histograms see chapter 3.10.

2.11 Flowcytometric Analysis of p53 in Murine Leukaemia Cell Lines DP16-1 and C88.

2.11.1 Introduction

It has become increasingly apparent that oncogenesis is a result of deregulation of the mechanisms involved in multicellular organisms that control normal cell growth and differentiation.[75] Frequent mutation of p53 is crucial and often an obligatory step in carcinogenesis. Wild type p53 acts as a tumour suppresser gene while the mutated p53 has oncogenic potential.[18,76] Wild p53 gene inhibits oncogenic transformation and can prevent colony formation by tumour cells.[18] It participates in induction of cell death by apoptosis.[75] In murine leukaemia cell lines (MEL), there is a general lack of p53 expression. Lymphoid cell lines characteristically over-express p53.[76] Also wild type p53 could not be stably expressed by a p53 negative cell line.[77]

2.11.2 Material and Methods

Two murine leukaemia cell lines DP16-1 and C88 were grown in a humidified culture cabinet containing 5% CO_2 and at 37°C in Eagles medium (Gibco, UK). Eagles medium was supplemented with L-Glutamine, 10% Foetal calf serum (Gibco, UK), 100IU Penicillin and 100µg Streptomycin. The cells were subcultured every second day using fresh medium. When growing at an exponential phase at a concentration of $1x10^6$

cells/ml, they were harvested. The cell were washed twice in x1PBS and fixed in 70% Ethanol/x1PBS for 1hr on ice. The cells were washed twice in x1PBS. $1x10^6$ cells were suspended in 1ml of 0.25% Triton-X-100/x1PBS for 15 minutes, to facilitate permeabilisation. They were spun down at 400g in a microcentrifuge, the supernatant removed and resuspended in 500µl of 20% normal rabbit serum/TBS to prevent non specific binding of antibody. They were spun at 400g in a microcentrifuge and the supernatant removed. 75µl, 100µl or 150µl of neat ascitic fluid derived murine anti p53 (either 240 or 246 epitope provided by Dr. I. Lang - Aberdeen University) was added to the test samples for both cell lines DP16-1 and C88. Incubation was carried out for one hour in a water bath at 37°C.

Some controls were also included:

1) Blank only sample

2) FITC only sample

3) Isotype antibody control sample. (TNP-Pharmigen, USA)

The isotype control was used by adding 10µl to $1x10^6$ cells after 1hr. The cells were washed twice in 1% BSA/x1PBS. 40µl of FITC (Rabbit anti mouse-F0313-104) diluted 1/20 in 0.25% Triton-X-100/x1PBS was added and incubation was carried out for 30 minutes at room temperature. The cells were washed twice and analysed by a FAC-STAR flowcytometer.

For results - histograms - see chapter 3.11.

2.12 Effects of X-ray Irradiation on p53 Expression on Leukaemia Cell Lines DP16-1 and C88.

2.12.1. Introduction

In tissue culture systems, levels of p53 have been noted to rise in response to various DNA damaging agents.[78,79] This rise is mediated by post transcriptional mechanisms and is associated with transcriptional activation of p53 responsive genes (Kern et al. 1991).[78] p53 exerts a tumour suppressive effect by activating those genes. *In vivo* experiments where human or mouse skin has been irradiated with UV have shown a dramatic rise in p53 protein giving a link between p53 protein and response to DNA damage.[78]

DNA damage is a regular occurrence in a cells lifestyle. The mechanisms for repairing DNA damage are ancient in evolution and some elements are remarkably conserved from yeast to man. Repairing DNA damage is essential as DNA damage may contribute to 80% of human tumours (Doll et al. 1981).[18] It has been proposed that p53 (wild type) plays a role in causing cell cycle arrest in response to DNA damage to allow cells to repair damaged DNA template.[18,77]

An experiment was carried out on DP16-1 and C88 cell lines to assess p53 levels in response to X-ray irradiation. It was intended to prove further that the C88 cell line had a mutated p53 with no response to irradiation and to see if in DP16-1 p53 levels increased like a wild p53 would in response to irradiation.

2.12.2 Material and methods

Two murine leukaemia cell lines DP16-1 and C88 were grown in culture in a humidified culture cabinet containing 5% CO_2 and 37°C in Eagles medium (Gibco, UK). The culture medium was supplemented with L-Glutamine, 100IU of Penicillin and 100µg Streptomycin. Every day the culture medium was replaced with fresh medium. While growing at an exponential phase and at a concentration of $2x10^6$/ml they were irradiated with X-rays. The irradiation machine was Faxitron (Hewlett Packard, UK) delivering 70 Rads per min. For the C88 - 1500 Rads were administered to 10mls. of cells in a culture flask while for the DP16-1 1000 Rads were administered. For the control samples no irradiation was administered. After 7hrs. in culture both irradiated and non irradiated samples were harvested.

The cells were washed twice in x1PBS and fixed in 70% Ethanol/x1PBS for 1hr. on ice. Cells were washed twice in x1PBS and suspended in 0.25% Triton-X-100/x1PBS for 15 minutes to facilitate permeabilisation. They were spun down at 400g in a microcentrifuge. The supernatant was removed and cells resuspended in 500µl of 20% N.R.S (Normal rabbit serum)/TBS to prevent non specific binding of the antibody. They were spun down and the supernatant was removed. For the irradiated and normal control samples 75µl of an anti p53-242 epitope (Pan epitope provided by Dr. Lang of Aberdeen

University) was added to $1x10^6$ cells. For both cell lines 10µl of isoantibody TNP (Pharmigen, USA) was used. Incubation was carried out at 37°C in a water bath for 1hr.

Controls included:

1 Blank only sample.

2 FITC only sample.

3 Iso antibody control sample. (TNP-Pharmigen -USA)

Cells were washed twice in BSA/x1PBS after 1hr incubation. Except for the blank they were counterstained with 40µl of FITC (Rabbit anti mouse F313-1O4) diluted in 1:20 in 0.25% Triton-X-100/x1PBS. Incubation was carried out at room temperature for 30 minutes. They were washed twice in x1PBS. They were, except for the blank and FITC samples, also counterstained with Propidium iodide, final concentration 10µg/ml. Analysis was carried out by means of a FAC-STAR flowcytometer with laser beam tuned in at 488nm.

For results see chapter 3.12.

2.13 Cell Differentiation in Murine Leukaemia Cell Line C88. The Role Played by c-myc and p53 and the Link with Apoptosis as Confirmed by DNA Ladders.

2.13.1 Introduction

Various reports have shown the ability of erythroleukaemia cell line to differentiate under inducing agents ie DMSO (Dimethyl Sulfoxide) and HMBA (hexamethylenebisacetamide).[81,82,83,84] Exposure to those agents leads to recapitulation of the normal erythrocyte differentiation program[82] (see fig. 72). α and β globulin mRNA, α and β haemoglobin synthesis increases 10-100 fold over a 5 day period.[83,84] There is also accumulation of a chromatin associated protein $1P^1/_{25}$, also known to be identical to HI_0 protein. This is associated with cell proliferation and differentiation.[81] A number of biosynthetic enzymes undergo changes similar to the ones observed for the *in vivo* erythrocyte differentiation.

Commitment to differentiate under inducing agents i.e. DMSO and HMBA follows a latent period of 12-18hrs.[83,84] Commitment to differentiation is defined as the ability for

the cells to initiate a program of terminal differentiation even in absence of the inducer. Previous reports have shown that induced MEL cell line differentiation is a multistep process and involves proto-oncogene products like c-myb, c-myc, c-Fos and p53 have been detected in the nucleus where they could play a role in cell replication.[81] (see Fig. 72).

c-myc in the early precommitment stage 8-10 hrs. show a decrease which after this period rises to normal levels.[83] However using a blocker of differentiation i.e. Dexamethasone, does not prevent decrease in c-myc during differentiation but reverses c-myc deregulation.[83] p53 oncogene has also been shown to decrease during differentiation using DMSO.[85] Knowing that the oncogenes c-myc and p53 play a crucial role in cell proliferation as well as apoptosis in the case of wild p53, it was decided to examine the possible link between cell differentiation and cell death by apoptosis.

2.13.2 Material and Methods

C88 cells murine leukaemia cell line were grown in culture using Eagles medium supplemented with 10% Foetal calf serum, 100IU of Penicillin, 100μg of Streptomycin and L-Glutamine. The cells were grown in a culture cabinet at 37°C and containing 5% CO_2.

While growing at an exponential phase and at a concentration of $1x10^6$ cells per ml, DMSO (Dimethyl sulfoxide, Sigma, UK) was added to a final concentration of 2% DMSO. To the control samples no DMSO was added. The cells were left to grow in culture and some samples were taken off after 24hrs, 48hrs. and 72hrs. for DNA extraction and gel electrophoresis as previously described. Also samples were taken off at 2hrs. 10hrs. and 24hrs for the estimation of p53 levels.

Flowcytometric assessment of p53 and c-myc status was carried out as follows:

The cells from culture after appropriate incubation periods were washed twice in x1PBS. They were fixed for one hour on ice in 70% Ethanol/x1PBS. They were adjusted to a concentration of $1x10^6$ cells per ml and 0.25% Triton-X-100/x1PBS and left for 15 minutes to facilitate permeabilisation. They were spun down in a microcentrifuge at 400g

and resuspended in 500µl of 20% normal rabbit serum/TBS to reduce non specific binding of antibody. After 20 minutes they were spun down in a microcentrifuge at 400g and supernatant removed $1x10^6$ cells were labelled with 75µl of neat ascitic fluid derived anti p53-242 epitope (provided by Dr. Lang Aberdeen University).

Alternatively, some were labelled with 10µl of neat anti c-myc (Cambridge Research Biochemicals). Incubation was carried out for 1 hour in a water bath at 37°C. The cells were washed twice in 1% BSA/x1PBS. Cells were labelled with FITC (Rabbit antimouse -F313-104, DAKO, UK) dissolved in 40µl of 0.25% Triton-X-100/x1PBS and left at room temperature for 30 minutes - Controls included a blank and an FITC only sample. After 30 minutes cells were washed twice in x1PBS. For the c-myc experiment, they were also counterstained with PI final concentration 10µg/ml. They were then analysed for FITC and PI fluorescence, using a FAC-STAR flowcytometer with laser beam tuned in at 488nm.

For results see chapter 3.13.

2.14 Experiment to Induce and Demonstrate Cellular Differentiation of a Murine Leukaemia Cell Line C88 Using DMSO and Cytospin Preparations

2.14.1 Introduction

Cellular differentiation is a process by which a cell under influence of various stimuli and growth factors become transformed into a different phenotype. Various reports have shown the ability of polar/apolar compounds like DMSO (Dimethyl Sulfoxide) and HMBA (Hexamethylene bisacetamide) to induce cellular differentiation but the mechanism of their effects is obscure.[83,84,85] These compounds contain an apolar group linked to one or more polar molecular groups so that their essential feature is hydrophobicity and a high dipole moment and their target is plasma membrane.

Previous studies have indicated that a change in electrical potential across plasma membrane occurs when these compounds are used to induce leukaemia cell lines to differentiate.[86] These compounds, at their effective concentrations, were noted to absorb on charged surfaces of cell membranes causing a shift in transmembrane potential. A

linear correlation was found between the shift and induction of differentiation. The inducers have been used to mimic the action of erythropoietin.

2.14.2 Material and Methods

C88 cell line was grown in culture using Eagles medium (Gibco, UK) supplemented with 10% Foetal calf serum, 100 IU Penicillin, 100µg Streptomycin and L-Glutamine. They were grown in a culture cabinet at 37°C and containing 5% CO_2. Fresh culture medium was added every day. When growing at a density of $2x10^6$ cells, induction of differentiation was carried out by adding DMSO to 10mls of culture and a final culture concentration of 2%.

For the control sample - no DMSO was added. Also 5% DMSO concentration was tried and longer incubation periods of 48hrs. and 72 hrs. were tried. The cells were diluted in 3% BSA/x1PBS for a final concentration of $1x10^5$ cells/ml. The BSA helps the cells to stick on the slides, 100µl aliquots of the cell suspension were put in a cytospin centrifuge (Cytospin-3, Shandon, UK) which had slides to catch the cell preparations. The cytospin program was carried out at 800 rpm for 15 minutes. The cell preparations on the slides were removed from the cytocentrifuge and allowed to dry in air for 15 minutes. They were fixed by putting them in a Hellendahl jar containing acetone and absolute Ethanol in a ratio of 1:1. Fixation periods of varying lengths were tried but a 15 minute fixation period appeared adequate and the slides were left to dry.

The fixed cells on the slides were counterstained with Hematoxylin as follows.
The slides were immersed in tap water for two minutes to rehydrate the cells. They were immersed in an Eosin solution for 1 minute and this was washed off in a tap water bath. The slides were then immersed in a Haematoxylin solution for 2 minutes and washed in a tap water bath. The slides were dehydrated through increasing grades of ethanol 95% and 99% for 10 minutes each time. They were passed through a xylene bath for 10 minutes and mounted with coverslips using DPX (BDH) medium. The morphology of the cells was examined under a microscope.
For results see chapter 3.14.

CHAPTER 3

RESULTS

3.1. A Study of Correlations Between Apoptosis Proliferation and BCL-2 Expression in non-Hodgkin's Lymphomas

The results of apoptotic indices (ISEL), proliferation index (mRNA Histone) and Bcl-2 expression both for the low grade and high grades NHL are represented in tables 4, 5 and 6.

On the whole, the highest apoptotic indices and proliferation rates were seen in the high grade tumours. The occasional high grade tumours also had low proliferation rates. More of the low grade tumours had Bcl-2 expression (see tables 4, 5 and 6). The graph in fig.1 of Histone mRNA versus apoptotic indices (ISEL) and Bcl-2 expression, further confirms these observations more clearly with a good delineation of the low grade tumours with low apoptotic rates, low proliferation rates and more of them expressing Bcl-2. The graph also shows that all the high grades tumours do not fall on the imaginary straight diagonal, but are in three distinct sub groups. The ones above the diagonal have the highest mRNA histone and apoptotic rates, whereas the ones below, have low proliferation and apoptotic rates. The ones that falls on the imaginary diagonal are in between. This can be explained by the fact that the high grade tumours are a heterogeneous group.

TABLE 4 **Diagnostic classification, mRNA, ISEL and BCL-2 indices for High grade NHL**

No.	Diagnosis	Histone Count per 1000 cells	ISEL Count per 1000 cells	Bcl-2
1	2° CENTROBLASTIC	137	133	2
2	2° CENTROBLASTIC	500	135	1
3	2° CENTROBLASTIC	173	133	2
4	CENTROBLASTIC	203	87	3
5	CENTROBLASTIC	220	72	0
6	PERIPHERAL T CELL	364	118	2
7	T-LYMPHOBLASTIC	434	68	0
8	B-IMMUNOBLASTIC	245	54	0
9	CENTROBLASTIC	202	69	0
10	CENTROBLASTIC	390	53	0
11	CENTROBLASTIC	400	77	2
12	Ki-1 + T CELL lymphoma	121	45	2
13	HIGH GRADE CENTROBLASTIC	336	69	0
14	CENTROBLASTIC	348	83	0
15	B-IMMUNOBLASTIC	205	47	0
16	CENTROBLASTIC	234	125	0
17	CENTROBLASTIC	178	120	3
18	CENTROBLASTIC	247	82	0
19	CENTROBLASTIC	505	82	0
20	CENTROBLASTIC	234	122	0

TABLE 5 Diagnostic classification, mRNA, ISEL and Bcl-2indices for Low grade NHL

No.	Diagnosis	Histone Count per 1000 cells	ISEL Count per 1000 cells	Bcl-2 Positivity
21	T-CELL RICH, B-CELL LYMPHOMA	121	20	2
22	CENTROBLASTIC/CENTROCYTIC	67	37	0
23	CENTROBLASTIC/CENTROCYTIC	79	32	0
24	CENTROCYTIC/CENTROBLASTIC	103	37	3
25	CENTROCYTIC/CENTROBLASTIC	129	24	1
26	LYMPHOCYTIC	120	14	1
27	LYMPHOCYTIC	60	25	3
28	ptcl LENNERT'S lYMPHOMA	114	21	1
29	CENTROCYTIC/CENTROBLASTIC	60	10	0
30	CENTROBLASTIC/CENTROCYTIC	128	24	3
31	LYMPHOCYTIC	52	12	3
32	CENTROCYTIC/CENTROBLASTIC	83	22	3
33	LYMPHOCYTIC	107	35	0
34	CENTROCYTIC/CENTROBLASTIC (DIFFUSE)	132	34	1
35	CENTROCYTIC/CENTROBLASTIC (DIFFUSE)	138	16	3
36	T CELL RICH B CELL LYMPHOMA	130	22	2
37	CENTROCYTIC/CENTROBLASTIC	110	28	3
38	LYMPHOCYTIC	87	25	0
39	LYMPHOCYTIC	62	14	3
40	CENTROCYTIC/CENTROBLASTIC	110	30	0

Table 6 **The immunohistochemical results for proliferation rates and apoptotic indices and Bcl-2 in low and high grade NHL.**

Low Grade NHL Diagnosis	**No. of Cases**	**Proliferation index -range**	**Apoptosis index range**	**No. cases BCL-2 positive**
ML lymphocytic	6	52-120	12-35	4
ML follicular centrocytic/centroblastic	9	67-129	16-37	6
ML follicular/diffuse centrocytic/centroblastic	2	132-138	16-34	2
ML PTCL Lennert's lymphoma	1	114	21	2
ML B cell rich T-cell lymphoma	2	121-130	20-22	0
Low grade NHL totals and means	20	99.6	24.1	14
High Grade NHL Diagnosis				
ML B centroblastic	13	173-505	53-135	4
ML B centroblastic secondary	2	137-500	133-135	2
ML B immunoblastic	2	205-245	47-54	0
ML T cell lymphoma Ki-1 positive	1	121	45	1
ML T cell peripheral	1	364	118	1
ML T lymphoblastic	1	434	68	0
High grade NHL totals and means	20	283.8	88.7	8

TABLE 7 **Prognostic data as it relates to Proliferation, apoptotic and Bcl-2 expression indices in High grade NHL.**

High grade NHL				Prognosis					
No.	Hist	Tunnel	Bcl2	sur. in M	Alive	Dead	PR	CR	NR
1	137	133	2	83	1		3		
2	500	135	1	29	1		3		
3	173	133	2	12		2			5
4	203	87	3	37	1			4	
5	220	72	0	22	1			4	
6	364	118	2			2	3		
7	434	68	0	30	1			4	
8	245	54	0	57		2	3		
9	202	69	0	63	1			4	
10	390	53	0	59	1		3		
11	400	77	2	34	1			4	
12	121	45	2	23	1		3		
13	336	69	0	19		2	3		
14	348	83	0	26		2	3		
15*	205	47	0						
16	334	125	0	39		2			5
17	178	120	3	23	1			4	
18	247	82	0	22	1			4	
19	505	82	0	61	1			4	
20	234	122	0	8		2			5
Totals HG NHL					12/20	7/20	8/20	8/20	3/20

CODE 1=Alive

2=Dead

3=Partial remission(PR)

4=Chronic remission(CR)

5=No remission(NR)

* =Information not available

TABLE 8 **Prognostic data as it relates to Proliferation, apoptotic, and Bcl-2 expression indices in the Low grade NHL.**

	Low grade NHL			Prognosis					
No.	Hist.	Tunnel	Bcl2	Sur.-in-M	Alive	Dead	PR	CR	NR
21	121	20	2	26	1		3		
22	67	37	0	18	1		3		
23	79	32	0	122		2	3		
24*	103	37	0						
25	129	24	1	31	1			4	
26	120	14	1	34	1			4	
27	60	25	3	33	1		3		
28	114	21	1	18	1			4	
29	60	10	0	17	1		3		
30	128	24	3	18	1		3		
31	52	12	3	66	1		3		
32	83	22	3	29	1		3		
33	107	35	0	18	1		3		
34	132	34	1	119	1		3		
35	138	16	3	30	1			4	
36	130	22	2	110	1			4	
37	110	28	3	86	1			4	
38	87	25	0	38		2			
39	62	14	3	39	1			4	
40*	110	30	0						
Totals LG NHL					16/20	2/20	10/20	7/20	0/20

CODE 1=Alive

2=Dead

3=Partial remission(PR)

4=Chronic remission(CR)

5=No remission(NR)

* =Information not available

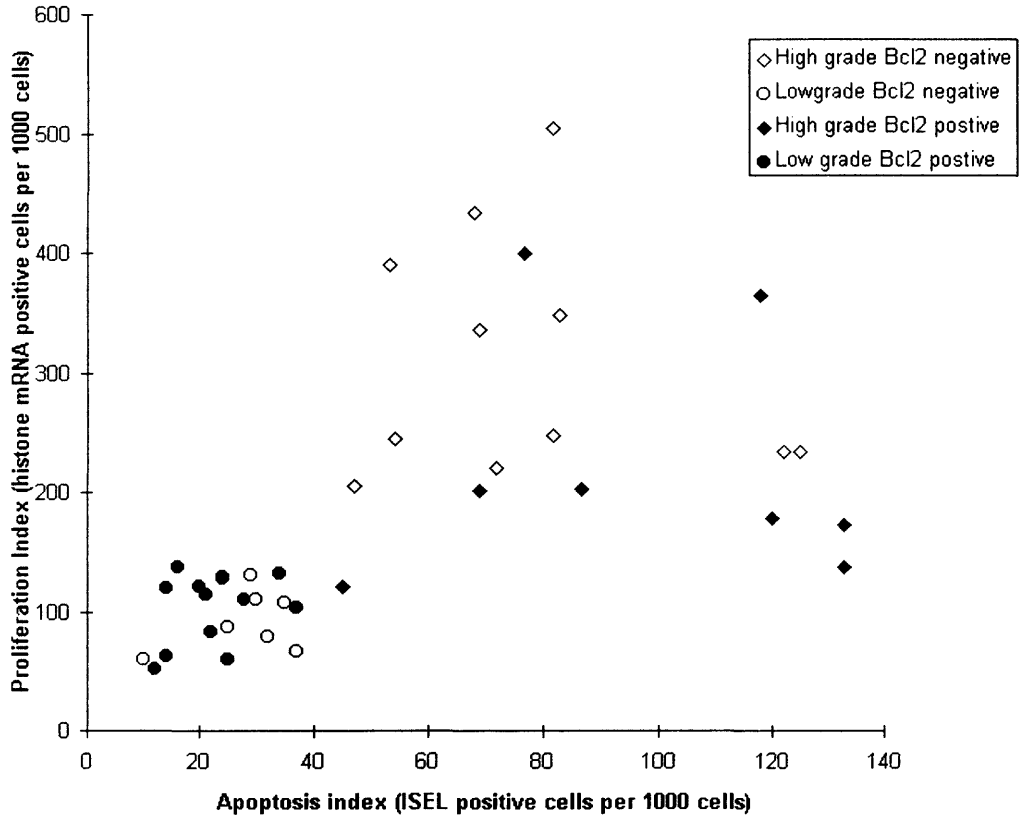


Figure 1 A plot of mRNA histone vs ISEL and Bcl-2 for the HG and LG NHL

Apoptotic index as assessed by ISEL

Black staining of nuclei was seen at apoptotic sites (see Plates III and IV in the appendix). As shown in Tables 4, 5 and 6 the high grade tumours had the highest apoptotic rates 68-133/1000 cells with a mean of 88.7. On the other hand the low grades had apoptotic rates 10-37/1000 cells with a mean of 24.1. These results were highly significant when the T-test was applied (P= <0.0001).

Cell proliferation assessment by mRNA Histone staining

Positive cells were identified by their brown/black staining of the cytoplasm (see Plates V and VI in the appendix). For the high grade some staining was seen in the macrophages. Tables 4, 5 and 6 show the results for proliferation index measurements. High grade tumours had the highest levels of cell proliferation as assessed by mRNA Histone, with a range of 121-505/1000 cells and with a mean of 283/1000 cells. The low grade tumours had significantly lower levels of mRNA histone indices of 52-135/1000 cells with a mean

of 99/1000 cells (T test P = <0.0001). A plot of apoptotic index versus mRNA Histone (Fig.1) shows a good correlation between the two.

Bcl-2 Staining

Plates VII and VIII (see appendix), show Bcl-2 staining of the high and low grade non Hodgkin's lymphomas Bcl-2 staining is shown in Tables 4, 5 and 6 The results show that 8 out of 20 high grade NHL stained for Bcl-2, and 14 out of 20 low grade NHL stained . More of the low grade NHL cases showed stronger Bcl-2 staining (8 out of 20 grade 3) compared to the high grades (2 out of 20 grade 3). Here was a positive correlation (correlation coefficient r=0.606) between apoptotic index and proliferation index and our results showed that the low grade NHL had stronger Bcl-2 expression with more of them showing Bcl-2 expression compared to the high grade cases (Tables 4, 5, 6 and Figure 1). There was no correlation between apoptotic index and Bcl-2 staining for high grade lymphomas

Prognostic Information

The results show a strong correlation between Bcl-2 expression and survival (P = <0.043). Most mortalities occurred in the Bcl-2 negative patients (see Table 7 and 8).

3.2 Flow Cytometric Demonstration of Apoptosis in a Human Leukaemia Cell Line (HL-60) and Raji Cell Line.

For the HL-60 cell line apoptotic activity as shown by the sub G_1 peaks was demonstrated in this cell line even at very low concentrations of Actinomycin D (0.25μg/ml) and increased with increasing concentrations of the drug (see figs. 2-6). Apoptosis was confirmed by DNA ladders (see chapters 2.5, 3.5 and plate X). The S-phase which characterises the proliferative compartment of the cell cycle, was progressively diminishing with an increasing degree of apoptosis. There is also a gradual reduction in the height of the G_1 phase. There was an obvious reduction in the S-phase as apoptotic activity increased. For the Camphotecin treated samples apoptotic activity was seen in the 2hr and 3hr samples. No significant apoptotic activity was seen in the 1hr. sample. Increasing duration of incubation with this drug caused increased apoptotic activity with similar effects on the reduction in G_1 height in the S-phase (see figs. 7-10). Apoptotic

activity was confirmed by DNA ladders (see plate IX and X). For EM, see plate XXVII, XXVIII and XXIX

For the RAJI cell line apoptotic activity was seen in significant amounts beginning at Cycloheximide concentration of 100μg and increased to maximum levels at 1g concentration (see figs 11-15). Apoptotic activity at all the concentrations of Cycloheximide used was confirmed by means of DNA ladders (see plate XII), for EM see plates XXX and XXXI.

3.3 Demonstration of Apoptotic Activity in Murine Leukaemia Cell lines-DP16-1 and MEL-C88

For the DP16-1 cell line apoptotic activity as evidenced by the sub G_1 peak was seen at all the concentrations of the drug used. There was evidence of increasing apoptotic activity with increasing concentration the drug (see figs.18-21). There was evidence of a decrease in the S phase in all samples in which apoptosis was induced which was maximum at the highest concentration of Thapsigargin used. For the C88 cell line, induction of apoptosis required a longer incubation period i.e. 24hrs. compared to 6hrs. for the DP16-1.

Apoptotic activity was seen at all the concentrations of the drug used and maximum at the highest concentration of the drug using Thapsigargin 40μg. There was also a progressive reduction in the S phase paralleled by an increase in the sub G_1 peaks (see figs. 22-26). Further confirmation of apoptosis was achieved by doing DNA ladders and EM on the sorted samples. For the C88, see chapter 2.5 and 3.5 for gel electrophoresis and for EM, plate XXXII (for normal control), and plate XXXIII for the treated sample. For the DP16-1 for gel electrophoresis see chapters 2.5 and 3.5. For EM see plate XXXII (normal control) and plate XXXV - for the treated sample.

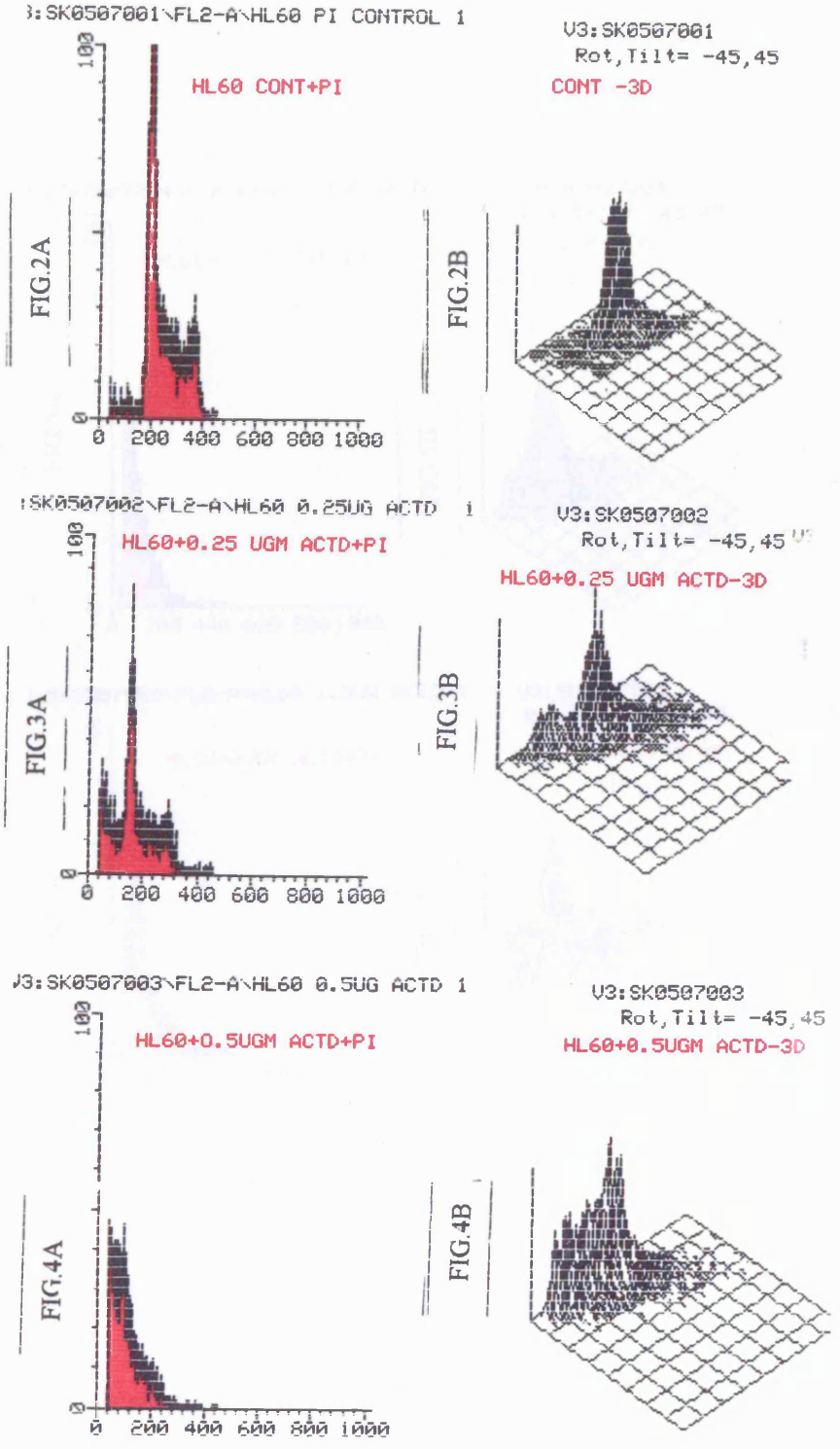
3.4 Flow Cytometric Demonstration of Apoptotic Activity in a human Leukaemia Cell Line HL-60 and a murine Leukaemia Cell Line C88 Using an End Labeling Technique with Digoxigenin-11-dUTP

The results clearly indicate that the end-labelling technique has worked well as the enzyme treated samples have been clearly separated from the Digoxigenin-11-dUTP only

treated samples (see fig. 29 C88 and fig.30 for HL-60). The reaction to incorporate Digoxigenin-11-dUTP is enzyme dependent as increasing concentration of the enzyme allows an increasingly bigger separation of labelled from unlabelled samples. For the HL-60, the fluorescence peaks due to FITC and the enzyme labelled nucleotides were the furthest and clearly separated from the control samples with no enzyme but only nucleotide. Also, these were clearly separated from the blank and FITC only samples. The incorporation of Digoxigenin oligonucleotide probe into the DNA breaks (3′ hydroxyl ends of DNA) requires the TdT enzyme. PI staining showed apoptotic peaks in HL-60 cell line and apoptotic activity.

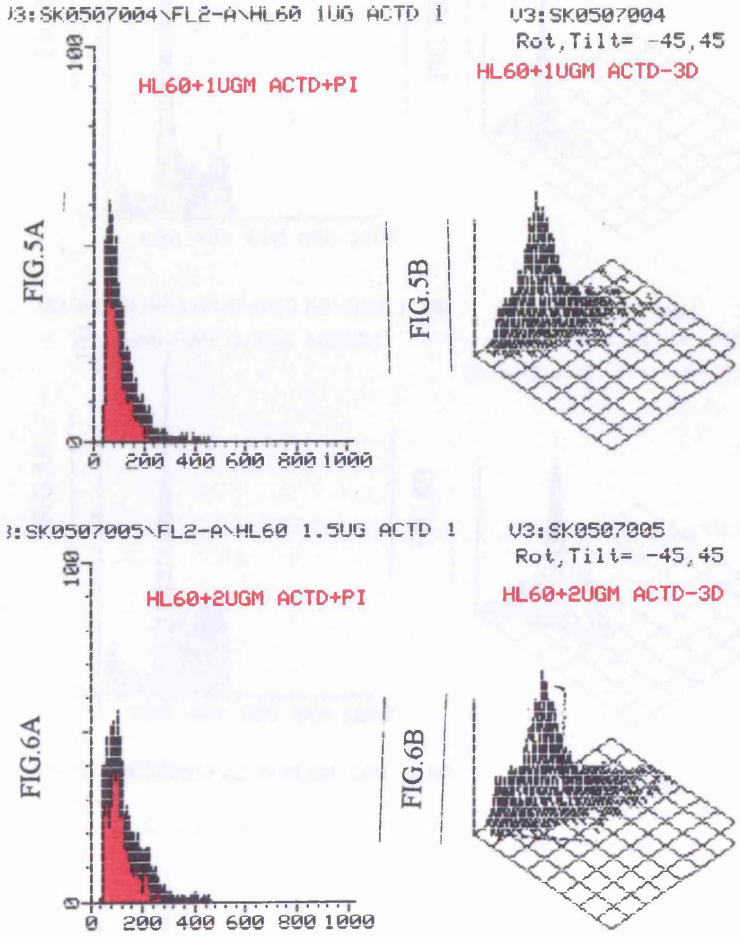
For the C88 the reaction works well at both concentrations of the Digoxigenin -11-dUTP oligonucleotide probe as both histograms overlap each other. For the C88 cell line, the fluorescent peaks of the enzyme and nucleotide labelled samples, were clearly well delineated from the nucleotide only labelled samples, as well as blank and FITC only controls. The end-labelling technique, appears to have worked well at all the three concentrations of the TdT (Terminal Deoxy nucleotidyl transferase) enzyme, i.e. (5IU, 10IU and 20IU).

The fluorescence peak for the 20 units of TdT enzyme appears further, indicating that the labelling process worked better at this concentration. The apoptotic activity was confirmed in both C88 and HL-60 cell lines by means of DNA ladders.
See in the appendix, plate XIX for the C88 and plate XVIII for the HL-60



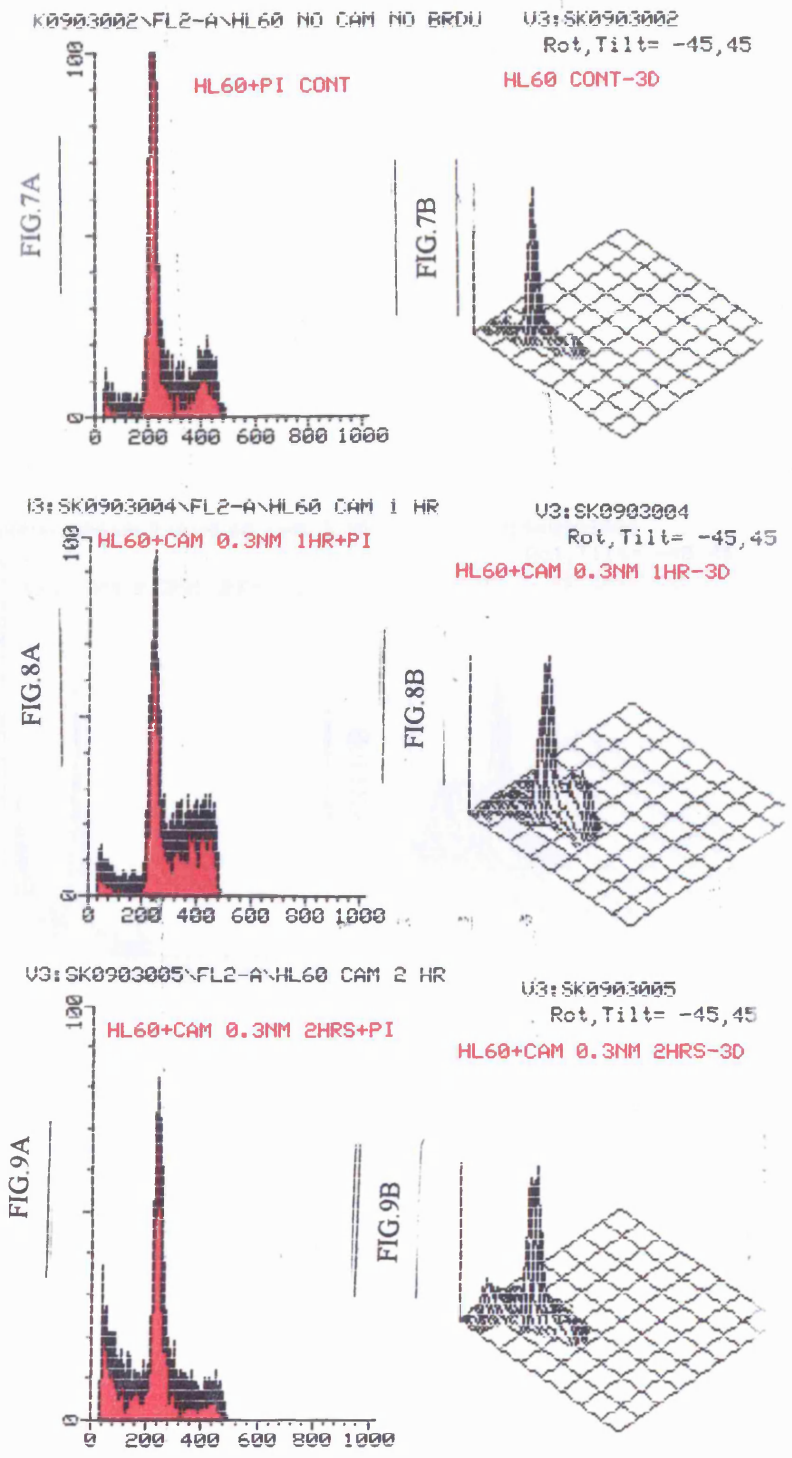
HL-60

Figs 2 -6. DNA frequency histograms of HL-60 cell line. Fig.2 is normal control. Figs 3-6-represent samples treated with varying concentrations of Actinomycin D (0-25μg-2μg) for 5hrs, fixed with 70% Ethanol and stained with PI. Figs 3A-6A shows increasing apoptosis as manifested by the sub G_1 peaks, with the increasing concentration of Actinomycin D. There is also a corresponding diminishing S phase. Figs. 2B-6B represent a three dimensional display of similar data.



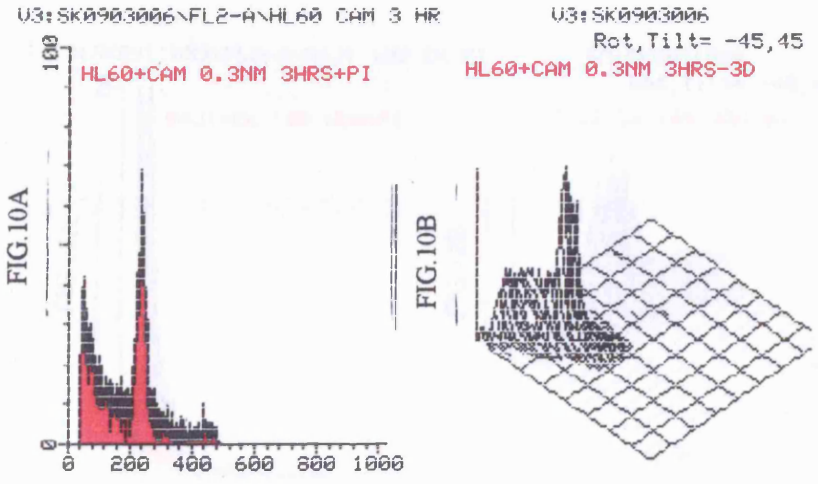
HL-60

Figs 5-6. DNA histograms of HL-60 cell line treated with 1μgm and 2μg of Actinomycin D respectively, and stained with PI. Apoptotic peaks are evident in the sub G_1 phase there is also a reduction in the S phase. Figs.5B-6B represent a 3 dimentional display of similar information.



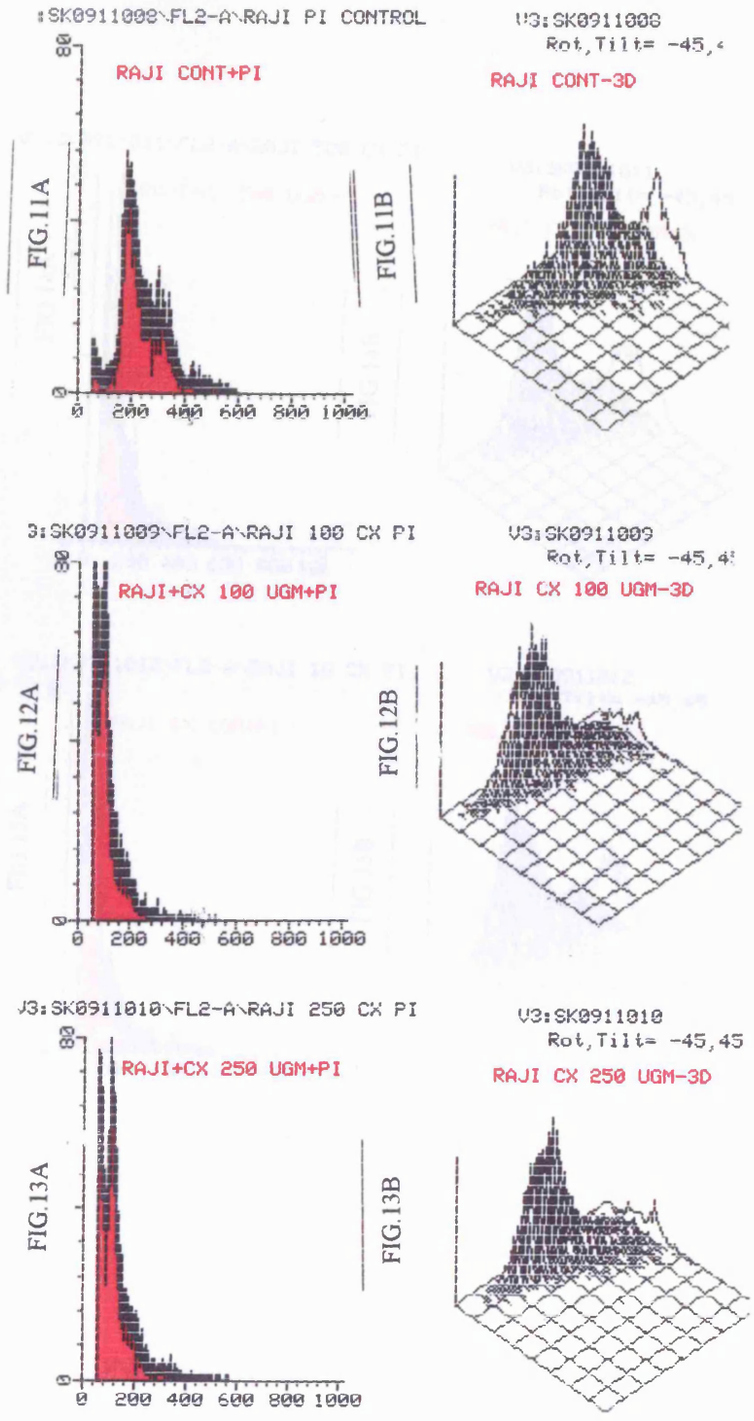
HL60

Figs. 7-10. DNA frequency histograms of HL-60 cell line. Fig.7 is normal control. Figs. 8-10 represent samples treated with (Camphotecin 0.3NM) for 1-3hrs. They were fixed with 70% Ethanol and stained with PI. Figs.8 A-10A show increasing apoptosis as manifested by the sub G_1 peaks, with the increasing concentration of Camphotecin. There is also a corresponding diminishing S phase. Figs.7B-10B represent a three dimensional display of similar data.



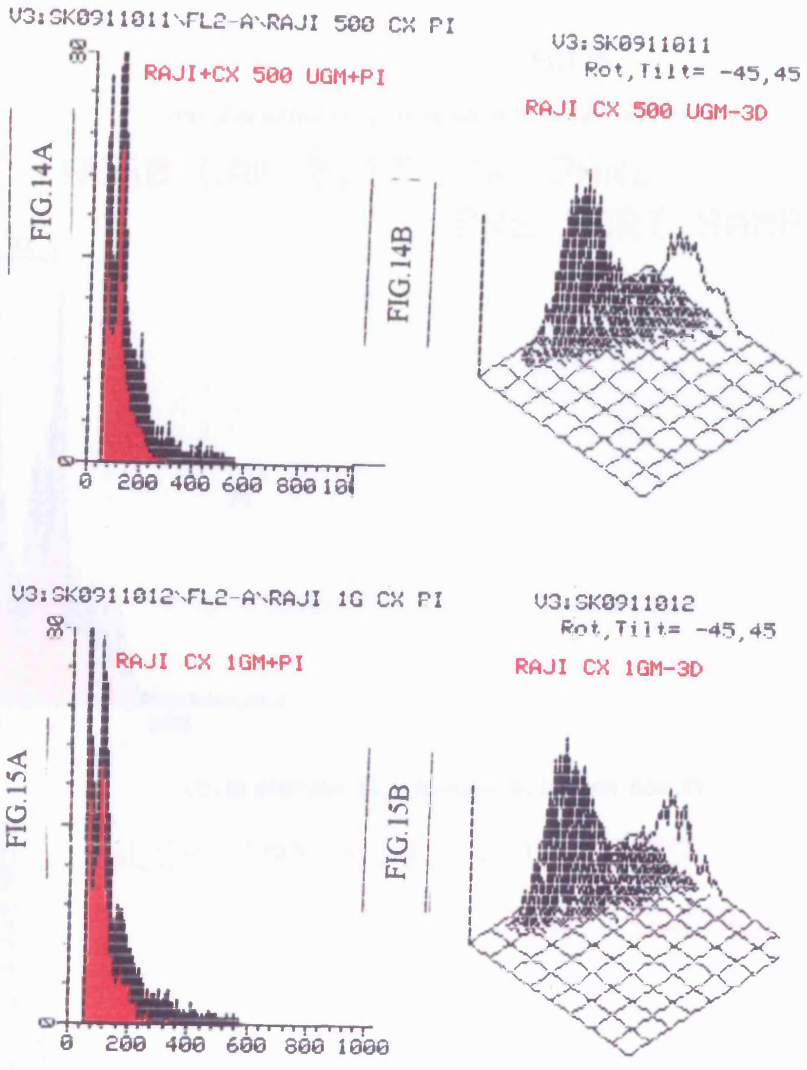
HL-60

Figs 10A - B. DNA histograms of HL-60 cell line treated with 0.3NM of Camphotecin for 3hrs and stained with PI. Apoptotic peaks are evident in the sub G_1 phase there is also a reduction in the S phase. Fig.10B represent a 3 dimentional display of similar information.



Raji

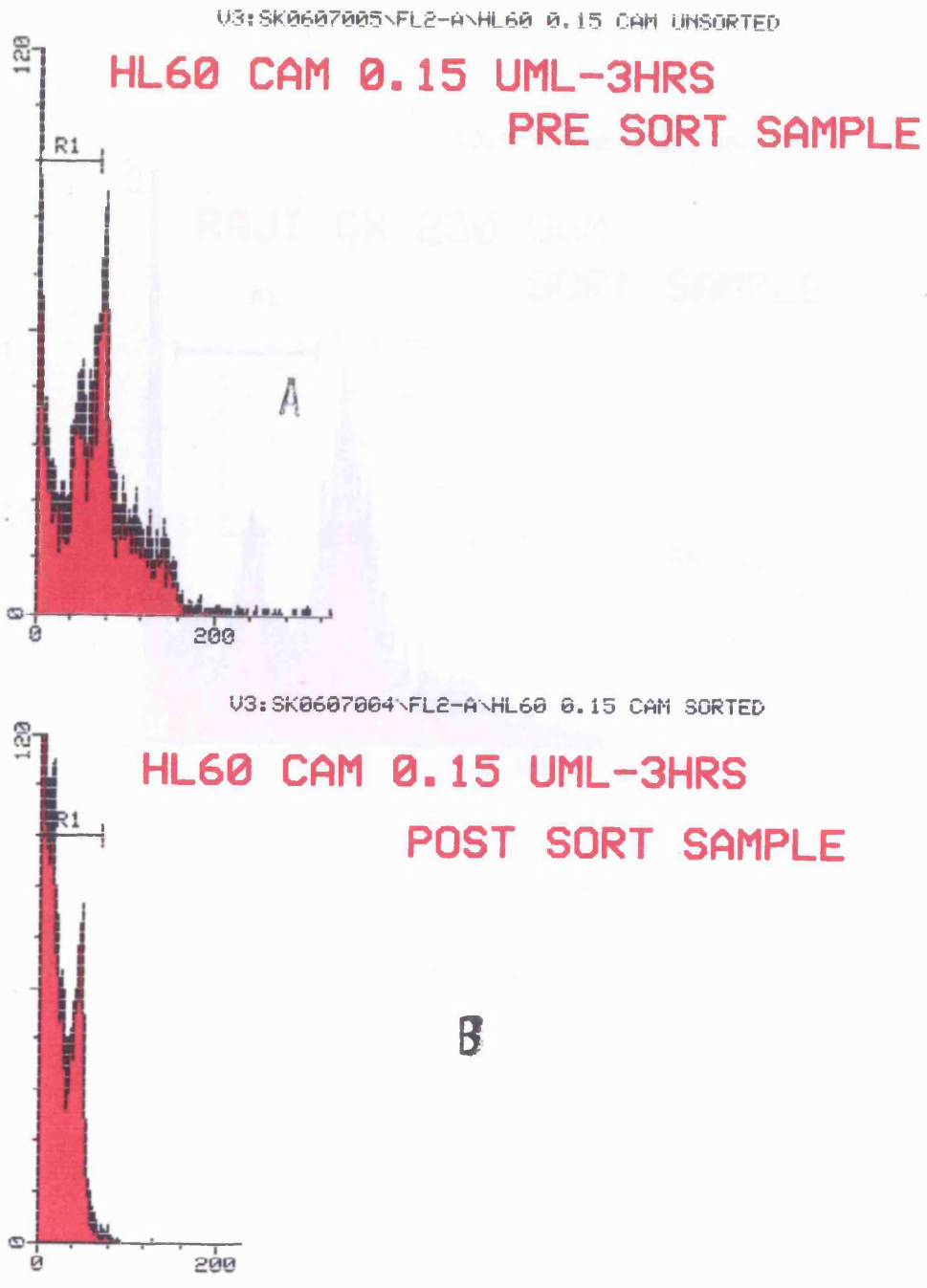
Figs. 11-15. DNA frequency histograms of Raji cell line. Fig 11 is normal control. Figs. 12-15-represent samples treated with varying concentrations of Cycloheximide (100µg-1g) for 72hrs. They were fixed with 70% Ethanol and stained with PI. Figs 12A-15A show increasing apoptosis as manifested by the sub G_1 peaks, with the increasing concentration of Actinomycin D. There is also a corresponding diminishing S phase. Figs 11B-15B represent a three dimensional display of similar data.



Raji

Figs. 14A-15A. DNA histograms of Raji cell line treated with 500µg and 1g respectively, of Cycloheximide for 72hrs and stained with PI. Apoptotic peaks are evident in the sub G_1 phase there is also a reduction in the S phase. Fig.14B -15B represent a 3 dimentional display of similar information.

FIG.16



HL60-Sort

Figs 16A and Fig 16B. DNA frequency histograms of HL60 cell line. They were treated with Camphotecin 0.15μM for 3hrs, fixed with 70% Ethanol and stained with PI. Fig 16A is the pre-sort sample. There are big apoptotic peaks in the sub G_1 phase
Fig 16B is the post sort sample. It clearly represents the sorted apoptotic sub G_1 peaks

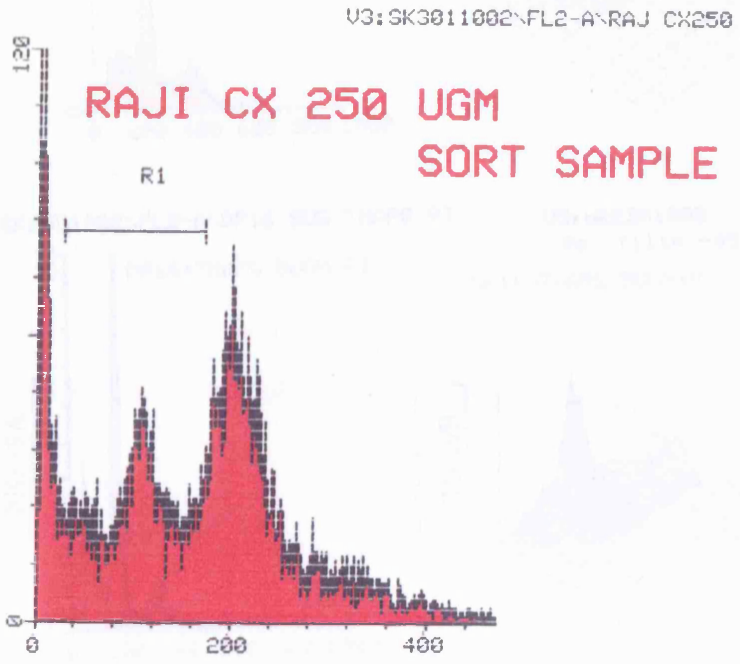
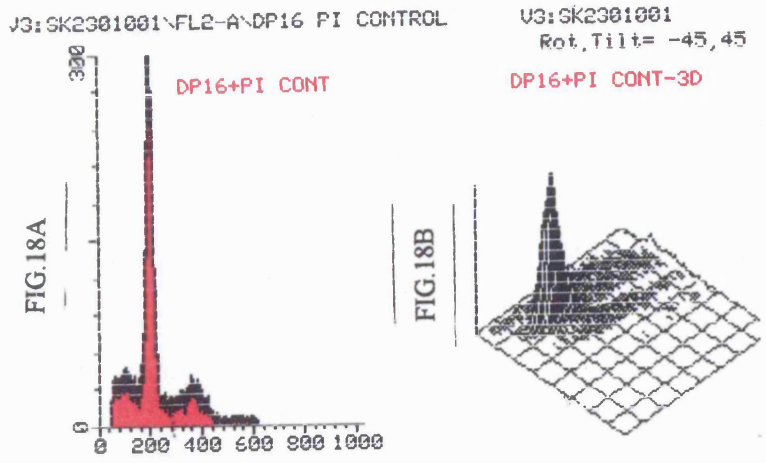
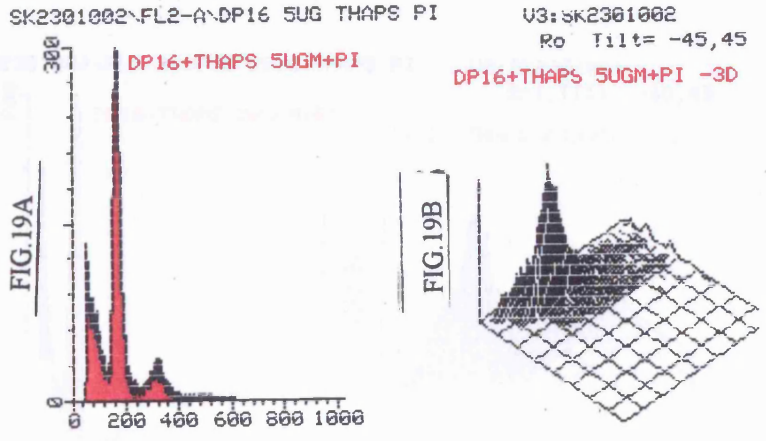


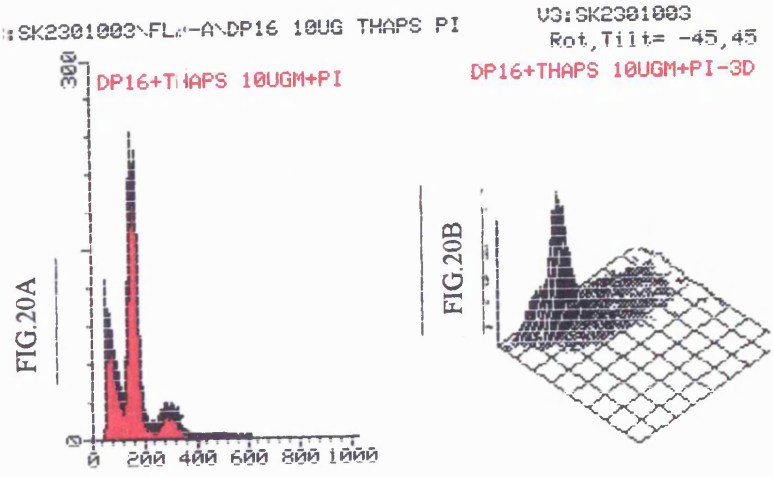
FIG.17

Raji Sort sample

Fig 17. Represents DNA frequency histograms of Raji cell line. The cell were treated with 250µg of Cycloheximide for 72hrs ,fixed with 70% Ethanol and stained with P.I. There are big apoptotic peaks in the sub G_1 phase. A region R1, was drawn around the sub G_1 peaks and cells in this region were sorted.

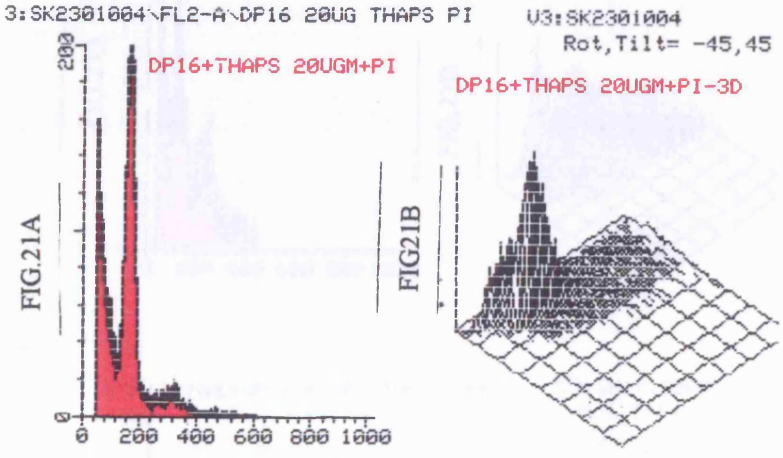






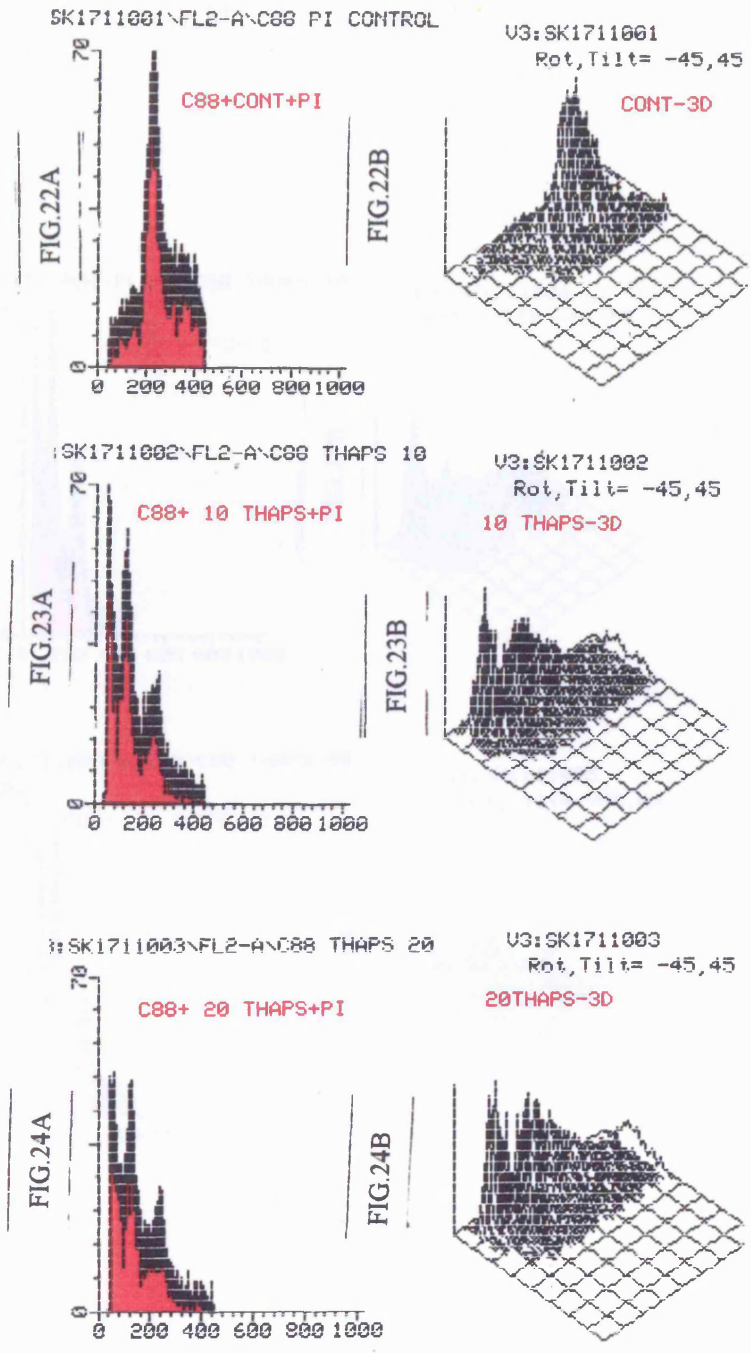
DP16-1Cell Line

Figs 18-21. DNA frequency histograms of DP16-1 cell line. Fig.18 is normal control. Figs 19-21-represent samples treated with varying concentrations of Thapsigargin (5μg-20μg) for 6hrs, fixed with 70% Ethanol and stained with PI. Figs 19A-21A show increasing apoptosis as manifested by the sub G_1 peaks, with the increasing concentration of Thapsigargin. There is also a corresponding diminishing S-phase. Figs. 18B-21B represent a three dimensional display of similar data.



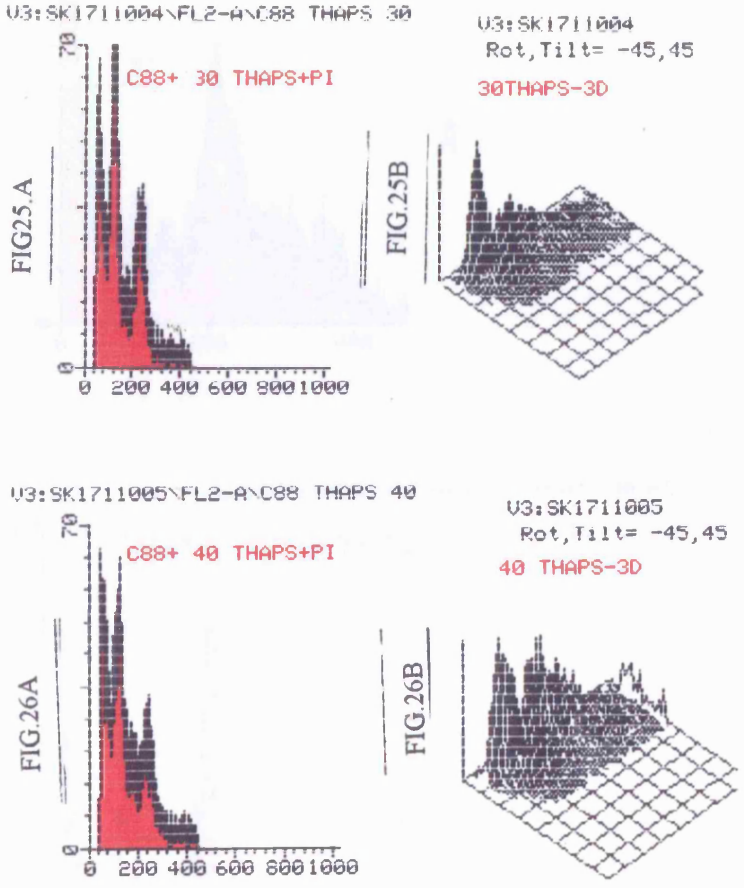
DP16-1Cell Line

Figs 21A - B. DNA histograms of DP16-1 cell line treated with 20μg of Thapsigargin and stained with PI. A tall apoptotic peak in the sub G_1 phase is evident. There is also a reduction in the S-phase. Fig.21B represents a 3 dimensional display of similar information.



C88 Cell Line

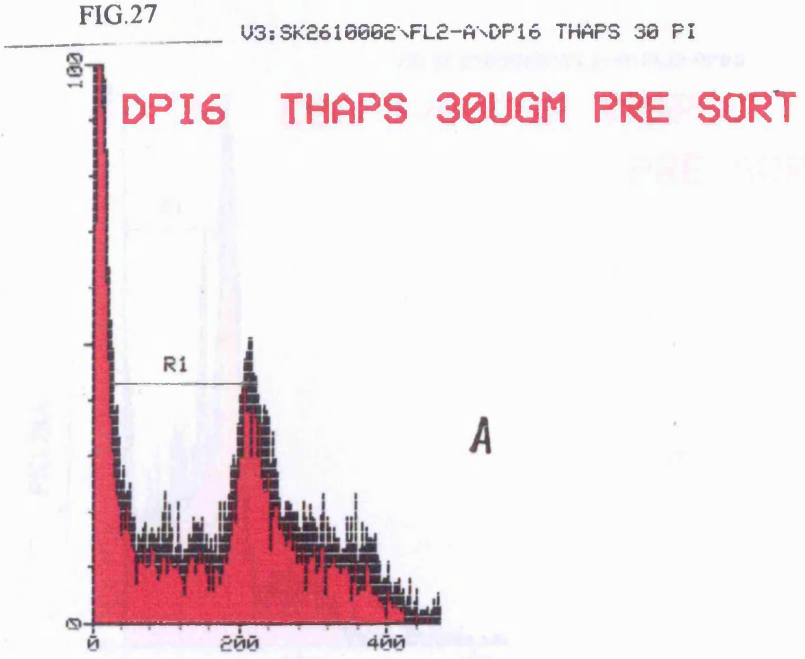
Figs 22-26. DNA frequency histograms of C88 cell line. Fig.22 is normal control. Figs 23-26-represent samples treated with varying concentrations of Thapsigargin (10μg-40μg) for 24hrs, fixed with 70% Ethanol and stained with PI. Figs 23A-26A show increasing apoptosis as manifested by the sub G_1 peaks, with the increasing concentration of Thapsigargin. There is also a corresponding diminishing S-phase. Figs. 23B-26B represent a three dimensional display of similar data.

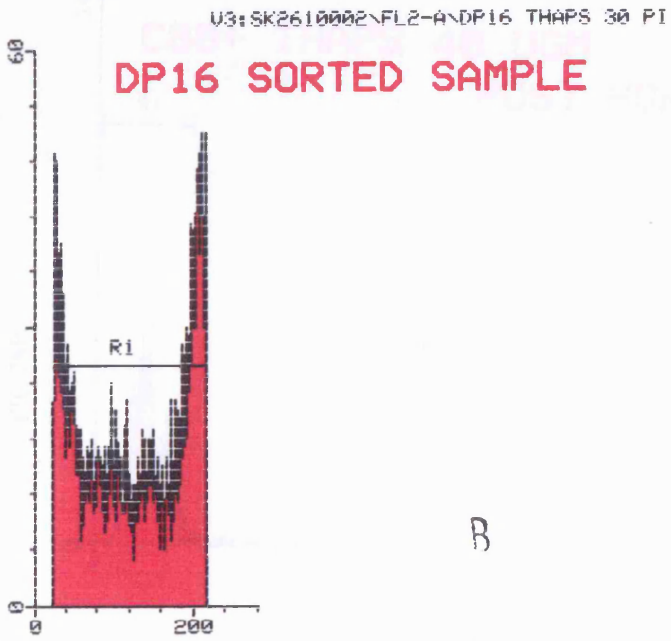


C88 Cell line

Figs 25A -26 A. DNA histograms of C88 cell line, treated with 30μg and 40μg respectively, of Thapsigargin for 24 hrs and stained with PI. Apoptotic peaks are evident in the sub G1 phase there is also a reduction in the S-phase. Figs.25B-26B represent a 3 dimensional display of similar information.

FIG.27

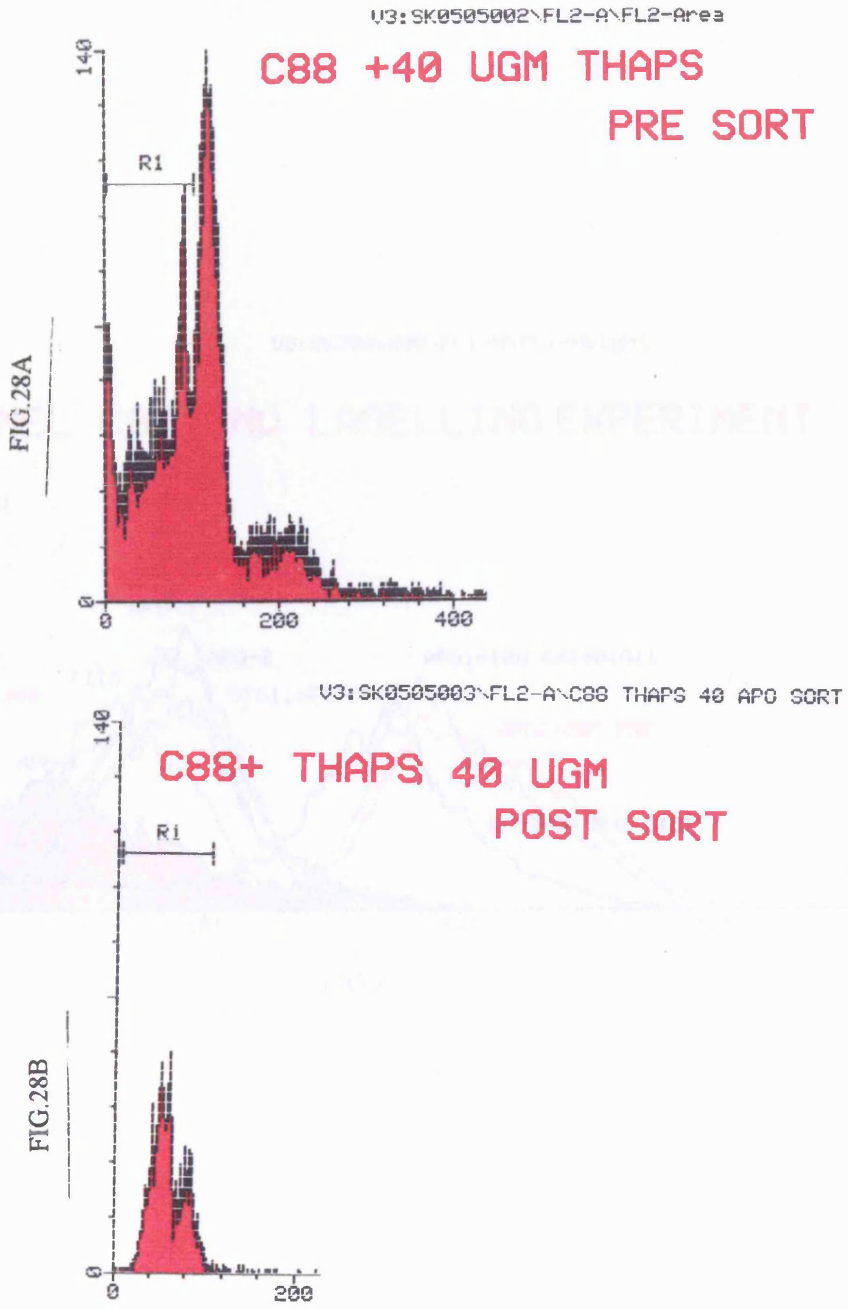




DP16-1 SORT

Fig 27A and Fig 27B. DNA frequency histograms of DP16-1 cell line. They were treated with 30μg Thapsigargin for 6hrs, fixed with 70% ethanol and stained with PI. Fig 27A is the pre-sort sample. There are big apoptotic peaks in the sub G_1 phase. A region R1 was drawn and cells in this area were sorted.

Fig 27B is the post sort sample. It clearly represents the sorted apoptotic sub G_1 peaks.



C88 SORT

Fig 28A and Fig 28B. DNA frequency histograms of C88 cell line. They were treated with 40μg Thapsigargin for 24hrs, fixed with 70% ethanol and stained with PI.
Fig 28A is the pre-sort sample. There are big apoptotic peaks in the sub G_1 phase
Fig 28B is the post sort sample. It clearly represents the sorted apoptotic sub G_1 peaks.

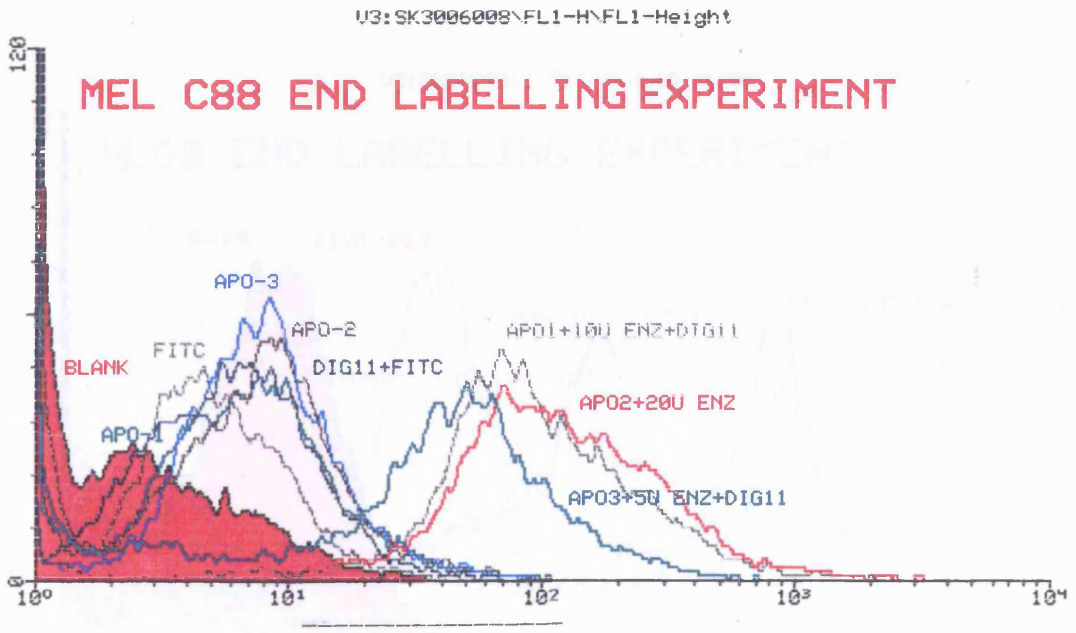


FIG.29

C88 cell line - End Labelling

Fig 29. Shows histograms of C88 cell line End labelling technique with Digoxigenin-11-dUTP. On the x-axis is FITC fluorescence and the y-axis is cell number. The blank is represented by the red histogram. All the histograms overlapping the red (blank) are controls to which no enzyme had been added as well as the FITC only control. All the controls are well separated from the test samples to which the Digoxigenin-11-dUTP and TdT enzyme concentrations of 51U, 10IU as well as 20IU had been added. This shows that the End labelling method has worked. Increasing enzyme concentration gives better separation

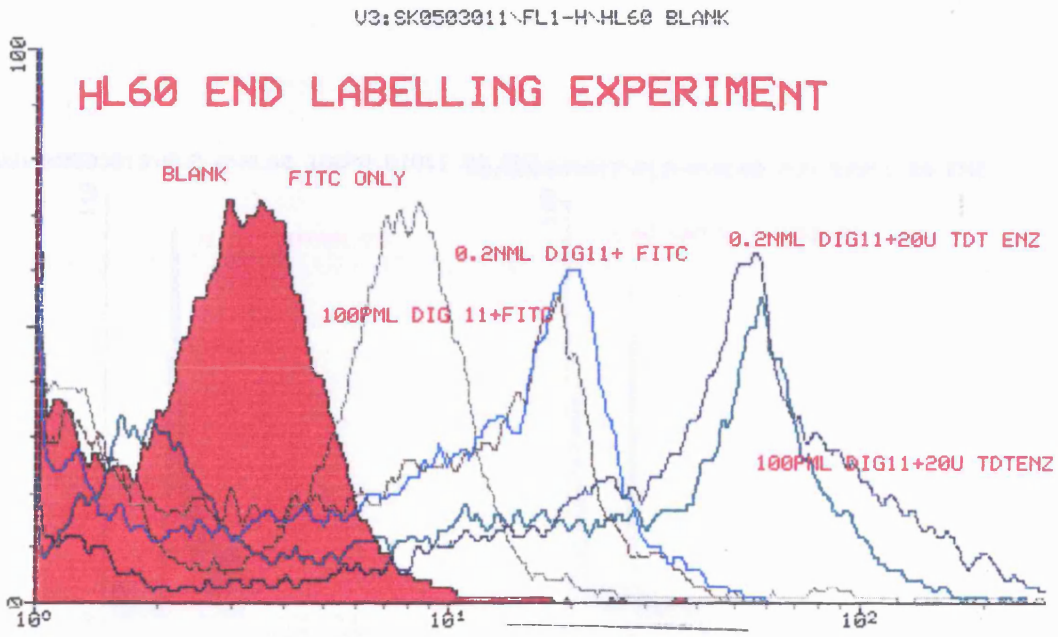


FIG.30

HL-60-Cell line - End Labelling

Fig 30. Shows histograms of HL-60 cell line End labelling technique with Digoxigenin-11-dUTP. On the x-axis is FITC fluorescence and the y-axis is cell number. The blank is represented by the red histogram. All the histograms overlapping the red (blank) are controls to which no enzyme had been added as well as the FITC only control. All the controls are well separated from the test samples to which the Digoxigenin-11-dUTP at concentrations of 0.2nM and 100pm as well as 20IU of TdT. enzyme had been added. This shows that the End labelling method has worked at both concentrations of Digoxigenin-11-dUTP.

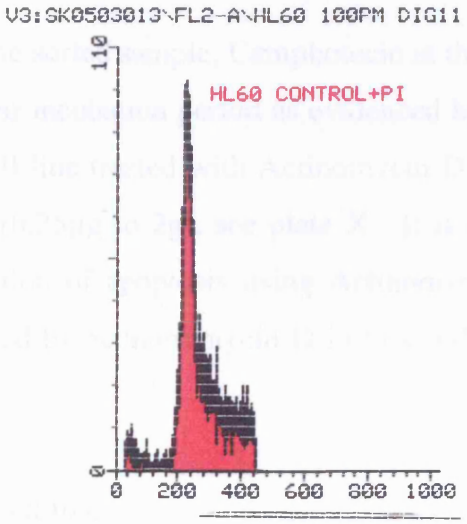


FIG.31

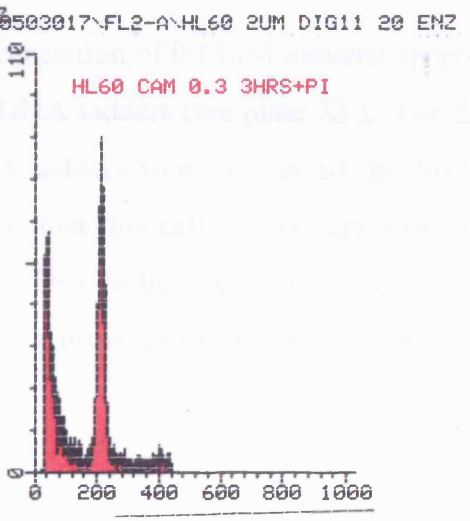


FIG.32

Fig 31. DNA histogram of HL-60 cell line fixed with 70% Ethanol and stained with PI (normal control). Fig 32:DNA histogram of HL-60 cell line incubated with 0.3μ Camphotecin for 3hrs, fixed with 70% Ethanol and stained with PI. Apoptotic activity represented by sub G_1 peak is evident.

3.5 DNA Extraction and Gel Electrophoresis on the Sorted and non Sorted Samples of HL-60, Raji, C88 and DP16-1

HL-60 cell line

When Camphotecin was used to induce apoptosis at concentrations of 0.15μM for 2 and 3hrs and 0.3μM for 3hrs, DNA ladders could be demonstrated. Bands of DNA are clearly evident in the photomicrographs at 2hrs and 3hrs, whereas none are seen in the 1hr or control sample There were no ladders at 1hr incubation period (see plate IX). The induction of apoptosis in this cell line characterised by DNA ladders at 2hrs and 3hr further confirms that the sub G_1 peaks seen in the histograms in earlier experiment (Chapter 3.2), are indeed apoptotic peaks.

For the sorted sample, Camphotecin at the concentration of 0.15μM induced apoptosis for the 3hr incubation period as evidenced by the DNA ladders (see plate XI.). For the HL-60 cell line treated with Actinomycin D. DNA ladders were seen at all the dose ranges used (0.25μg to 2g), see plate X. It is obvious that this cell line is very susceptible to induction of apoptosis using Actinomycin D. This further confirms apoptotic activity induced by actinonomycin D in this cell line in a previous experiment (see chapters 2.2 and 3.2)

Raji cell line

For the non sorted samples, DNA ladders were seen at all the concentrations of Cycloheximide used (100μg, 250μg, 500μg and 1g) with a 72hr incubation period. See plate XII. For the sorted sample treated with Cycloheximide 250μg DNA ladders were demonstrated. See (plate XIII). This further confirms that the sub G_1 peaks in the histograms in Chapter 3.2 are indeed apoptotic peaks.

DP16-1 cell line

DNA ladders were demonstrated at all the concentrations of Thapsigargin used (5μg, 10μg, 20μg and 30μg (see plate XIV). For the sorted sample treated with Thapsigargin 30μg DNA ladders were demonstrated (see plate XV). This further confirms that the apoptotic activity seen in the histograms, as represented by sub G_1 peaks is genuine (see Chapter 3.3).

C88 cell line

Apoptotic activity seen on histograms in Chapter 3.3 was confirmed in the non sorted samples treated with Thapsigargin at all the concentrations used for the 24hr. incubation period ie,10μg, 20μg, 30μg and 40μg. See plate (XVI). For the sorted sample apoptotic activity was confirmed at the concentration of Thapsigargin (30μg) used. See plate XVII

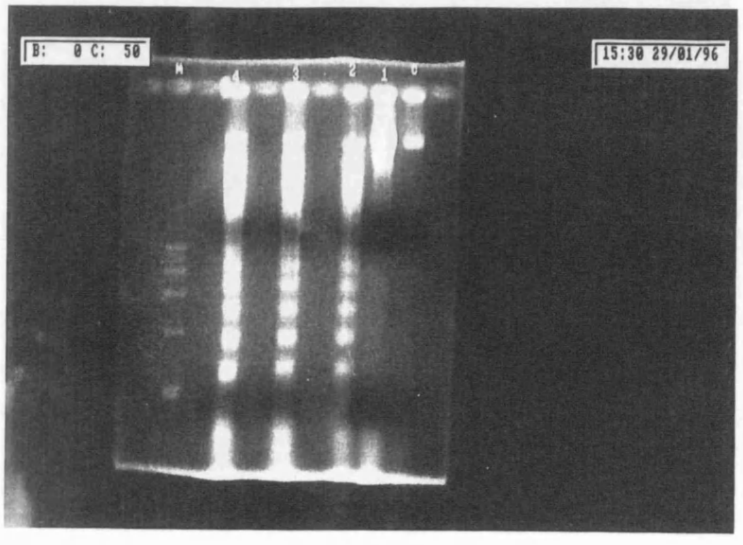


Plate IX

Gel electrophoresis - HL-60 cell line treated with Camphotecin 0.15μM at varying incubation periods.
C - Control DNA
Lane 1 - 1hr. DNA
Lane 2 - 2hrs. DNA
Lane 3 - 3hrs. DNA
Lane 4 - 3hrs. DNA 0.3μM Camphotecin
Lane 5 - marker DNA (Physex/Hind III)

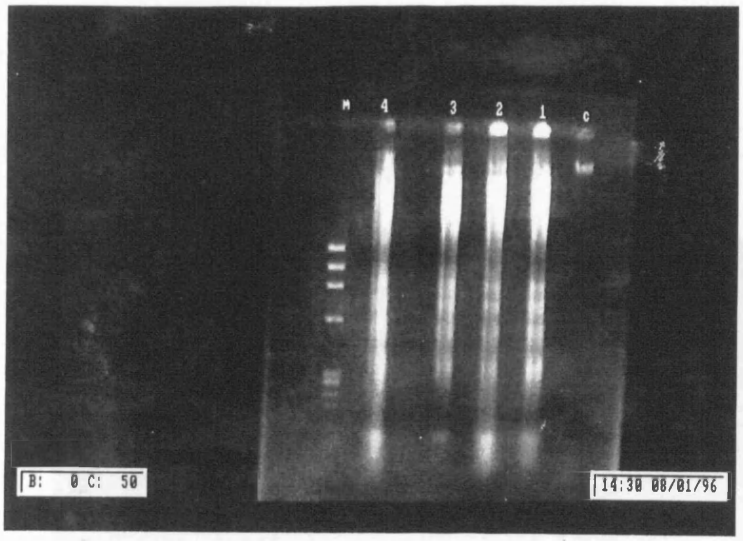


Plate X

HL-60 cell line treated with varying concentrations of Actinomycin D
Lane 1-0.25µgms
Lane 2- 0.5µgm
Lane 3- 1µgm
Lane 4- 2µgms
M - Marker DNA(Physex/Hind III)

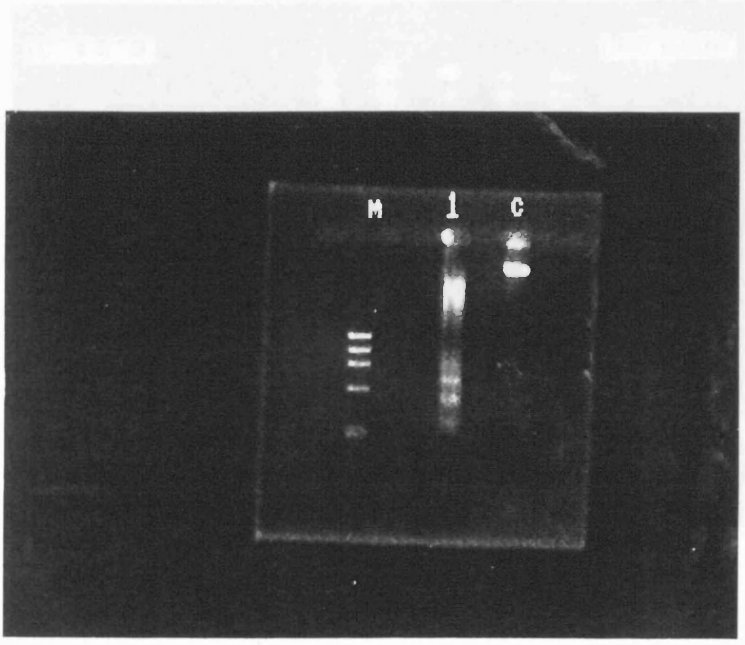


Plate XI

Gel electrophoresis.
Sorted sample HL-60 cell line treated with Camphotecin 0.I5μM for 3hrs.
C - Control DNA
Lane - 1 - Sorted sample
M - Marker DNA (Physex/Hind III)

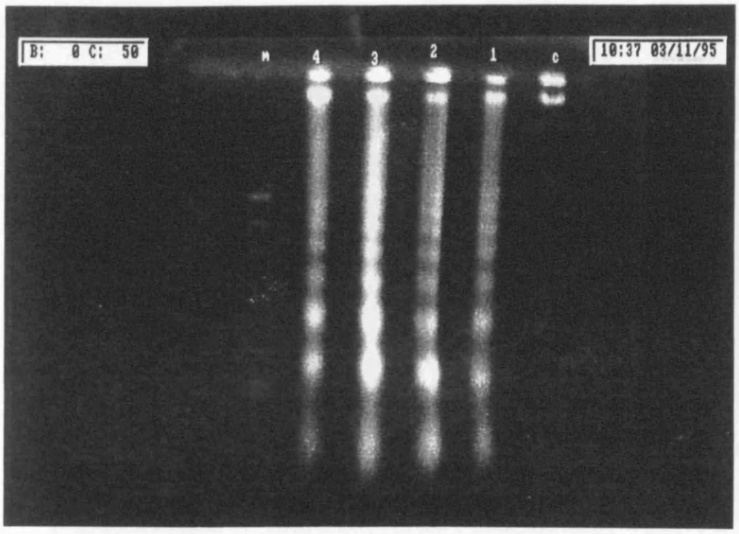


Plate XII

Gel electrophoresis.
Raji cell line treated with varying concentration of Cycloheximide (Cx.) for 72hrs.
C - Control DNA
Lane - 1 - Cx- 100µgms
Lane - 2 - Cx- 250µgms
Lane - 3 - Cx- 500gms
Lane - 4 - Cx- 1000µgms
M - Marker DNA (Physex.Hind III)

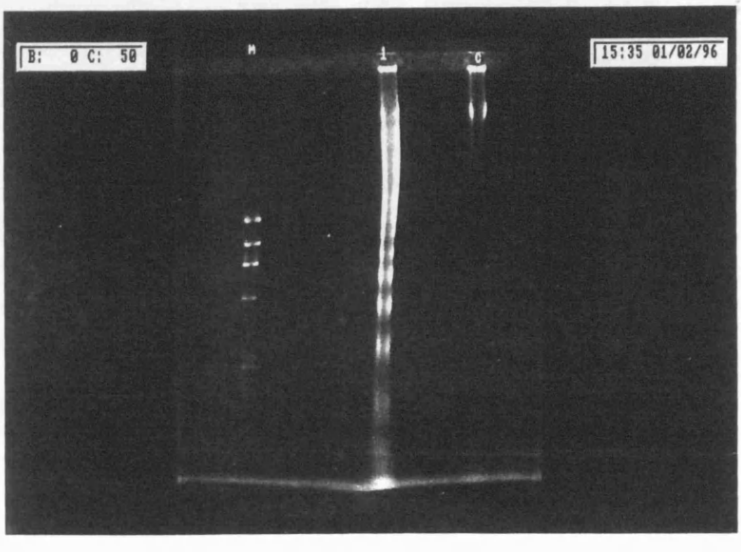


Plate XIII

Gel electrophoresis.
Raji cell line -sorted sample treated with
Cycloheximide(Cx) 250µgms - 72hrs.
C - Control DNA
Lane 1- Sorted sample DNA
M - Marker DNA

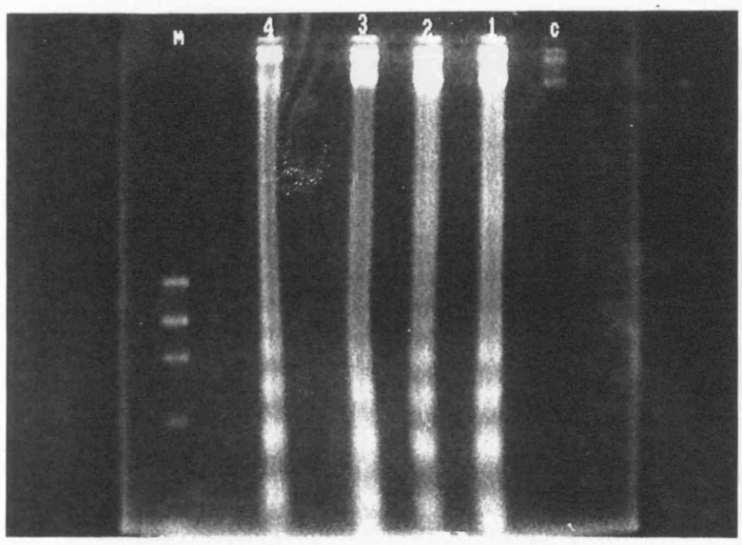


Plate XIV

Gel electrophoresis.
DP16-1 -cell line treated with varying concentrations of Thapsigargin- 6hr. incubation period.
C - Control DNA
Lane 1- 5µgms
Lane 2 - 10µgms
Lane 3 - 20µgms
Lane 4 - 30µgms
M - Marker DNA (Physex/Hind III)

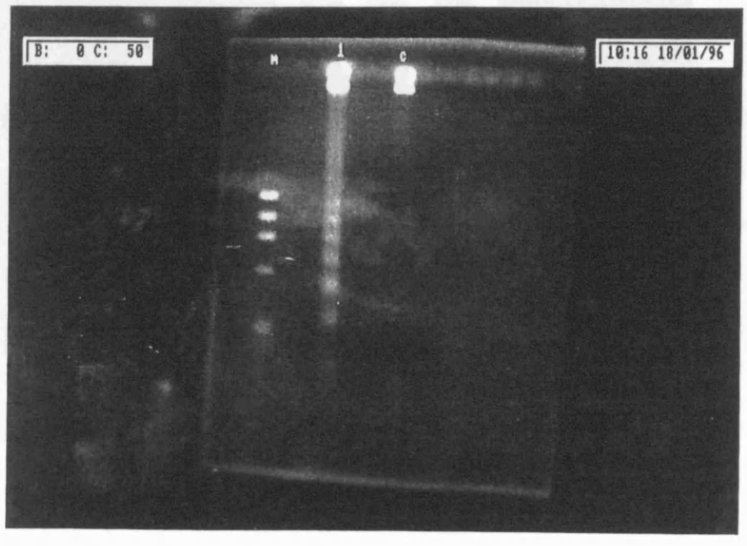


Plate XV

Gel electrophoresis.
DP16-1 cell line treated with Thapsigargin-
30µgms for 6hrs. - sorted sample.
C - Control sample
Lane 1 - Sorted sample
M - Marker DNA (Physex/Hind III)

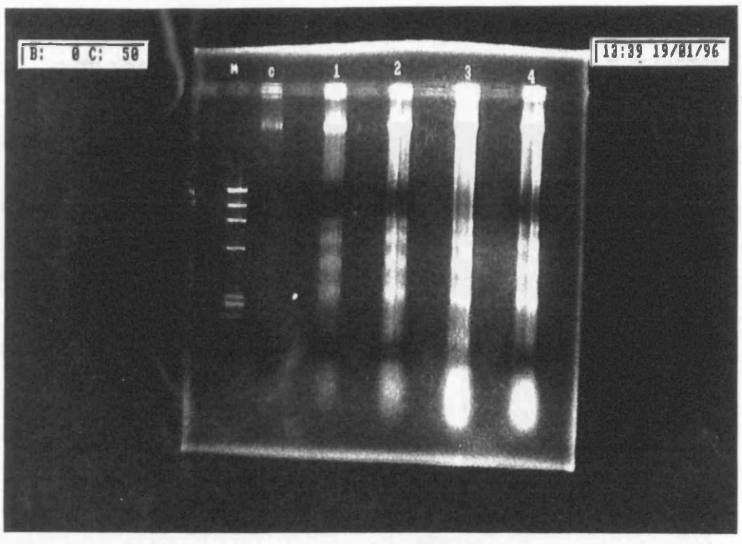


Plate XVI

Gel electrophoresis.
C88 cell line treated with varying concentrations of Thapsigargin for 24hrs.
C - Control DNA
Lane - 1- 10µgms
Lane - 2- 20µgms
Lane - 3- 30µgms
Lane - 4- 40µgms
Lane - 5- Marker DNA(Physex/Hind III).

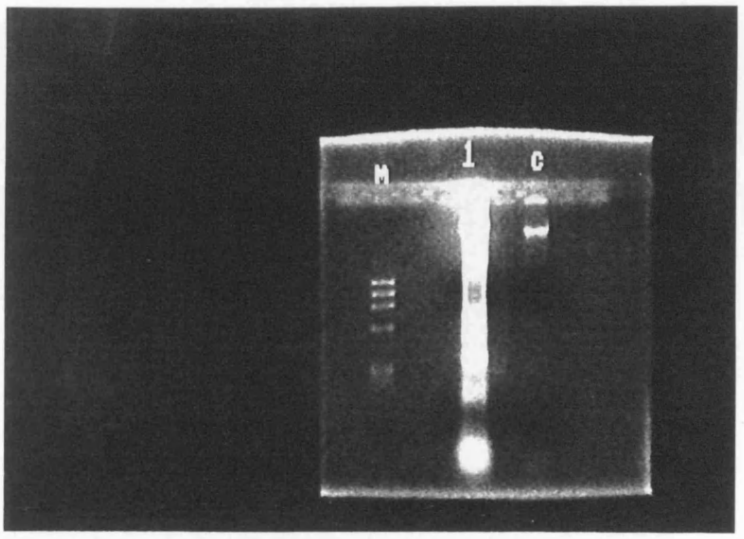


Plate XVII

Gel electrophoresis.
C88 cell line sorted sample.
Treated with Thapsigargin 30µgms- 24hrs
C - Control DNA
Lane - 1 - Sorted sample DNA
M - Marker DNA (Physex/Hind III)

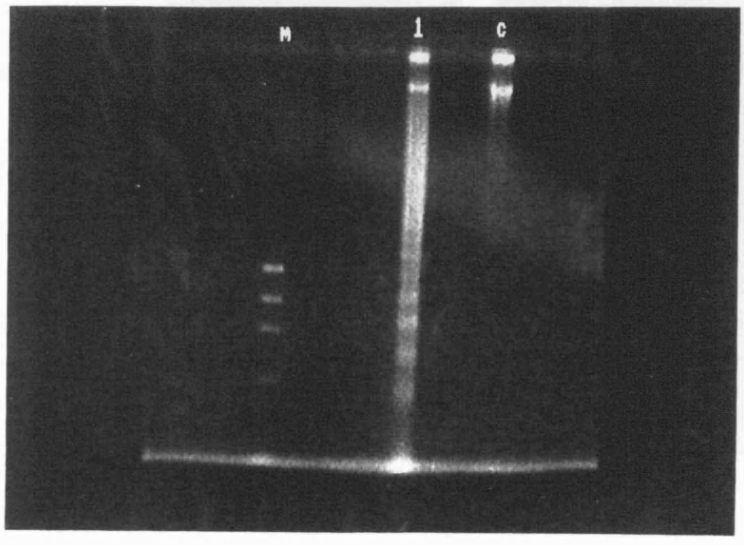


Plate XVIII

Gel electrophoresis - HL-60 cell line (End labelling, expt.) treated with Camphotecin 0.3μM -3hrs.

C - Control DNA

Lane 1- treated sample DNA (Hind III)

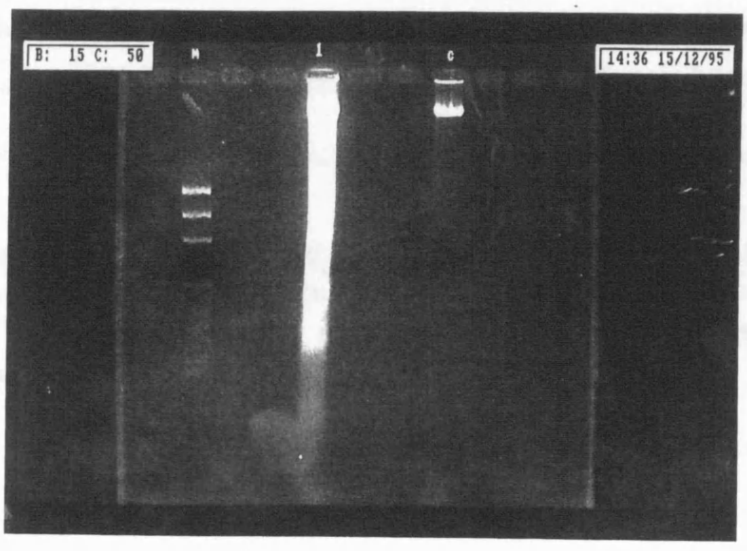


Plate XIX

Gel electrophoresis
C88 cell line (End labelling , expt.) treated with
30µgms Thapsigargin- 24 hrs.
C - Control
Lane -1 - treated sample DNA
M - Marker DNA (Physex/Hind III)

3.6 Assessment of Cell Proliferation and Correlating it with Apoptosis in a Human Promyelocytic Cell line (HL-60) Using PCNA (Proliferating Cell Nuclear Antigen).

The histograms (figs.33A-37A) show increasing activity with increasing dose and duration of the incubation period with Camphotecin. Maximum apoptotic activity is seen at 3hrs. with Camphotecin concentration of 0.15nM and 0.3nM. Dot plot displays of PCNA -FITC versus PI fluorescence show PCNA distribution throughout the cell cycle.

As the level of apoptotic activity increases, as demonstrated on PI histograms, there is a reduction in the proliferating compartment, i.e. S-phase and accumulation of the cells in the sub G_1 phase. This is more clearly demonstrated in the dot plot displays (figs-34B-37B). It appears that apoptosis negatively correlates with proliferative activity as detected by PCNA.

3.7 Flow cytometric Measurement of Cell Proliferation and Correlating it with Death by Apoptosis in HL-60 (A Human Promyelocytic cell line) and DP16-1 (A Murine Proerythroblastic cell line) Using Bromodeoxyuridine Incorporation Technique.

For the HL-60, the technique of Bromodeoxyuridine incorporation appears to have worked well as seen in fig. 38. The sample with BrUrd. and anti BrUrd. is showing maximum FITC fluorescence compared to the controls. Apoptosis has been successfully induced by camphotecin as evidenced by sub G_1 peaks in all the PI histograms of the treated samples (figs. 39A-42A). No apoptotic peaks are seen in the control (fig. 39A). The contour plots as well as dot plots of PI versus FITC fluorescence show BrdUrd uptake at all incubation periods but there is a progressive reduction of the S-phase which is the proliferative compartment of the cell cycle (see figs. 39B and C-fig 42B and C). This is paralleled by the increasing apoptotic activity seen on the PI histograms, as evidenced by increasing heights of apoptotic sub G_1 peaks (see figs. 39A-42A).

For the DP16-1 cell line, Bromodeoxyuridine incorporation has been very successful as shown in fig.43. The fluorescence peak of the test sample to which BrdUrd and anti BrdUrd antibody has been added is well separated from the controls. Increasing incubation periods with Thapsigargin have produced increased apoptotic activity as seen

in the PI stained histograms -figs. 46A-48A. There are no apoptotic peaks in the control sample fig 44A.

Apoptotic activity is seen as increasing height in the sub G_1 apoptotic peaks. There is also a decrease in the S- phase with progressive lengths of incubation with Thapsigargin (Cal Biochem-Nov Biochem-UK.) -an apoptosis inducing drug, (see figs. 46B-49B). Confirmation of apoptotic activity in both cell lines was done by doing DNA ladders (see plates XXI and XXII).

3.8 The effects Of X-ray Irradiation on The Cell Cycle and Cell Proliferation of Human (Raji) and Murine leukaemia Cell Lines-DP16-1 and C88

Raji Cell Line

The Propidium Iodide histograms of DNA profile of the irradiated Raji cells show some minimal radiation changes (see fig. 50A-G). These are manifested by loss in G_1 height at levels above 300 rads and a small increase in G_2 peaks. There does not appear to be any arrest in the progression of the cell cycle in this cell line even at 2000 rads (fig 50G). These results indicate that this cell line is radiation resistant. This can be explained by the fact that this cell line is known to carry a mutated p53.

C88 Murine Cell Line

The Propidium Iodide DNA profiles of the C88 cell lines 8hrs. after irradiation (figures 51A-E) show the progressive loss of the G_1 height with progressive accumulation of the cells in the G_2-M phase. This would indicate that this cell line has no effective mechanism of inducing a G_1 arrest in response to DNA damage. For instance it does not have a wild p53. The DNA histograms from the 24hr. post irradiation show increasing diminution of G_1 height with increasing doses of irradiation and lack of G_1 arrest with progressive accumulation of cells in the G_2-M.

Also progressive increase in the sub G_1 phase due to apoptosis is evident with increasing doses of radiation (see fig. 53A-57A). For EM see plate XXXII normal and plate XXXVI irradiated C 88 cells.

The diminution in the S-phase compartment, as reflected by decreased BrdUrd uptake with increased radiation doses, is clearly demonstrated in the dot plot displays see (figs. 54B-8B). There is also evidence of increasing accumulation of the cells in the sub G_1 phase as the dose of radiation is increased. The dot plots of Bromodeoxyuridine - PI labelled cells reflect the changes described for the PI only histograms. Additionally, one sees more distinctly a progressive decrease in S-phase paralleled by a progressive increase in the sub G_1 phase where apoptotic cells accumulate. This was confirmed by doing DNA ladders which were seen in the 24hr. sample at the 250, 500 and 1000 rads. The DNA fragmentation was more prominent at the higher doses 500 and 1000 rads. See chapter 2.5 and 3.5.

DP16-1 Cell Line.

Compared to the control, the DNA histograms show radiation changes with increasing loss in the G_1 height and an accumulation of the cells in the G_2.M. (see figs.52A-E). These changes are seen from as little as 100 rads. This cell line appears to be sensitive to irradiation when compared to the Raji cell line. This cell line, however, is unable to arrest at G_1 phase because most likely it does not have a functional wild type of p53.

3.9 Experiment to Demonstrate Bcl-2 Oncogene Expression in two Murine Leukaemia Cell Lines DP16-1 and C88 Using a Flow Cytometric Technique.

The flow cytometry detection of Bcl-2 in the DP16-1 cell line has worked well as demonstrated in the fluorescence histograms (fig. 58). It is evident that for this cell line the histogram of the test sample is well separated from the controls and the centre of its peak is just before the log 10^2. This cell line therefore has Bcl-2 oncogene. It appears that the experiment works equally well at both dilutions of phycoerythrin, as there is a definite overlap of the histogram displays both at 1/250 and 2/250 dilutions.

The C88 show Bcl-2 expression as shown by the fluorescence histograms (see fig 59). The fluorescence spectrum of the cells with anti-Bcl-2 antibodies are well separated from P.E. only, isotype or blank sample. The mid point of the fluorescence peak of P.E. fluorescence in the C88 cell line is very much further beyond 2nd log-10^2 compared to

one due to DP16-1 (see histograms). As the same number of cells and concentration of antibodies were used the intensity of fluorescence spectrum due to C88 indicate that this cell line has greater Bcl-2 reactivity. This is in agreement with previous reports[68].

3.10 The Role of c-myc in Apoptosis in HL-60 and DP16-1 Cell Lines as Investigated by a Flow Cytometric Technique.

(DP16-1) Murine Leukaemia cell line

The fluorescence histograms (fig 61) show that the FITC fluorescence of the samples to which anti c-myc antibodies (control and the sample with Thapsigargin) had been added are clearly well separated and are the furthest from the blank only FITC and isotype sample. There is a reduction in the FITC fluorescence in the apoptotic sample compared to the control

(HL-60) Human promyelocytic leukaemia cell line.

Figure 61 is the HL60-FITC fluorescence histograms display. It shows that the blank and FITC only sample are very close to the origin while the ones which anti c-myc antibody had been added, i.e. the apoptotic sample and the normal sample are the furthest away. It does appear that there is a decrease in the c-myc levels after induction of apoptosis as reflected by the decreased FITC fluorescence in the apoptotic sample. The PI only histogram clearly show apoptotic peaks in the apoptotic sample compared to the controls (fig 62A and B). For both cell lines, apoptosis was confirmed by DNA ladders (see plates XXIII and XXIV).

3.11 Flow Cytometric Analysis of p53 in Murine Leukaemia Cell lines DP16-1 -1 and C88.

C88 Cell line

For the C88 cell line at all the concentrations used it appears that mutated p53 as detected by the 240 epitope is present, as the histograms of the test samples are well separated from the blank isotype control and FITC only sample. The 150µl volume of the anti p53 antibody gives the best separation (see fig.63). For the 246 epitope which recognises the wild type, the histograms of fluorescent spectrums of the test samples are not as well separated from controls as is the case with the 240 epitope (see fig 64). It can be

concluded that in the C88 cell line, the mutated p53 is the dominant oncogene but the cell line has also a wild type allele recognised by the anti p53-246 epitope.

The DP16-1 Cell line

The histogram of both anti p53 240 and 246 epitopes appear to overlap (see fig.65). They are also well separated from the control samples. The inclusion of the isotype control sample is to ensure that genuine p53 detection occurs. On these results it appears that this cell line has detectable epitopes of p53 (246 and 240) with no dominant one.

3.12 Effects of X-ray Irradiation on p53 Expression on Leukaemia Cell Lines, DP16-1 and C88.

C88/53 Irradiation histograms

The histograms show a complete overlap of the irradiated and non irradiated sample indicating that there is no rise in p53 levels (see fig 66). Propidium Iodide stained histograms on this cell line show irradiation changes with loss of G_1 height lack of G_1 arrest and relative increase in G_2 phase (see fig.67)

DP16-1 -1 cell line

The histograms show that administration of 1000 Rads to DP16-1 causes only a slight increase in detectable p53 levels as there is a big overlap in the fluorescence histograms of the irradiated and non-irradiated samples (see fig. 68).

3.13 Cell Differentiation in Murine Leukaemia Cell Line C88. The Role Played by c-myc and p53 and the link with Apoptosis as Confirmed by DNA Ladders.

Flow cytometric p53 assessment

There is a decline in p53 levels as seen in the fluorescence histograms. It is seen most clearly at 10hrs.incubation with 2% DMSO and approaching zero levels at the 24 hr. period. The FITC fluorescence spectrums of these two samples are very much reduced and very close to the blank only sample (see fig.69).

Flow cytometric c-myc Assessment

In fig. 69, the fluorescent histograms show FITC (DAKO UK) fluorescence on the X-axis and cell number on the Y-axis. They also show that c-myc levels as reflected by FITC fluorescence, are reduced in the cell sample to which 2% DMSO has been added over a 24hr period. The dot plot displays show a good cell cycle (see fig. 70A and B). The reduction in the c-myc levels shown in fig 69 on the same samples is not well illustrated in this form of display. This is most likely because the reduction in c-myc levels is very small.

DNA Extraction and Gel Electrophoresis

The gel electrophoretic strips showing DNA ladder pattern at the 24, 48 and 72hrs. and the DNA ladders representing DNA fragmentation are seen more clearly at the 48 and 72hrs. This would mean that more apoptosis is seen more clearly with increasing incubation periods with 2% DMSO. The control shows no evidence of DNA laddering- see plate XXVI

3.14 Experiment to Induce and Demonstrate Cellular Differentiation of a Murine Leukaemia Cell Line C88 Using DMSO and Cytospin Preparations

Compared to the control samples, the cells exposed to DMSO showed differentiation towards nucleated erythrocyte as evidenced by the reduction in their size and cytoplasm and loss of mitosis. These changes were evident under a Leitz microscope and were photographed at x400 magnification as well as x200 magnification (see plates XXXVII, XXXVIII and XXXIX). Increasing concentrations of DMSO, i.e. 5% accelerated the differentiation program as more cells were differentiated.

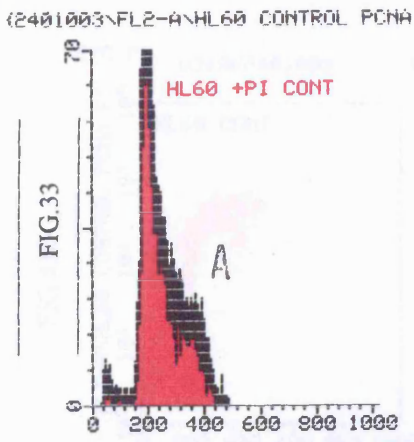


FIG.33

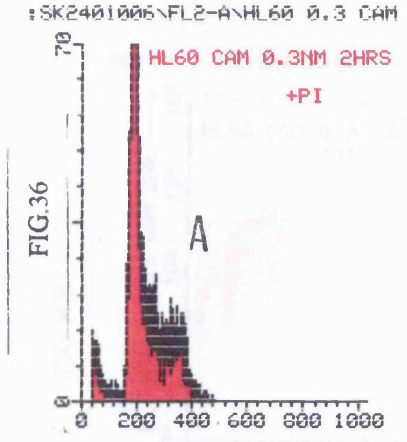


FIG.36

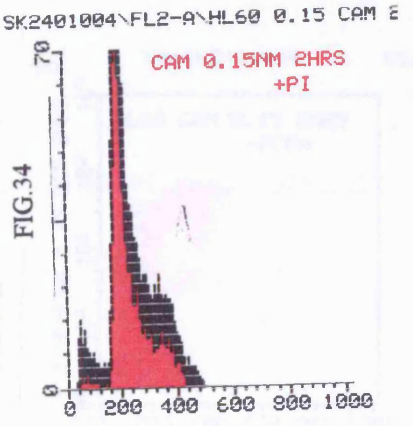


FIG.34

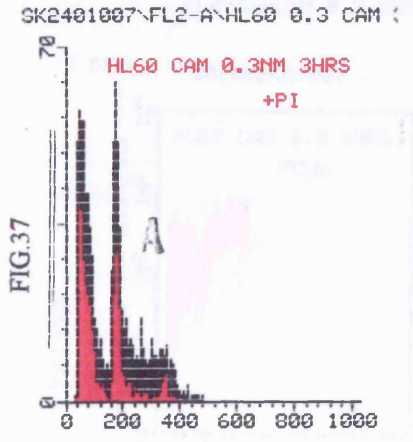


FIG.37

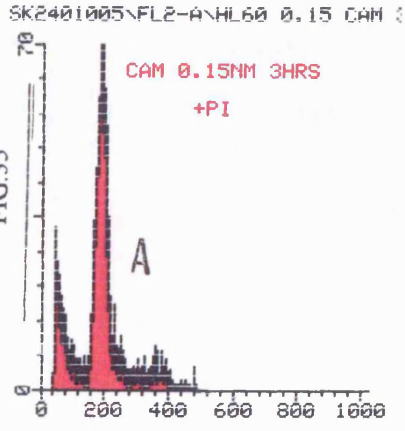


FIG.35

PCNA HL-60

Figs 33A-37A. DNA frequency histograms of HL-60 cell line treated with Camphotecin for varying incubation periods (2-3hrs) and concentrations, fixed with 70% Ethanol and stained with Propidium iodide. Compared to the control, Sub G_1 apoptotic peaks are evident in all the treated samples -fig 34A-37A. The tallest peaks appear with the longest incubation period of 3 hrs. Doubling the concentration of Camphotecin from 0.15nM-0.3nM gives a corresponding increase in the apoptotic peaks.

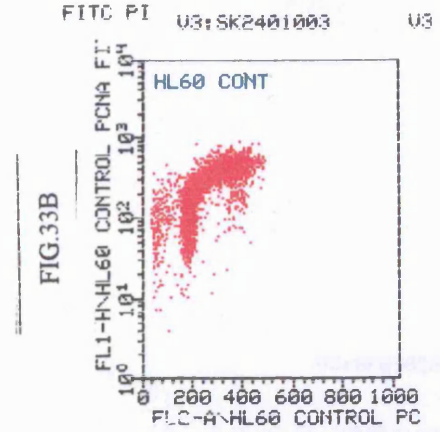


FIG.33B

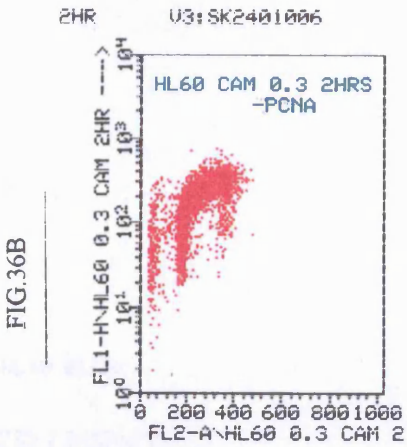


FIG.36B

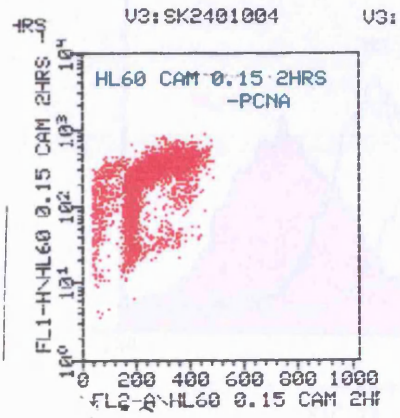


FIG.34B

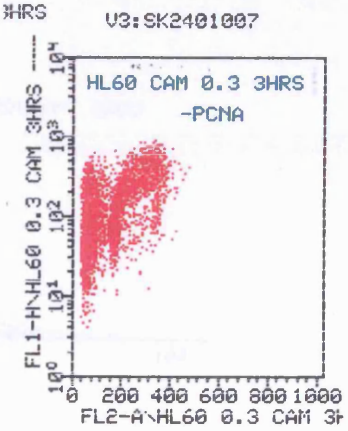


FIG.37B

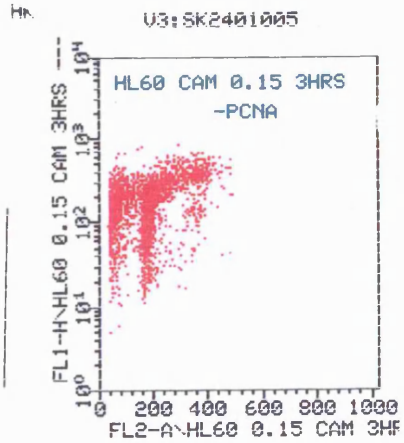


FIG.35B

PCNA HL60

Figs 33B-37B. These are DNA dot plot cytograms of PCNA expression in HL-60 after induction of apoptosis at varying incubation periods (2-3hrs) and concentration. On the y-axis is PCNA expression and on the x-axis is PI fluorescence. Compared to the control, there is diminishing PCNA expression in the S-phase with increasing incubation periods, which is maximum at 3 hrs.

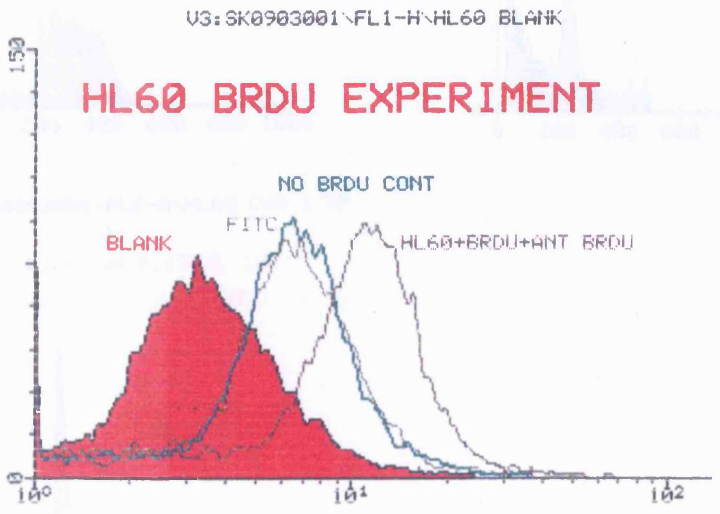


FIG.38

<u>BromodeoxyUridine incorporation</u>
<u>HL-60</u>

Fig 38. Shows FITC fluorescent histograms of a BrdUrd incorporation technique on HL-60 cells. On the x-axis is FITC fluorescence and the y-axis is cell number. The blank is represented by the red histogram. The FITC only control is the grey histogram. The sample with BrdUrd but no anti BrdUrd, is represented by the green histogram. The test sample is represented by the black histogram. The test sample fluorescent peak is further than the controls indicating that the BrdUrd incorporation has been achieved.

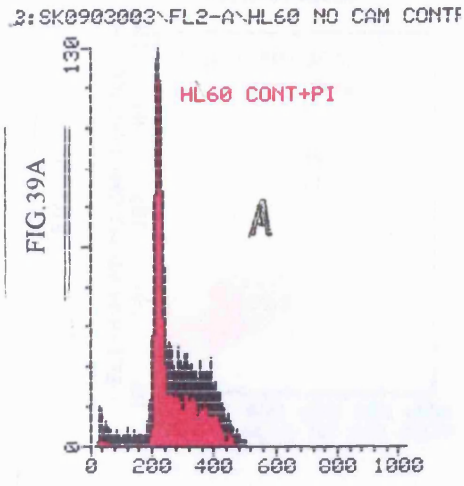


FIG.39A

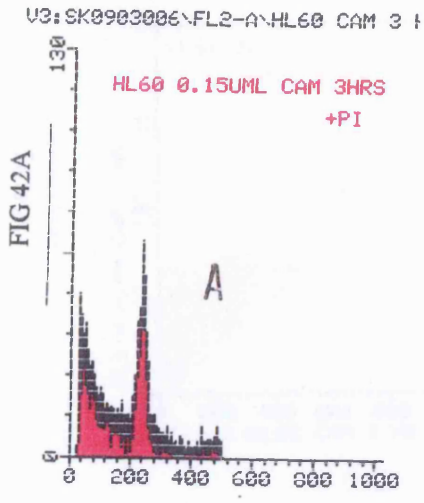


FIG 42A

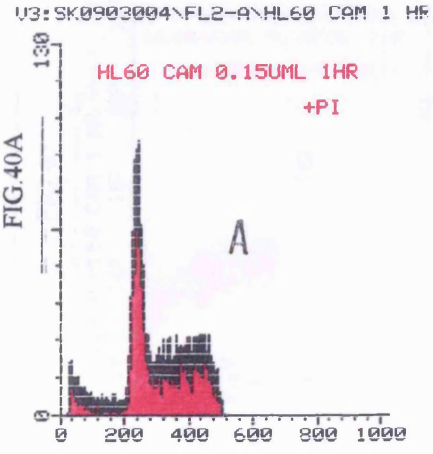


FIG.40A

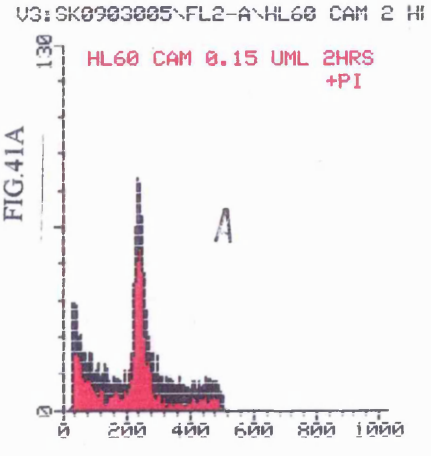


FIG.41A

<u>BromodeoxyUridine incorporation</u>

HL-60

Figs.39A-42A DNA frequency histograms of HL-60 cell line treated with Camphotecin (0.15nM), for varying incubation periods (1-3hrs.), fixed with 70% Ethanol and stained with Propidium iodide. Compared to the control, Sub G_1 apoptotic peaks are evident in all the treated samples -fig 40A-42A. The tallest peaks appear with the longest incubation period of 3 hrs.

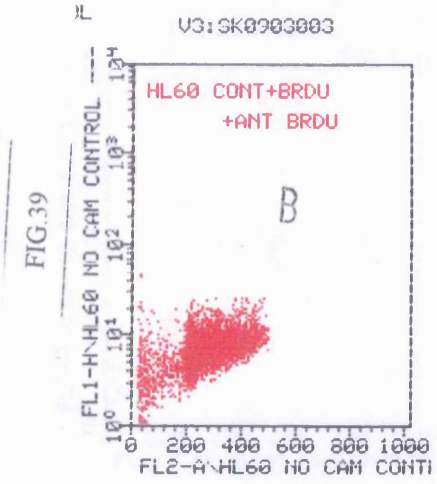


FIG.39

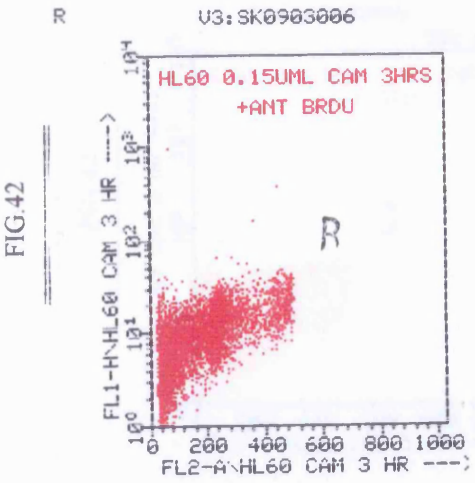


FIG.42

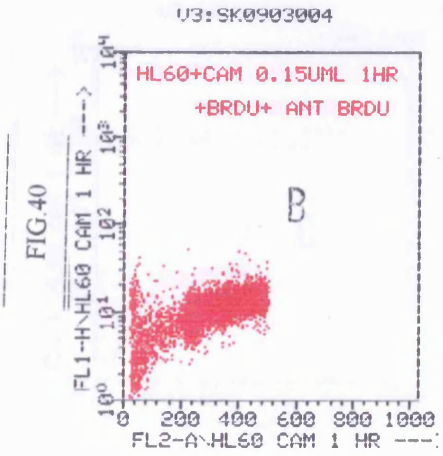


FIG.40

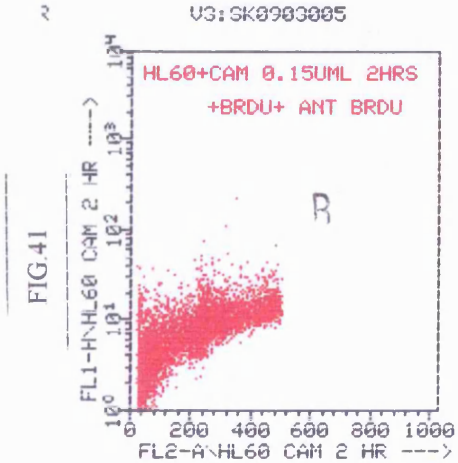


FIG.41

BromodeoxyUridine incorporation

Figs 39B-42B. These are corresponding dot plot cytograms of BrdUrd incorporation in HL-60 after induction of apoptosis at varying incubation periods (1-3hrs) with 0.15nM Camphotecin. On the y-axis is BrdUrd uptake and on the x-axis is PI fluorescence. Compared to the control, there is diminishing BrdUrd uptake in the S-phase with increasing incubation periods, which is maximum at 3 hrs.

FIG.39

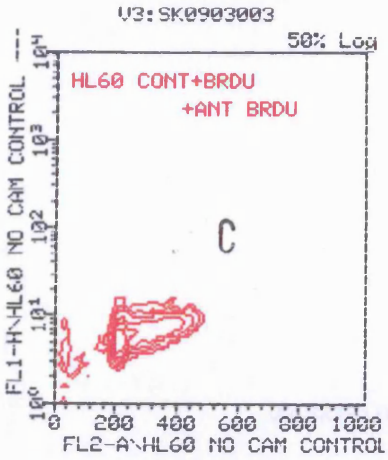


FIG.42

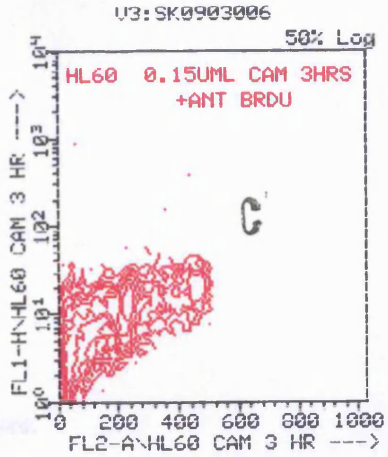


FIG.40

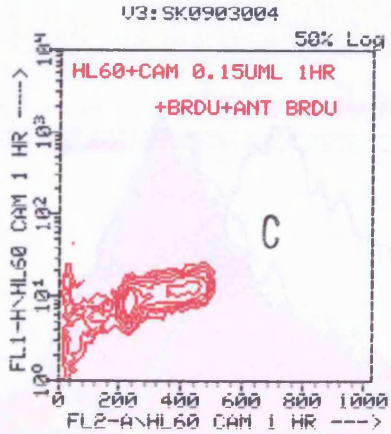
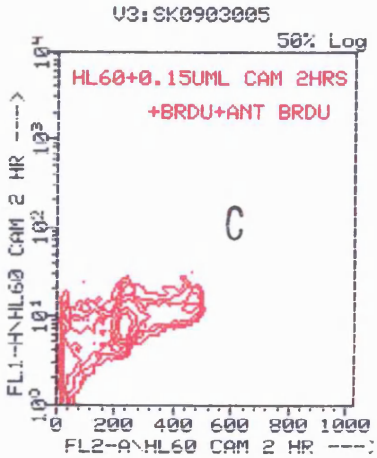


FIG. 41



BromodeoxyUridine incorporation

Figs 39C-42C. These are corresponding contour plot cytograms of BrdUrd incorporation in HL-60 after induction of apoptosis at varying incubation periods(1-3hrs.) with 0.15nM Camphotecin. On the y -axis is BrdUrd uptake and on the x axis is P.I. fluorescence. Compared to the control, there is diminishing BrdUrd uptake in the S phase with increasing incubation periods ,which is maximum at 3 hrs.

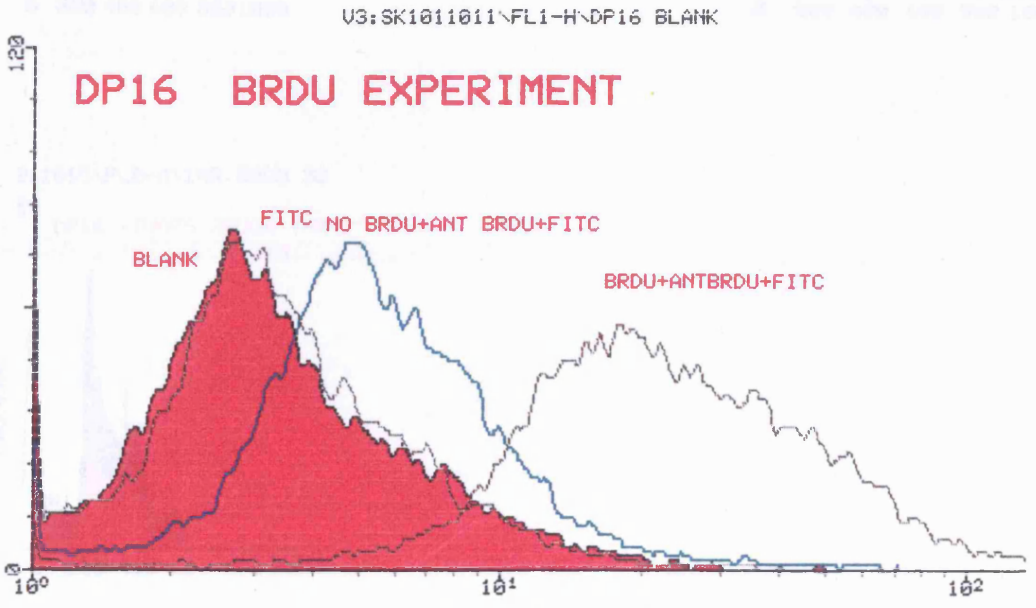


FIG.43

DP16-1 BrdUrd

Fig 43. Shows FITC fluorescent histograms of a Bromodeoxyuridine incorporation technique on DP16-1 cells. On the x-axis is FITC fluorescence and the y-axis is cell number. The blank is represented by the red histogram. The FITC only control is the black histogram overlapping the blank. The sample with BrdUrd but no anti-BrdUrd is represented by the green histogram. The test sample is represented by the grey histogram. The test sample fluorescent peak is further than the controls indicating that the BrdUrd incorporation has been achieved.

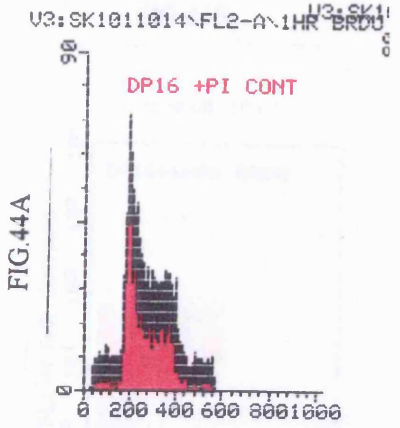


FIG.44A

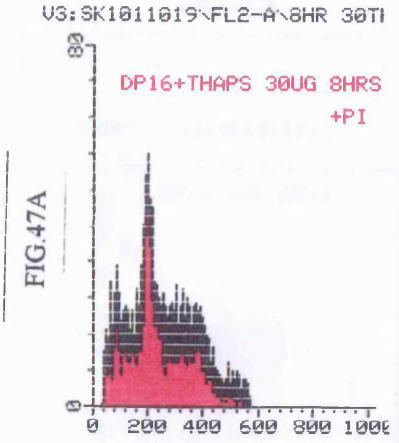


FIG.47A

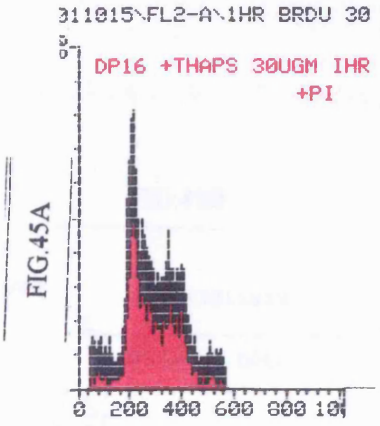


FIG.45A

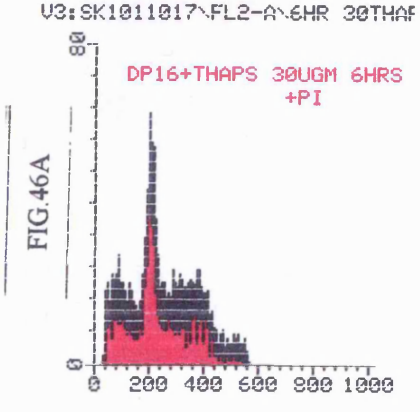


FIG.46A

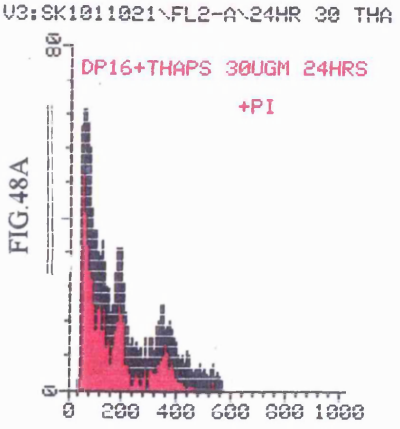


FIG.48A

Figs.44A-48A DNA frequency histograms of DP16-1 cell line treated with (30µgms of Thapsigargin), for varying incubation periods (6-24hrs), fixed with 70% Ethanol and stained with Propidium iodide. Compared to the control, Sub G_1 apoptotic peaks are evident in all the treated samples -fig 46A-48A. The tallest peaks appear with the longest incubation period of 24hrs.

FIG.44B

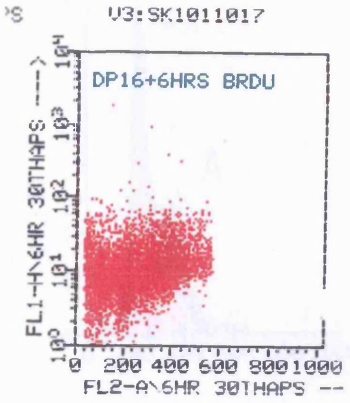


FIG.46B

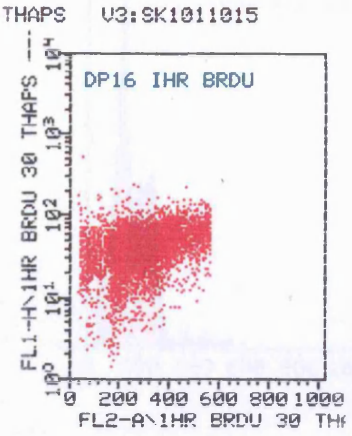


FIG.45B

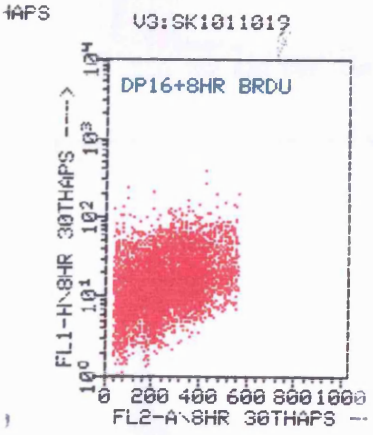
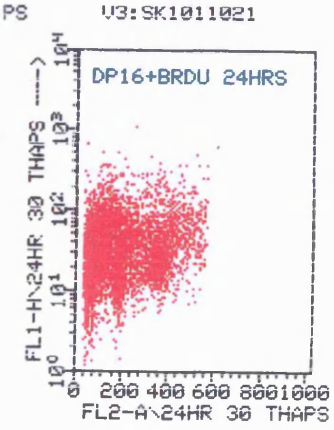
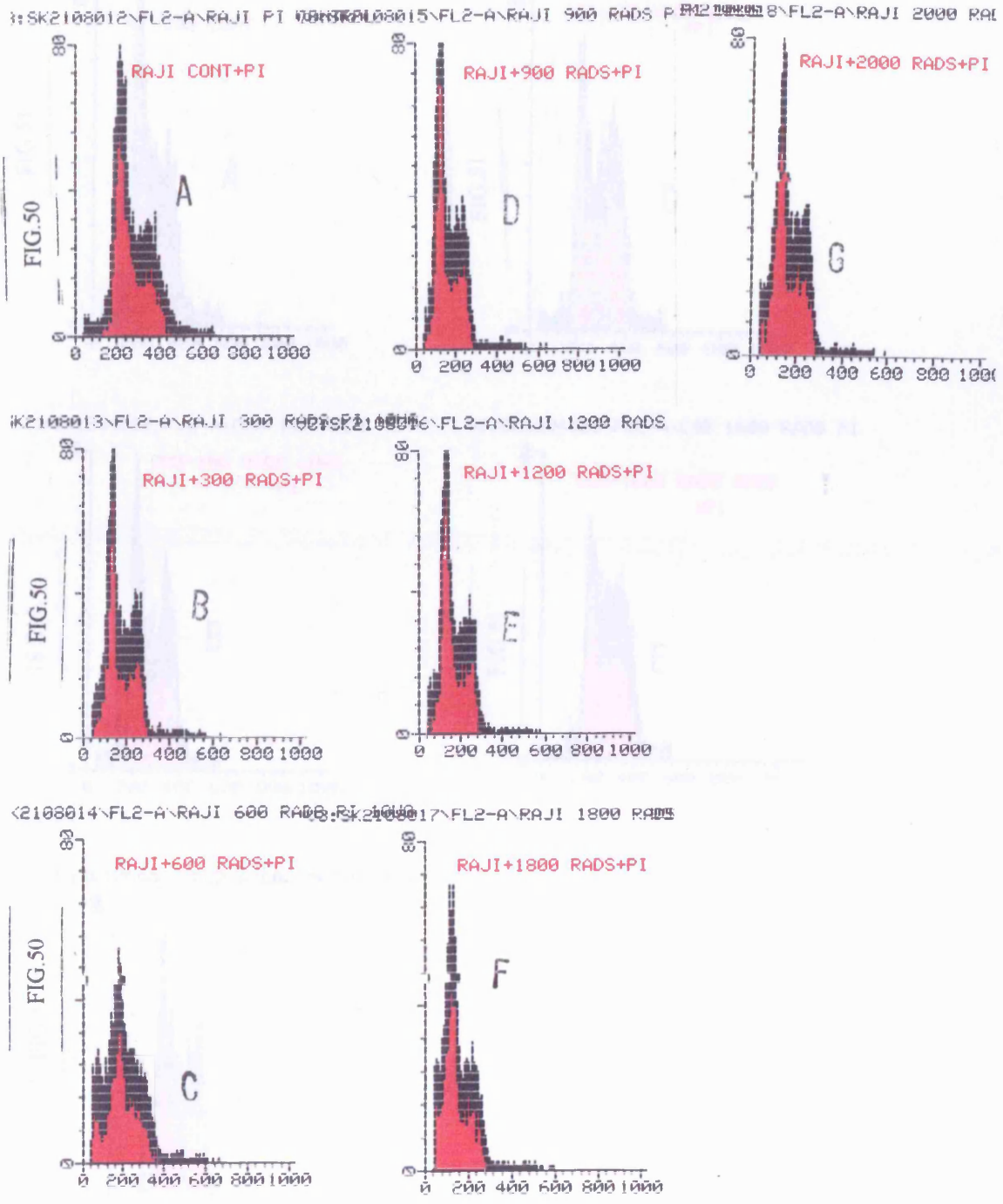


FIG.49



DP16-1 BrdU technique

Figs 44B-46B and 49. These are corresponding dot plot cytograms of Bromodeoxyuridine (BrdUrd) incorporation for the DP16-1 after induction of apoptosis at varying incubation periods (6-24hrs.) with (30µg of Thapsigargin). On the y-axis is BrdUrd uptake and on the x-axis is PI fluorescence. Compared to the control, there is diminishing BrdUrd uptake in the S-phase with increasing incubation periods which is maximum at 24 hrs.(fig 49).

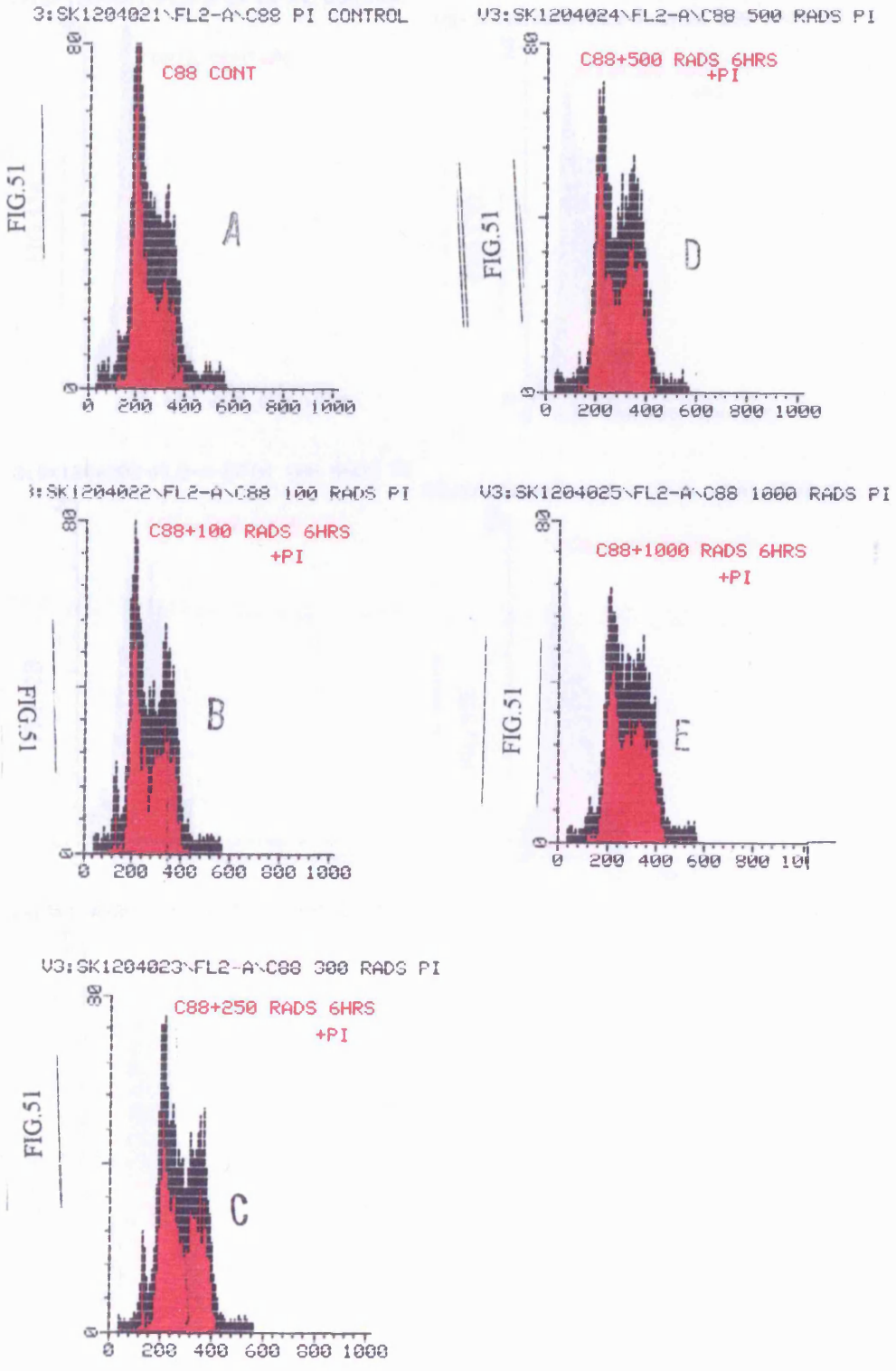


XRAY-Raji Cell Line

Fig 50 A-G. DNA frequency histograms of Raji cell line exposed to varying doses of irradiation (300-2000 rads), fixed in 70% Ethanol and stained with PI.

Fig.50A is normal control. Fig.51B (300rads) there is no effect.

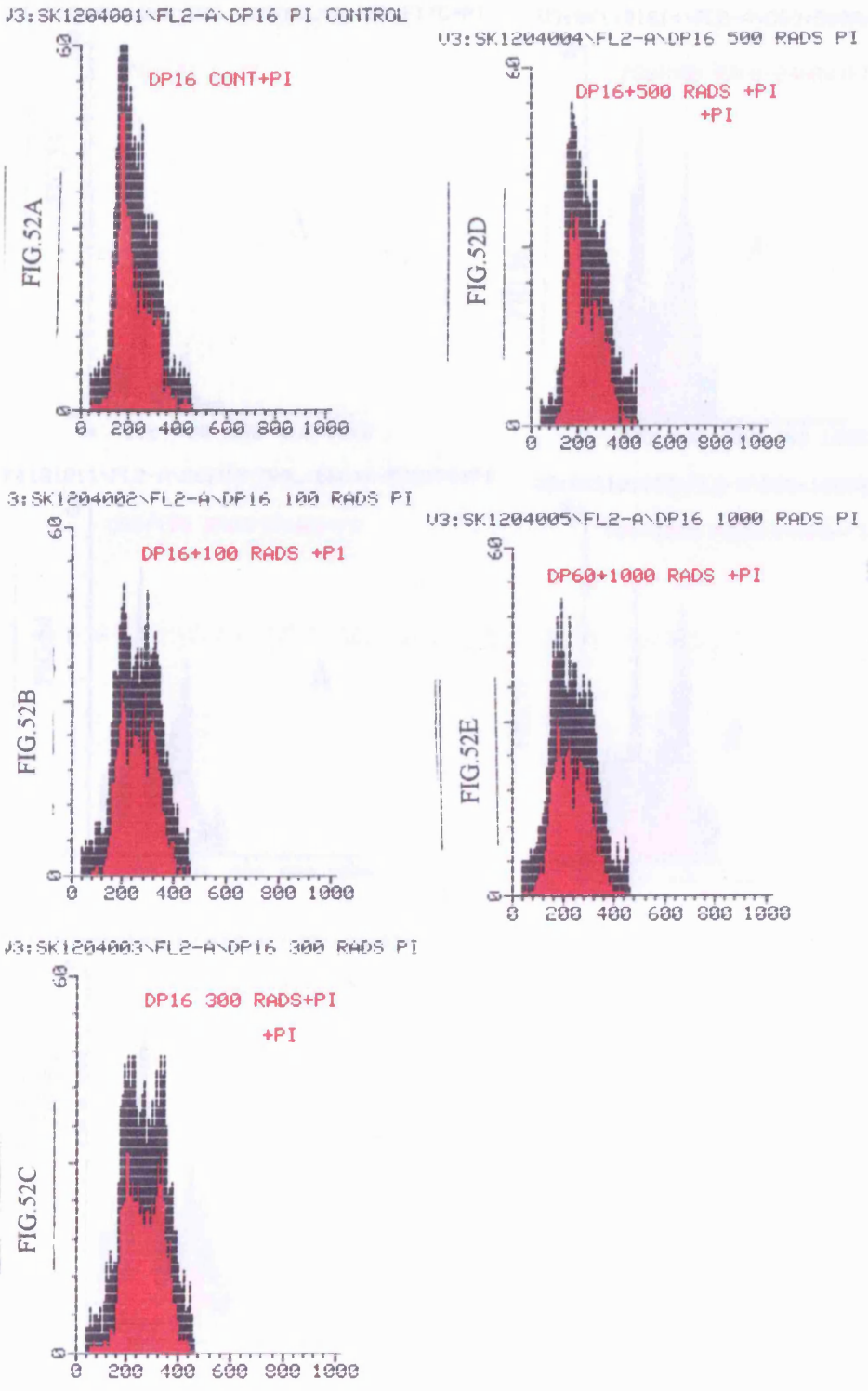
Fig 501C-51G (500-2000 rads) there are only very minimal changes characterised by a decrease in the G_1 height and a small increase in the G_2M height .



XRAY-C88 Cell Line

Fig 51A-E. DNA frequency histograms of C88 cell line exposed to varying doses of X-ray irradiation (100-1000 rads) left in culture for 6hrs, fixed in 70% Ethanol and stained with PI.

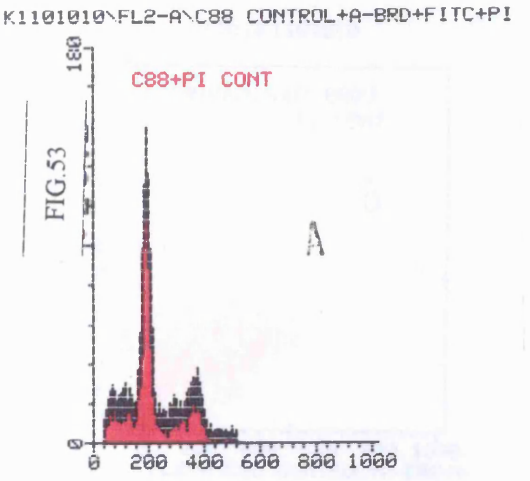
Fig.51A is normal control. All histograms with radiation doses from 100-1000 show radiation changes characterised by loss in height of the G_1 phase and an increase in the G_2M phase. There is no G_1 arrest.

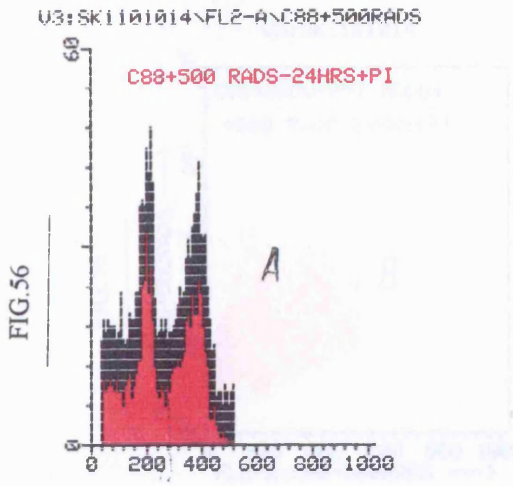


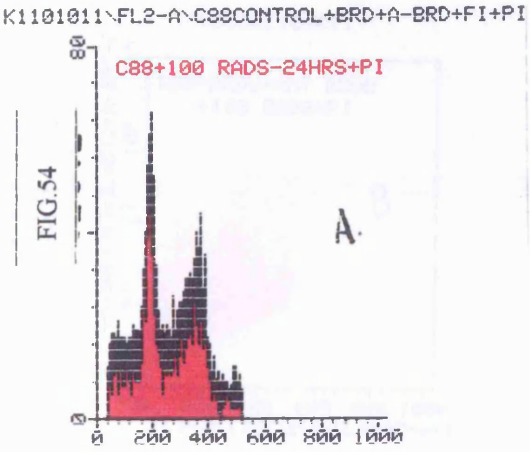
XRAY DP16-1 Cell Line

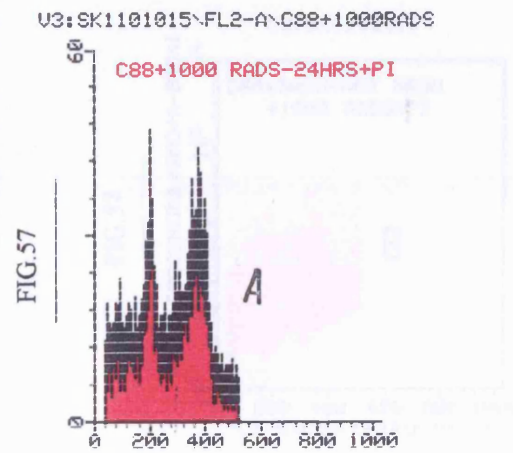
Fig 52A-E. DNA frequency histograms of DP16-1 cell line exposed to varying doses of X-ray irradiation (100-1000 rads) left in culture for 6hrs, fixed in 70% Ethanol and stained with PI.

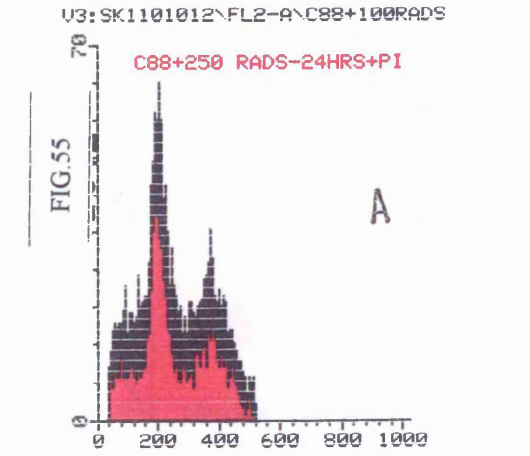
Fig.52A is normal control. All histograms with radiation doses from 100-1000 show radiation changes characterised by loss in height of the G_1 phase and an increase in the G_2M phase. There is no G_1 arrest.











C88 Cell Line X-ray and Bromodeoxyuridine incorporation

Figs 53A-57A. DNA frequency histograms of C88 cell line exposed to varying doses of X-ray irradiation (100-1000 rads) left in culture for 24hrs, fixed in 70% Ethanol and stained with PI.

Fig.53A is normal control. All histograms with radiation doses from 100-1000 show radiation changes characterised by loss in height of the G_1 phase and an increase in the G_2M phase. Apoptotic activity is seen from as little as 100 rads and increasing with radiation dose to reach maximum at 1000 rads. There is no G_1 arrest.

FIG.53

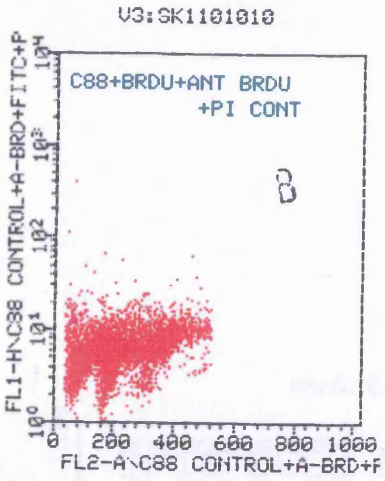


FIG.56

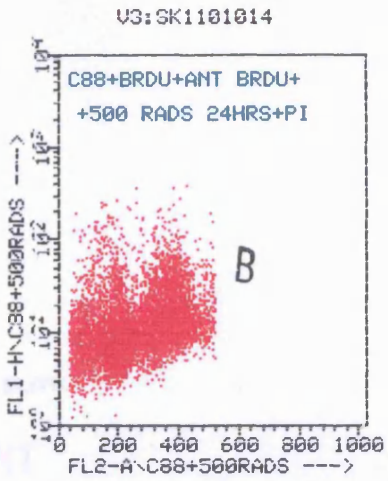


FIG.54

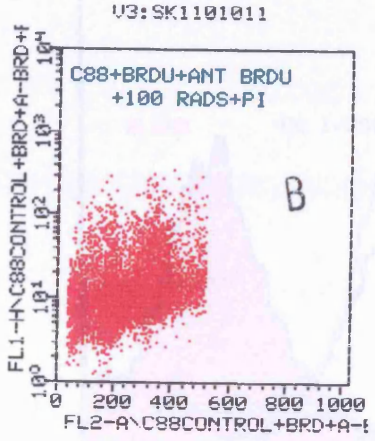


FIG.57

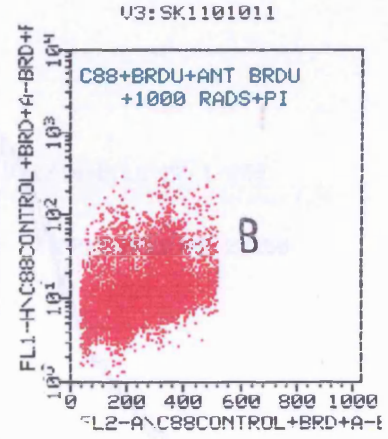
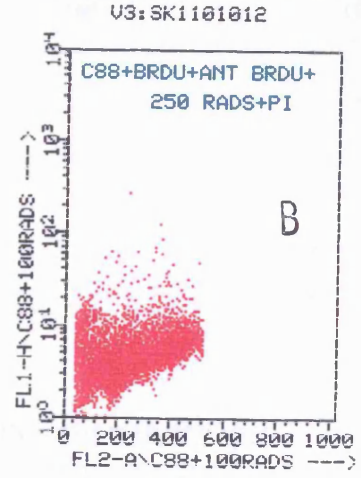


FIG.55



X-ray and Bromodeoxyuridine incorporation

Figs 53B--57B. Dot plot cytograms of C88 cell line. After X-ray irradiation 100-1000 rads and incubation in culture for 24hrs. BrdUrd incorporation was carried out (see method section) cells were then fixed 70% in Ethanol and counterstained with PI.
On the x-axis is PI fluorescence (DNA content) and on the y-axis is BrdUrd uptake.
Compared to the control Fig.53B there is a gradual reduction in the Bromodeoxyuridine during the S-phase which is maximum at 1000rads radiation dose (fig.57B).

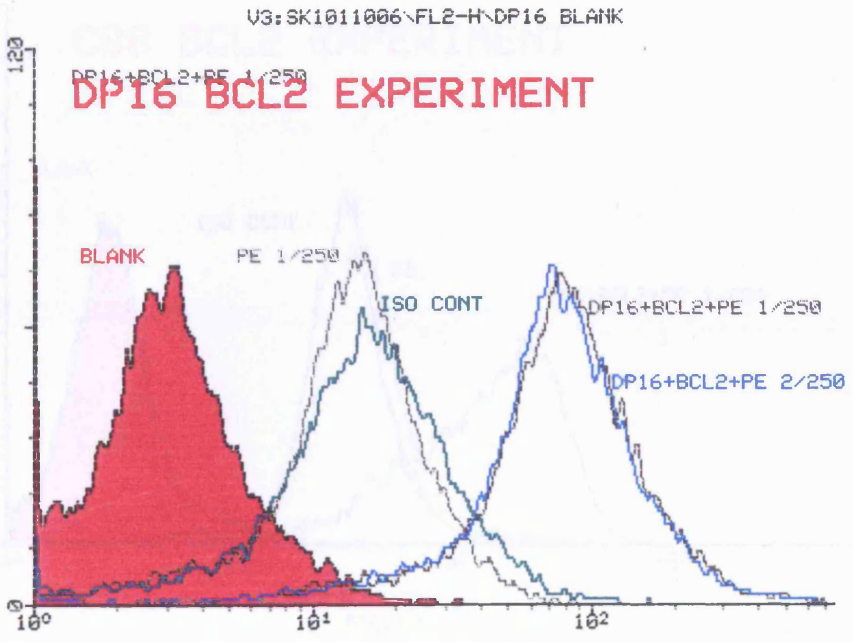


FIG.58

Bcl-2 detection in DP16-1

Fig 58. Shows Phycoerythrin fluorescence histograms of a Flowcytometric Bcl-2 detection technique on DP16-1 cell line. On the x-axis is Phycoerythrin fluorescence and the y-axis is cell number. The blank is represented by the red histogram. The phycoerythrin only, grey histograms is seen overlapping the isotype control fluorescence peak and both of them are very close to the blank. The fluorescence peaks for the test samples to which an anti Bcl-2 antibody and phycoerythrin (PE) used at 1/250 and 2/250 dilutions, are represented by the blue and black histograms respectively. These are well separated from the control samples indicating that DP16-1 cell line has detectable Bcl-2.

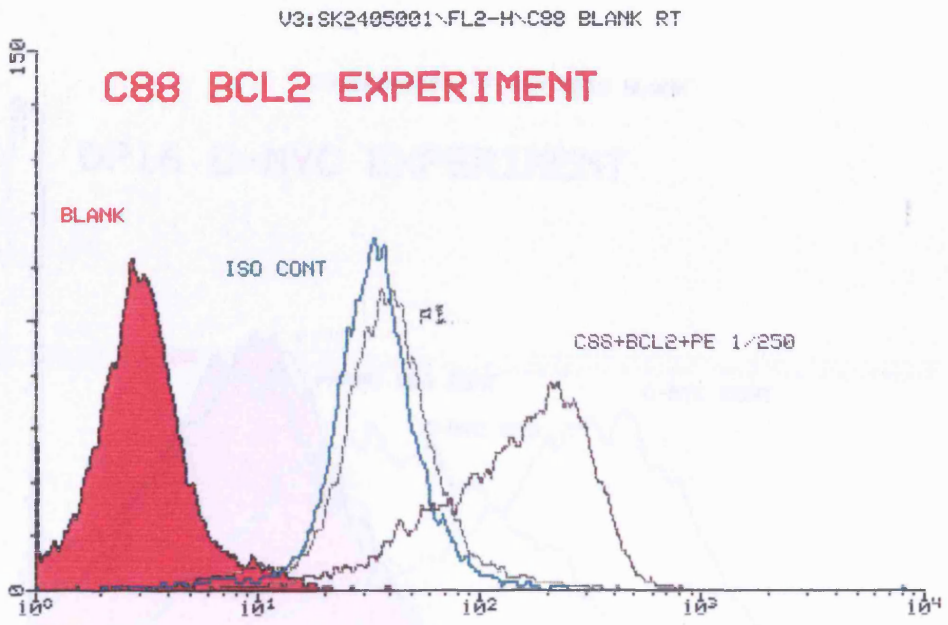


FIG.59

Bcl-2 detection in C88

Fig 59. Shows Phycoerythrin fluorescence histograms of a Flowcytometric Bcl-2 detection technique on C88 cell line. On the x-axis is Phycoerythrin fluorescence and the y-axis is cell number. The blank is represented by the red histogram. The phycoerythrin only histogram is overlapped by the isotype control (green histogram). The fluorescence peaks for both of them are very close to the blank. The fluorescence peaks for the test sample to which an anti Bcl-2 antibody and phycoerythrin (PE) used at 1/250 dilution, is represented by the black histogram. This is well separated from the control samples, indicating that the C88 cell line has detectable Bcl-2.

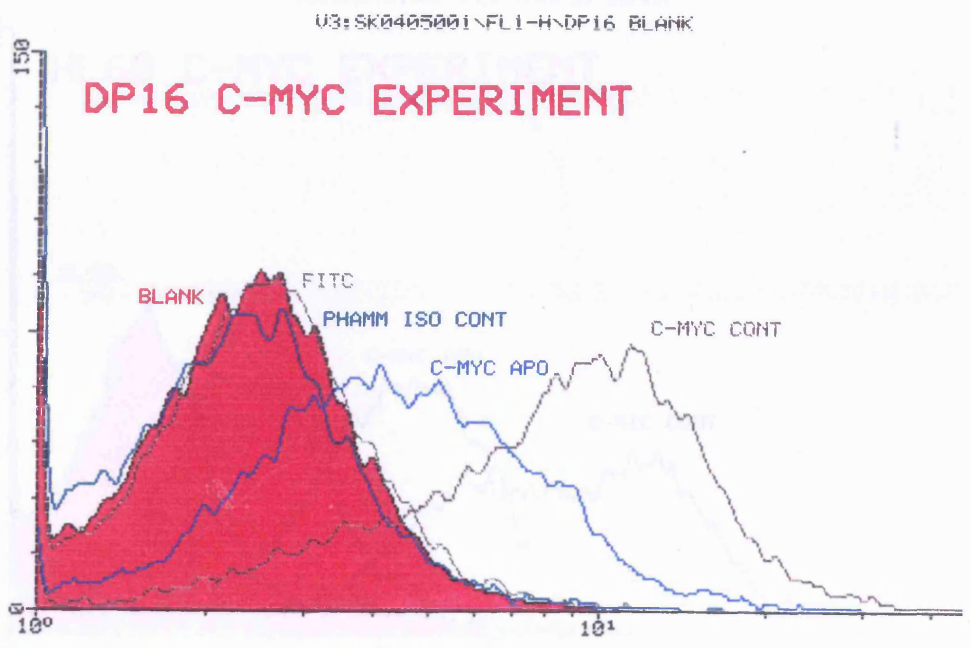


FIG.60

DP16-1 and c-myc detection

Fig. 60 shows FITC fluorescent histograms of a Flowcytometric c-myc detection technique before and after induction of apoptosis in a DP16-1 cell line. On the x-axis is FITC fluorescence and the y-axis is cell number. The blank is represented by the red histogram. The FITC only, is seen overlapping the Pharmigen isotype control. Their fluorescence peaks are also overlapping the blank. The fluorescence peaks for the test samples to which an anti c-myc antibody and FITC had been added and apoptosis had been induced , is represented by the blue histogram. The control to which both anti c-myc and FITC had been added ,without inducing apoptosis , is further on the x-axis than the c-myc control.There is therefore a reduction in c-myc levels after induction of apoptosis

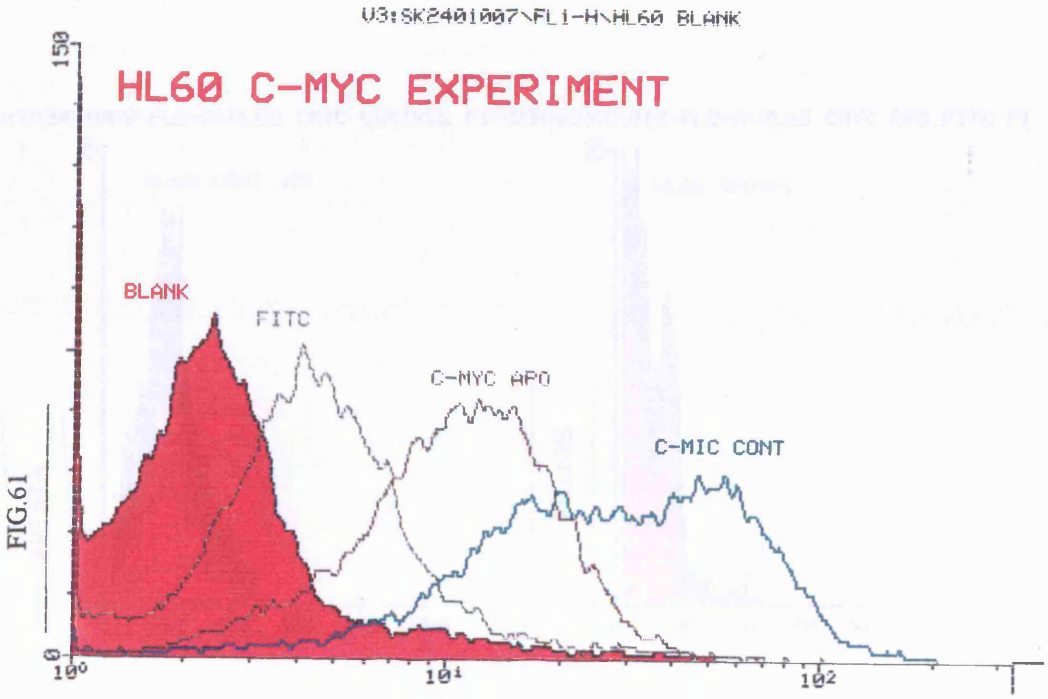
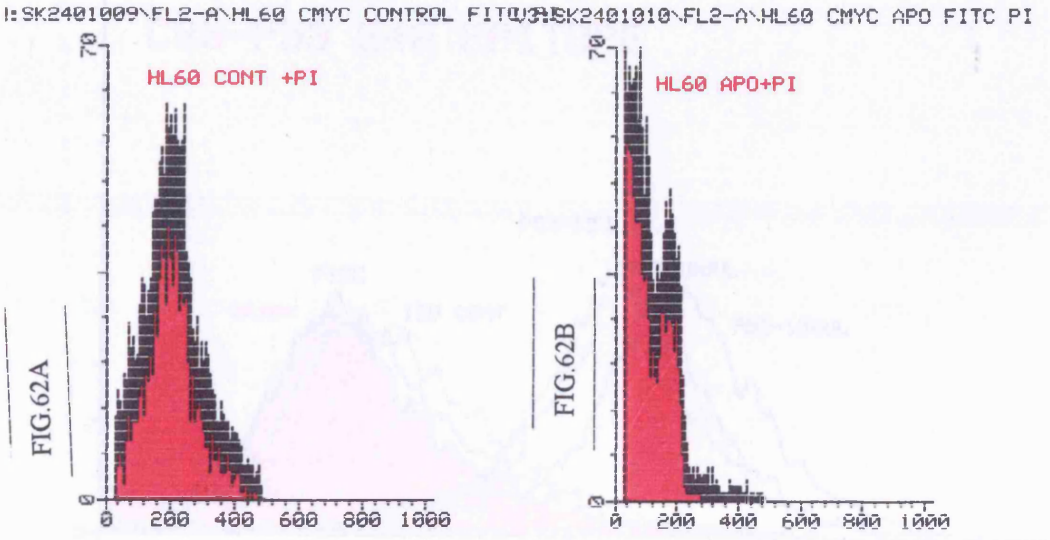


FIG.61

HL-60 and c-myc detection

Fig 61. Shows FITC fluorescence histograms of a Flowcytometric c-myc detection technique before and after induction of apoptosis in an HL60-cell line. On the x-axis is FITC fluorescence and the y-axis is cell number.. The FITC only, is seen overlapping the blank. The fluorescence peak for the test sample to which an anti-c-myc antibody and FITC had been added, and apoptosis induced, is next to the FITC. The control to which both anti-c-myc and FITC had been added without inducing apoptosis is the furthest on the x-axis. There is therefore a reduction in c-myc levels after induction of apoptosis.



Figs 62A-B. DNA frequency histograms of HL-60 cell line.
A .Normal control fixed with 70% Ethanol and stained with PI.
B. Incubated in culture with 0.3 Camphotecin for 3hrs ,fixed with70% Ethanol and stained with PI. A big apoptotic peak is seen in the sub G_1 position

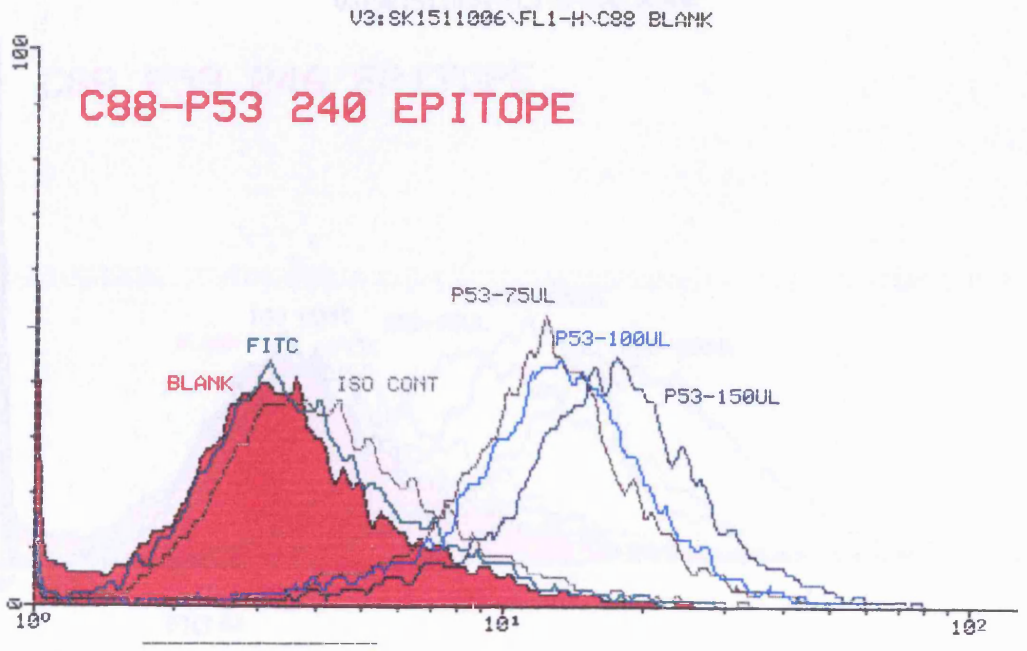


FIG.63

C88 p53 detection

Fig 63. Shows FITC fluorescence histograms of a Flowcytometric P53 240 (epitope) expression in a C88 cell line. On the x-axis is FITC fluorescence and the y-axis is cell number. The FITC only and the Isotype fluorescence peaks are seen overlapping the blank. The test samples to which anti P53 had been added at varying concentrations , are well separated from the controls. The highest concentration of 150µl gives the best separation. This cell line has mutated P53 detected by anti p53-240 epitope antibody.

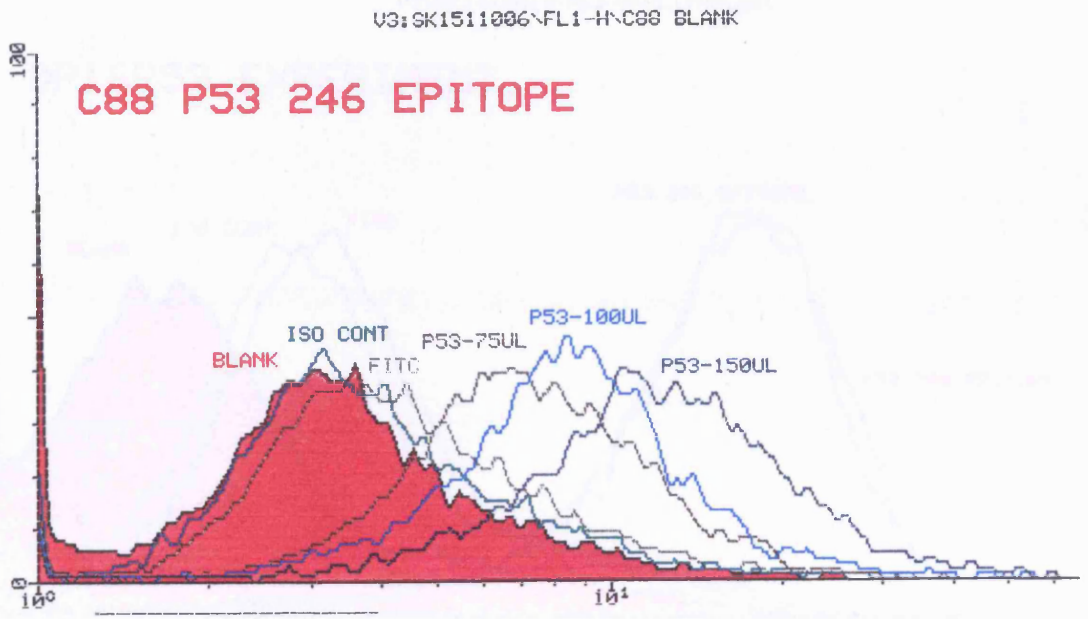


FIG.64

C88 p53 detection

Fig 64. Shows FITC fluorescence histograms of a Flowcytometric P53 246 (epitope) expression in a C88 cell line. On the x-axis is FITC fluorescence and the y-axis is cell number. The blank is represented by the red histogram the FITC only and the Isotype fluorescence peaks are seen overlapping the blank. The test samples to which anti-p53 had been added at varying concentrations. Their fluorescence peaks, are very close to the controls and some are overlapping the controls. This cell line has a weak expression of the wild P53 detected by anti-p53-246 epitope antibody.

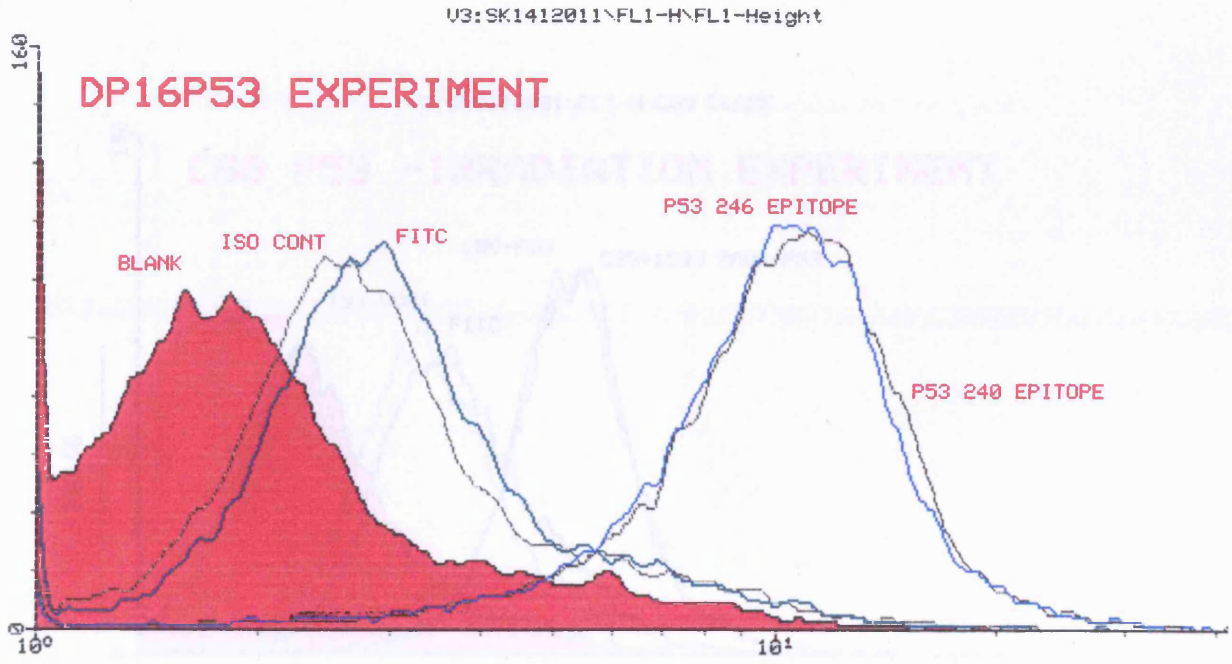


FIG.65

DP16-1 p53 detection

Fig 65. Shows FITC fluorescence histograms of a Flowcytometric.
P53 246 (epitope) expression in a DP16-1 cell line. On the x-axis is FITC fluorescence and the y-axis is cell number. The blank is represented by the red histogram. The FITC only and the Isotype fluorescence peaks are seen overlapping the blank. The test samples to which anti-p53 had been added 246 and 240 epitopes. are close but separate from the controls and their fluorescence peaks are overlapping. This cell line has detectable the wild p53 and mutated p53 alleles.

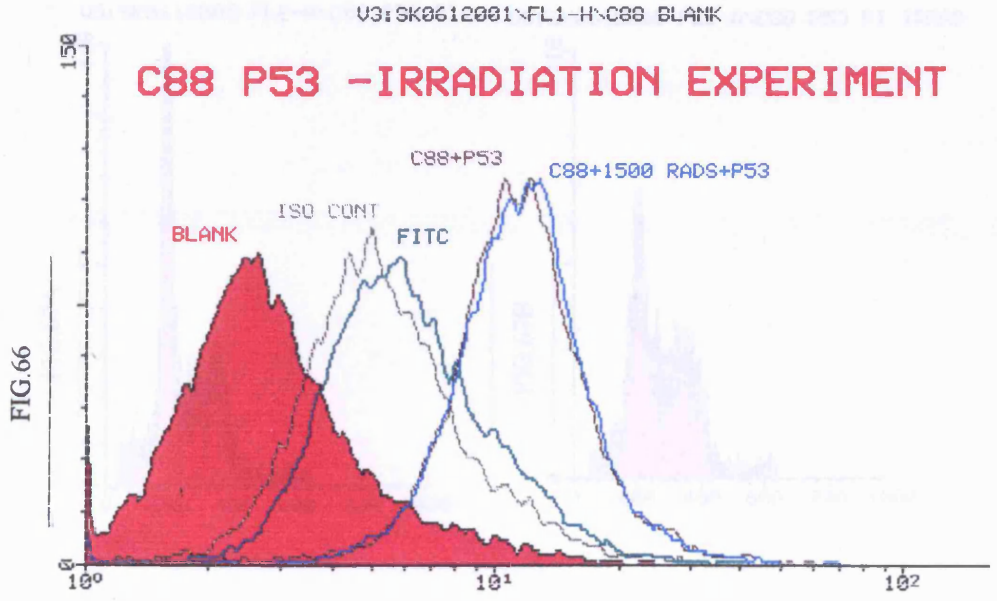


FIG.66

C88 P53 detection post X-ray irradiation.

Fig 66. Shows FITC fluorescence histograms of a Flowcytometric expression p53 246 epitope (mutated type) in a C88 cell line before and after irradiation. On the x-axis is FITC fluorescence and the y-axis is cell number. The FITC only and the Isotype fluorescence peaks are seen overlapping the blank. The test sample which had been irradiated with 1500rads and anti-p53 had been added, is overlapping the normal control which has not been irradiated. .Their fluorescence peaks, are the furthest on the x-axis. This cell line has mutated p53 which does not respond to irradiation.

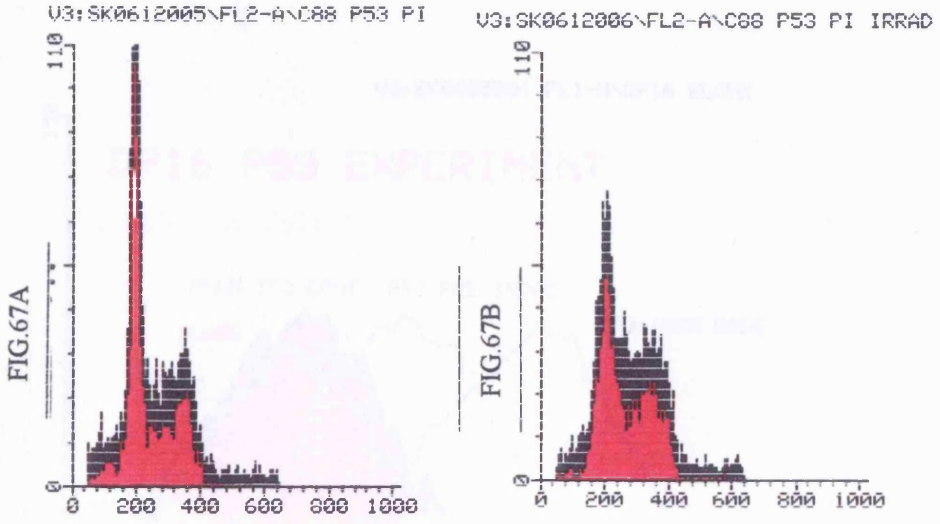


Fig 67A-B. DNA frequency histograms of C88 cell line.
A .Normal control fixed with 70% Ethanol and stained with PI.
B. Irradiated with 1500 rads , left in culture for 7hrs, fixed with 70% Ethanol and stained with PI. There is a reduction in the G_1 height and some increase in the G_2M.

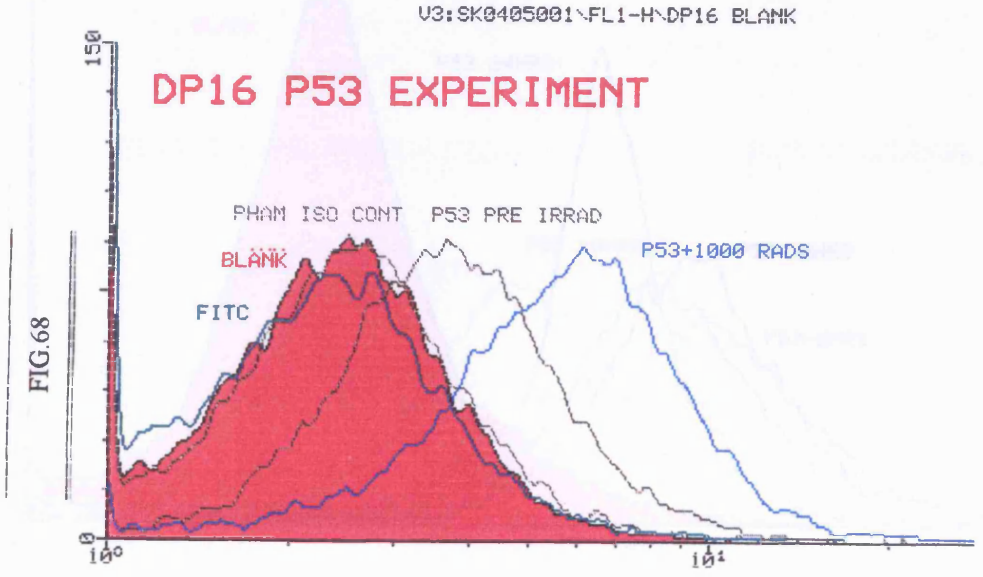
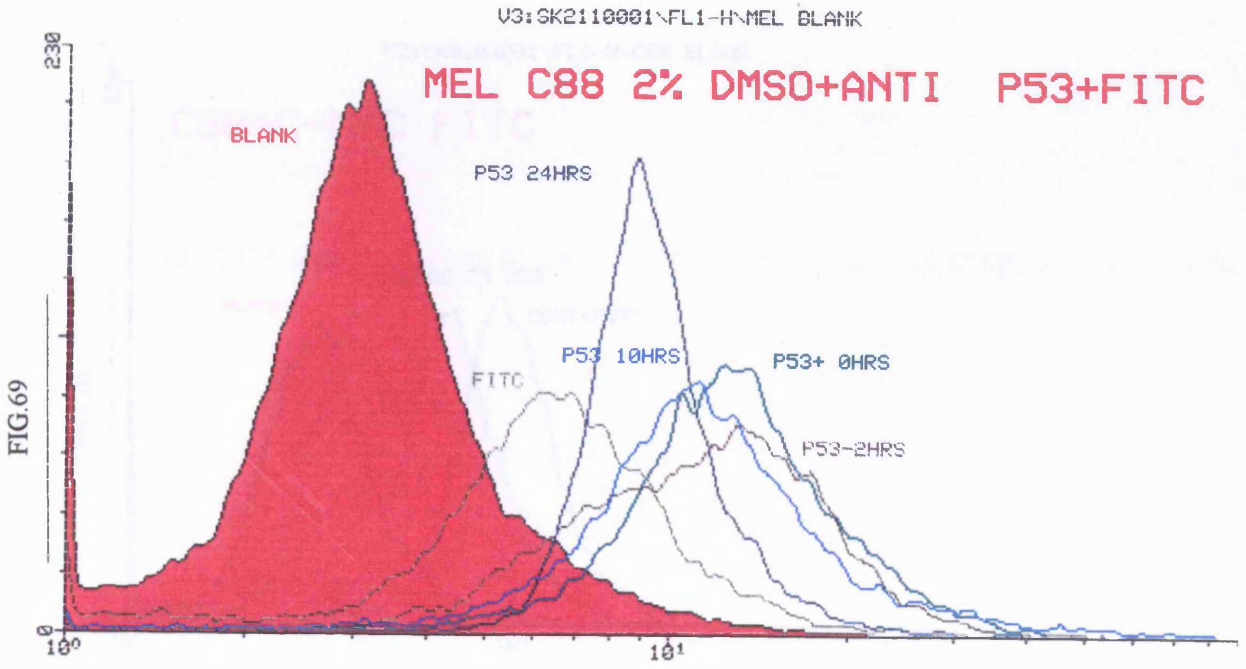


FIG.68

DP16-1 p53 detection post X-ray irradiation.

Fig 68. Shows fluorescence histograms of p53 240 epitope (wild type) expression in a DP16-1 cell line before and after irradiation. On the x-axis is FITC fluorescence and the y-axis is cell number. The FITC only and the Isotype fluorescence peaks are seen overlapping the blank. The test sample which had been irradiated with 1000 rads and anti-p53 had been added, is overlapping the normal control, which has not been irradiated. The irradiated sample fluorescence peak, is the furthest on the x-axis. The cell line has upregulated its level of p53 in response to irradiation. This makes it likely that it has wild p53, which has been detected by the 240 epitope.



C88 Cell line p53 detection post differentiation with DMSO

Fig 69. Shows FITC fluorescence histograms of p53 expression in a C88 cell line before and after DMSO treatments of varying durations. On the x-axis is FITC fluorescence and the y-axis is cell number. The FITC only and the Isotype fluorescence peaks are seen overlapping the blank. The fluorescence peak for the 0hr is well separated from the controls and is the furthest on the X-axis. The 2hr peak overlaps the 0hr meaning that there is no appreciable change in p53. at 2hrs. The p53 levels approach zero, as the 24hr. peak is very close to the blank.

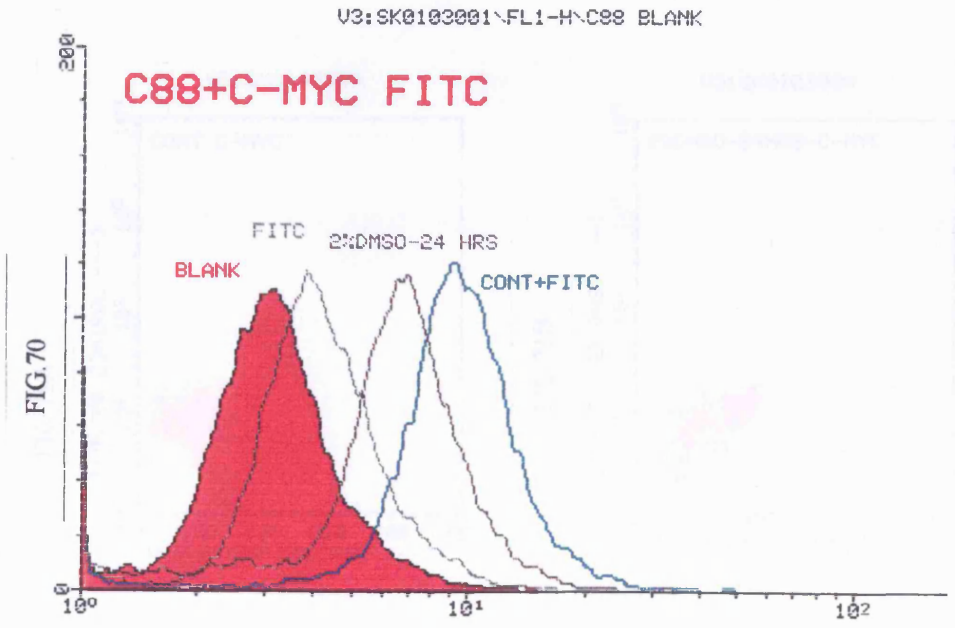


FIG.70

C88 Cell line c-myc detection DMSO post differentiation with

Fig 70. Shows FITC fluorescence histograms of c-myc expression in a C88 cell line before and after DMSO treatment. On the x-axis is FITC fluorescence and the y-axis is cell number. The FITC only fluorescence peak, is seen overlapping the blank. Next to this, is the test sample with 2% DMSO. The fluorescence peak for the control, is the furthest on the x-axis. There is a reduction in c-myc levels during differentiation with DMSO.

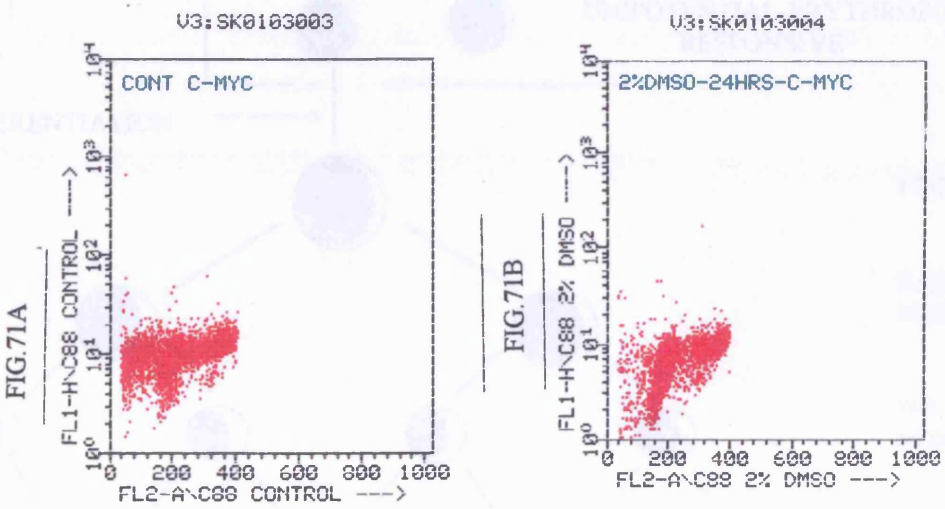


FIG.71A

FIG.71B

C88 Cell line c-myc detection DMSO post differentiation with

Fig 71 A. Shows dot plot cytogram depicting c-myc levels before DMSO administration. Fig 71B is a dot plot cytogram 24hrs post DMSO administration. There is some reduction in the c-myc levels after DMSO treatment.

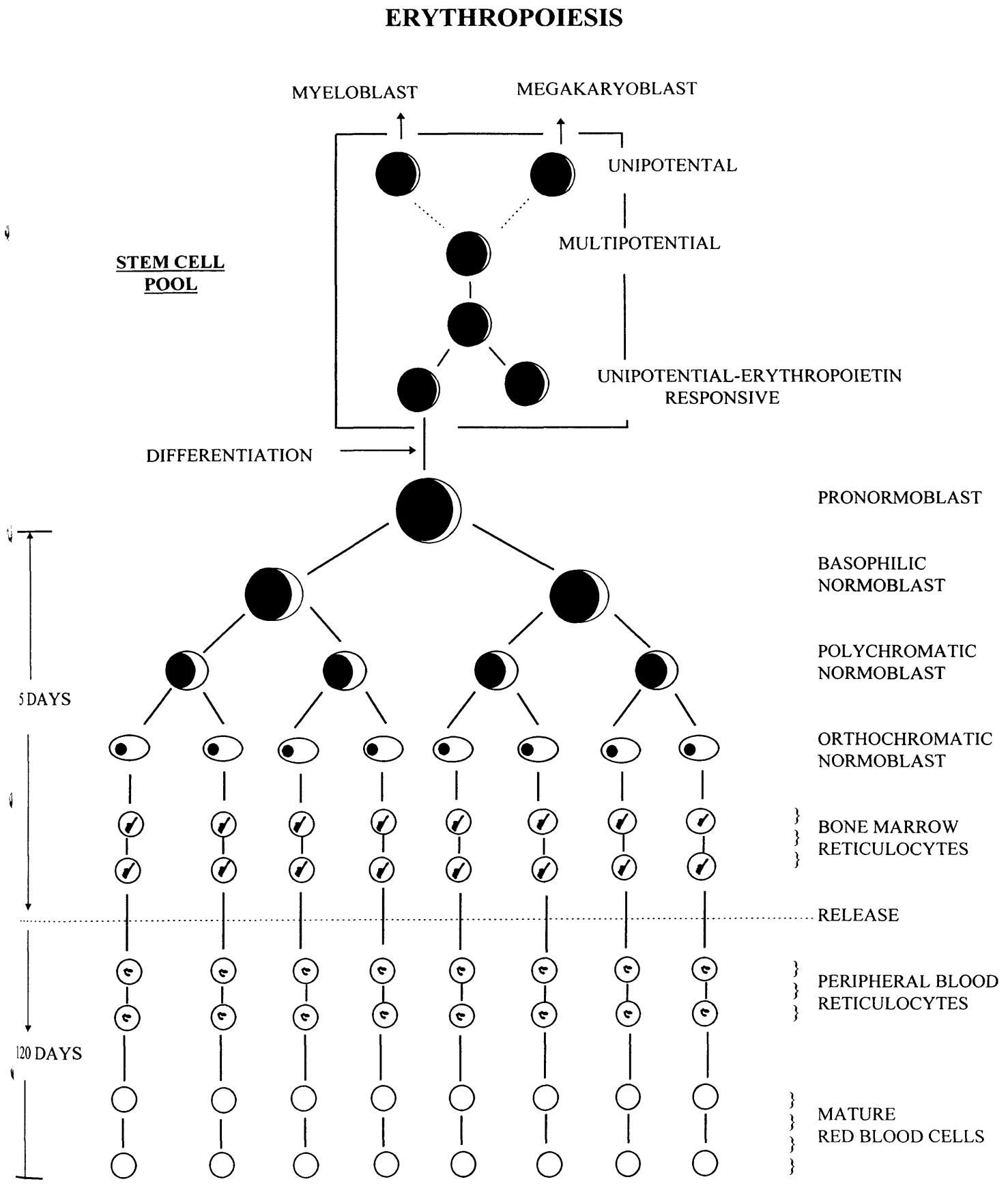


Fig. 72. Stages of differentiation of the erythrocyte

CHAPTER 4

DISCUSSION

4.1 A study of Correlations Between Apoptosis Proliferation and BCL-2 Expression in non-Hodgkin's Lymphomas.

In the management of lymphoma cases, it would clearly be useful to have information which would form the basis of reliable prognostic criteria. There have been a variety of classifications of non-Hodgkin's lymphomas, all of which claim to have prognostic value. The definition of broad categories of disease within lymphoma classifications has certainly proven useful but in individual cases it can be unreliable (Lauder, 1988).[85] One example of this is follicular lymphomas. They account for one third of non-Hodgkin's lymphomas in Western Europe and North America and while most of them follow an indolent course a small group are much more aggressive.

Some attempt has been made to divide small cell lymphomas into clinico-pathological groups based on estimation of the large proportions of neoplastic cells in a tumour but it has been difficult to separate follicular lymphomas into reproducible groups. On this basis, Beard and his co-workers introduced a quantitative criteria for counting large non-cleaved cells within the tumour to overcome these difficulties. Beard's method appeared to correlate best with prognosis when it was utilised for the working formulation and Lukes and Collin classification.[86] Even when differences between classification or difficulties in assessment of cell proliferation are disregarded, there are pathological and clinical factors which make comparisons between studies difficult. Looking at other biological parameters, as done in this study, offers a way forward. In this study I have assessed apoptotic activity using an oligonucleotide probe digoxigenin-11-dUTP incorporated by a terminal transferase enzyme at the sites of DNA breaks with the signal amplified by an indicator system, alkaline phosphatase.

The advantage of using digoxigenin is that it is not an endogenous substance to the human body like biotin. This technique has proved to be far superior in revealing apoptotic sites than Haematoxylin and Eosin or azure staining as used by Leochini L et al.[49] In this study the high grade tumours had the highest apoptotic rates. The study has shown a good correlation between the histone mRNA index of cell proliferation and apoptotic rate

as assessed by the end labelling technique (ISEL) and also with tumour grade (see figure 1).

Some studies have shown that several histone proteins are tightly coupled to DNA replication and could be used as markers of cell proliferation.[53,88] Ki-67 index has been shown by Gerdes et al in 1990 to be an excellent marker of growth fraction for normal and malignant tissues.[61] The mRNA histone technique has proven easy to carry out and is very reproducible and is far better in assessing cell proliferation than Ki-67 as it is S-phase specific. Previous studies have shown good correlation between S-phase proliferation rate and prognosis in lymphomas.[12,89]

In this study, I have been able to show in general that tumours with high mRNA histone count, also have the highest apoptotic rates. This indicates a link between high cell turn over and cell death by apoptosis. There has been no direct link between proliferation rate and survival. In this study Bcl-2 expression and its prognostic value has been assessed.

Interest in the Bcl-2 oncogene arose after the discovery of its role in translocations (14:18) in 85% of follicular cleaved small cell lymphomas and 20% of large cell diffuse lymphomas.[21,22] This translocation brings Bcl-2 from chromosome 18q21 to chromosome 14q:32 near to the Ig heavy chain.[91,92] This leads to deregulation of the gene causing inappropriately high levels as seen in malignancies. Further support for the oncogenic role is seen in transgenic mice experiments where those carrying Bcl-2 gene show extended survival of other B lymphoid cells and over a period go on to develop lymphomas.[31] The Bcl-2 oncogene is known to protect cells from apoptosis.[31,57] Previous studies have shown that expression of Bcl-2 is useful in distinguishing between benign and malignant lymphoid infiltrates.[92]

In the germinal centres of reactive lymphoid follicles, one seems to have a lot of death by apoptosis. These germinal centres are not protected by Bcl-2 and tend to be Bcl-2 negative while mantle zones or malignant follicles are Bcl-2 positive. This is similar to the studies of Mary Zutter.[87]

The detection of Bcl-2 in the present study has been enhanced by heat treatment by microwaving. This technique and others involving enzymatic treatment with Trypsin help to expose epitopes which are otherwise unavailable.[51]

In the present study, some lymphomas are Bcl-2 negative. Previous studies have shown these to have a bad outcome.[49] This study although limited in terms of numbers and short period of follow up (2-5yrs), has shown similar findings.

It has also shown that looking at other parameters in lymphomas such as Bcl-2 expression, apoptotic rates in addition to proliferation rates, one can, to a large extent delineate low grade from high grade tumours.

4.2 Flow cytometric Demonstration of Apoptosis in a Human Leukaemia Cell Line (HL-60) and Raji Cell Line.

The significance of my results in the successful induction of apoptosis and further confirmation by DNA ladders and EM on the sorted and non sorted samples, is in terms of therapeutic implications. Both Cycloheximide and Actinomycin D are established chemotherapeutic drugs against leukaemias, and the dose dependent apoptotic activity shown in these experiments can be exploited for therapeutic purposes, leading to a more efficient kill of leukaemic cells.

From the results of these experiments it can be concluded that apoptotic activity can be effectively induced in HL-60 cell line using Actinomycin D or Camphotecin. These are consistent with previous reports.[54,55]

HL-60 cell line derived from the peripheral blood of a patient with promyelocytic leukaemia has been used extensively to study promyelocytic leukaemia *in vitro*.[23] The HL-60 cell line is known to be very susceptible to apoptosis because of its amplified c-myc gene.[4] The cell line can therefore undergo apoptosis using only very small doses of Actinomycin D. Also increasing doses of either Actinomycin D or Camphotecin produced larger degrees of apoptosis.

The Raji cell line on the other hand needed large doses of Cycloheximide 100μg/ml or over for 72hr. incubation periods to undergo apoptosis. This is in agreement with previous reports.[3] For the HL60 on the other hand, apoptosis could be induced in this cell line using low doses of Cycloheximide (1μg/ml) over a 48 hr period.[54] The induction of apoptosis in both cell lines using Cycloheximide - an inhibitor of protein synthesis and in the case of HL-60 Actinomycin D, an inhibitor of RNA transcription, is further proof that in both cell lines, unlike in thymocytes, protein synthesis is not required for apoptosis to occur.

Camphotecin has successfully induced apoptosis in HL-60. Camphotecin is a topoisomerase inhibitor. Topoisomerase inhibitors are known to stabilise DNA cleavable complexes. This could explain their mechanism in inducing apoptosis. Previous reports have also shown that they trigger apoptosis in the S phase of the cycle.[100,101] For both cell lines an increasing degree of apoptosis as evidenced by an increase in the height of sub G_1 peak has been paralleled by diminution of S-phase (proliferative compartment).

4.3 Demonstration of Apoptotic Activity in Murine Leukaemia Cell lines DP16-1 and MEL-C88

For both C88 and DP16-1 cell lines, increasing apoptotic activity has been demonstrated by increasing height in the sub G_1 peaks as the dose of Thapsigargin has been increased (see figs. 18-21 for DP16-1 and 22-26 for C88). From these results it can be concluded that both DP16-1 and MEL-C88 cell lines undergo apoptosis when incubated with Thapsigargin, a drug that blocks the calcium- ATP pump.

It can be concluded that DP16-1 is more susceptible to apoptosis than C88 cell line, and the reason for this could lie in the fact that C88 has a mutated p53, which makes it resistant. On the other hand the DP16-1 cell line is known to have low levels of Bcl-2 which makes it more susceptible to apoptosis.[68] The Bcl-2 oncogene offers resistance to apoptosis.[95] For both C88 and DP16-1 cell lines increasing doses of Thapsigargin gives an increasing degree of apoptosis. Also (results not shown) increasing the incubation periods has a similar effect. For both cell lines calcium appears to be a necessary trigger for apoptosis. My results are a further contribution to the understanding of the

mechanism of apoptosis in those cell lines as so far there is no published information on the induction of apoptosis in those cell lines using Thapsigargin. Even though these are animal cell lines, the information derived showing that increasing doses of drugs produce more cell death by apoptosis can be exploited therapeutically.

4.4 Flow Cytometric Demonstration of Apoptotic Activity in a Human Leukaemia Cell Line HL-60 and a Murine Leukaemia Cell Line C88 Using an End Labeling Technique with Digoxigenin -11-dUTP

From these results it can be concluded that the end labelling technique has worked well for the HL-60 and C88 cell lines. This is shown by the fact that the histograms of the enzyme labelled samples are clearly well delineated from the ones to which only the control Digoxigenin oligonucleotide probe had been added. It has also been demonstrated by the results from the C88 cell line that the extent of separation, hence the intensity of the reaction, is very dependent on the enzyme concentration. Apoptotic activity in both cell lines has been confirmed by DNA ladders. The generation of DNA strand breaks during apoptosis by an endonuclease is said to be preceded by a proteolytic step. This has been shown to be inhibited by serine protease inhibitors like disopropyl fluorophosphate (DFP).[38] Previous end labelling reports on HL-60 cell line have used biotinylated dUT.[48] My work shows that Digoxigenin-11-dUTP is a very good alternative.

So far there are no published reports on end labelling carried out on C88 cell line. My work offers a further contribution to the literature available on the end labelling technique. The flow cytometric technique of end labelling of DNA 3′ OH ends of DNA using an oligonucleotide with an enzyme, offers a fast and sensitive method of demonstrating apoptotic activity in a large number of cells. The flow cytometer offers the advantages of speed, precision and reproducibility and statistical information could be derived. Leukaemia cells from patients can be labelled and assessment of apoptotic activity before and after treatment can be achieved. This enables monitoring of the response to treatment to be done by assessing the apoptotic activity which many chemotherapeutic agents produce.

4.5 DNA Extraction and Gel Electrophoresis on the Sorted and non Sorted Samples of HL-60, Raji, C88 and DP16-1

The experiment has achieved its objective to confirm that apoptosis had been successfully induced in these cell lines (HL-60, Raji, DP16-1 and C88) by means of DNA ladders. Also further confirmation of apoptosis on the sorted samples was done by means of EM pictures. Gel electrophoresis offers a way of visualising DNA fragments as a result of cleavage of DNA into fragments of 200 base pairs or multiples thereof by an endonuclease during apoptosis.

When an electric charge is applied to DNA which normally has a negative charge at neutral pH, the DNA tends to migrate towards the anode. The rate of migration of the DNA can be affected by the composition of the gel, the voltage applied, composition of the buffer and conformation of the DNA.[61] The smaller fragments of DNA would migrate furthest, while the larger fragments lag behind. Applying a bigger voltage causes a faster migration in gels that have higher resolution of smaller fragments.

Using marker DNA like Physex-1000 (Promega) which can mark base pairs with a range of 50-1000 and Hind III which detects a base range of 500 to 23000bp makes it possible to work out the size of the DNA fragments.

In lymphomas, sub G_1 diploid peaks can be easily confused with apoptotic peaks as they appear in the same position. The demonstration of DNA ladders on gel electrophoresis can help to overcome this problem. The relevance of these results on HL-60 and Raji cell line, is that Cycloheximide and Actinomycin D are already established chemotherapeutic agents. Their efficacy in inducing apoptosis has significant therapeutic implications as it now known that drugs kill tumour cells by the process of apoptosis.

4.6 Assessment of Cell Proliferation and Correlating it with Apoptosis in a Human Promyelocytic Cell line (HL-60) Using PCNA (Proliferating Cell Nuclear Antigen)

PCNA

Expression of PCNA during the cell cycle has been the subject of previous studies.[11,62,63] This method enables the measurement of the changes in the antigen during the cell cycle

and it is not necessary to have a synchronised cell population. Using this methodology has enabled me to correlate proliferative activity represented by S-phase as detected by PCNA and apoptotic activity. Using a flow cytometric approach allows the investigator to be objective and analyse a large number of cells with statistical precision. Different anti-PCNA antibodies on the market can produce varying results. The reason for this must be that these different antibodies recognise different epitopes. The results also can be affected by the method of fixation and permeablisation. There are 2 forms of PCNA. One type is tightly bound to nuclear chromatin. It is S phase specific and detergent resistant. The other one is the diffuse form, which is detergent labile. Using detergents like Nonidet-P40 and in my case, Triton-X100, it is possible to detect the S phase specific PCNA using monoclonal anti-PCNA antibody PC10.

In conclusion the PCNA results have demonstrated that, cell proliferation is negatively correlated with apoptotic activity, since increasing levels of apoptosis are associated with a reduction in the proliferative phase, as represented by the S-phase. The effect of Camphotecin in inducing apoptosis in this cell line, with increasing incubation periods and drug concentration, has been clearly demonstrated. Apoptotic activity was confirmed by DNA ladders (see plate XX).

PCNA is a reliable marker of cell proliferation in normal tissues and lymphomas.[11] It is useful to assess cell proliferation in tumours because it has been shown by previous workers that the aggressiveness of a tumour is related to its proliferation rate. The information gained from these experiments relating to proliferation rate and to apoptotic rate could be exploited for therapeutic manoeuvres.

4.7 Flow Cytometric Measurement of Cell Proliferation and Correlating it with Death by Apoptosis in HL-60 (A Human Promyelocytic Cell Line) and DP16-1 (A Murine Proerythroblastic cell line) Using Bromodeoxyuridine Incorporation Technique.

For the HL-60 cell line, Camphotecin (a topoisomerase I inhibitor) has produced DNA damage at the concentrations used, indicated by apoptosis as manifested by the sub G_1 peaks seen in the PI As expected, increasing incubation periods have produced more DNA damage (apoptosis). The S phase specificity of this drug is clearly evident in the

histograms and the dot plot displays by the diminishing S phase compartments (see figs. 39-43).

For the DP16-1 cell line apoptosis has successfully been induced using Thapsigargin. Calcium is a trigger for apoptosis and this drug by mobilising cytosol calcium, has demonstrated its ability to induce apoptosis in this cell line. Increasing incubation periods cause increased apoptotic activity with maximum apoptosis seen at the 24hr incubation period (see fig.50) The loss in the S-phase compartment is more pronounced at this period shown more clearly in the dot display (fig.50B).

From the BrdUrd incorporation experiments, it has been demonstrated for both cell lines that a decrease in the proliferative compartment (S-phase) has been paralleled by an increase in apoptotic activity as evidenced by an increase in sub G_1 peaks.

From these results, it can be concluded that apoptotic activity is negatively correlated with proliferative activity. Using a technique of BrdUrd incorporation, and simultaneously staining DNA with Propidium iodide, offers a unique opportunity for one to study the events in the cell cycle of DNA damage in response to chemotherapeutic agents or chemicals. For HL-60 (a human leukaemic cell line), its response to camphotecin and the S-phase specificity of this drug has been well demonstrated. A similar technique could be adopted on live patients. The work on the murine leukaemia cell line has shown similar principles of inducing apoptosis with increasing incubation periods and this principal is applicable to human trials.

4.8 The effects Of X-ray Irradiation on The Cell Cycle and Cell Proliferation of Human (Raji) and Murine Leukaemia Cell lines-DP16-1 and C88

Much of the earlier work on irradiation of human leukaemic cells was done on HeLa cells by calculating mitotic figures at different times. Painter and Robertson found that there was a division delay within 2hrs following exposure to 500 rads, which lasted at least 8hrs and disappeared by14·5hrs.[16,98] By pulse labelling the cells with tritiated thymidine and determining the percentage of labelled cells, they demonstrated a division delay of 4 - 8hrs following the dose.[16] Taylor subsequently showed that this accumulation was a

direct result of depression of DNA synthesis. While rate of entry into G_1- S-phases remained the same.

Yu and Sinclair found that Chinese Y79 cells showed a dose dependent mitotic delay following irradiation with 2·35- 7·1Gy.[99] It was greatest for the cells irradiated in the late S-phase and least in the G_1 phase and intermediate for the cells in the G_2 phase. Exposure of a variety of cells to ionising irradiation (X-rays, gamma rays) or UV irradiation results in a division delay which may have several components i.e, G_1 block, G_2 arrest and S-phase delay.

G_1 arrest is absent in many cell lines while G_2 arrest is present in all Eurkaryotic cells. G_2 arrest may involve suppression of cyclin B1 mRNA and or protein in some cell lines, and tyrosine phosphorylation of p34 Cdc2 in others. It has been postulated that G_2 delay would help cells to repair DNA damage before undergoing cell division.[16] Also, that if cells undergo division with damaged DNA, this can lead to malignancy.

The mechanisms for S-phase delay are not well known in man but in yeast the Rad gene tends to regulate DNA replication initiation and responds to irradiation since none of the Rad mutants show G_2-phase delay for doses each as large as 600 Rads.[16]

Radiation treatment is a well established mode of treatment for tumours. Some insight into factors governing sensitivity to radiation damage is useful for the clinicians. The aim of radiation therapy is to arrest cell growth, proliferation and induce cell death. In this case cell death by apoptosis has been confirmed by doing DNA ladders especially on the C88 cell line. Some tumours or cell lines are more radiation resistant.[66] Increasing doses of radiation as shown in these experiments have been followed by a progressive reduction in the growing phase - G_1 in the cell lines studied. There has been a failure to arrest in the G_1-phase as none of these cell lines have arrested in the G_1-phase and none of these cell lines are known to have a functional wild p53.

The relationship between p53 and G_1 arrest has been confirmed by O'Connor et al,[100] who could correctly predict the p53 status (wild type or mutant) in 15 of the 17 Burkitt's

lymphoma and lymphoblastoid cell lines by, determining whether they exhibited an intact G_1 arrest following administration of 6·3Gy. Much progress has been made since the classic studies of Tolmac and Painter who originally described the effect of radiation on cell cycle progression.[98] The effects of radiation and other chemotherapeutic agents on cell cycle are of tremendous interest to researchers in the hope that knowledge gained could be exploited to chemotherapeutic advantages.

From the results of my work on the C88 cell line, it can be concluded that, increasing doses of irradiation cause an increasing degree of apoptosis, and this has been confirmed by DNA ladders. The p53 mutation is important in resistance to apoptosis. This has been confirmed by my experiment on the RAJI cell line with known mutated p53. This very important information offers a potential for p53 manipulation to enhance radiation sensitivity.

4.9 Experiment to Demonstrate Bcl-2 Oncogene Expression in two Murine Leukaemia Cell Lines DP16-1 and C88 Using a Flow Cytometric Technique.

The Bcl-2 gene was discovered because of its involvement in the chromosomal translocation t(14:18) commonly found in lymphomas.[32] This cytogenetic abnormality places Bcl-2 gene from chromosome 18 into juxtaposition with the transcriptionally active immunoglobulin heavy-chain locus on chromosome 14 resulting in inappropriately high levels of Bcl-2 gene expression in B-lymphocytes.[32] Bcl-2 protein has been shown to delay or block programmed cell death (apoptosis) in a variety of circumstances.[95,101,102] Apoptosis is characterised by degradation of nuclear DNA of the cell into oligonucleosomal length fragments due to activation of an endogenous endonuclease. Apart from its physiological role in maintaining tissue homeostasis, it is clinically important because many of the anti cancer drugs kill cancer cells by the process of apoptosis.

High levels of Bcl-2 have been shown to protect lymphoid cells from death and DNA fragmentation used by anti neoplastic drugs.[32] Bcl-2 has been shown to co-operate with c-myc to immortalise pre B cells.[103] James Ryan et al 1994 using DP16-1, a cell line known to express very low levels of Bcl-2, introduced Bcl-2 transcripts into the cell line

by electroporation. He found that Bcl-2 delayed p53 induced apoptosis and prolonged the survival of the murine leukaemia cell line - DP16-1.[104] Previous reports indicate that cell lines with Bcl-2 are more resistant to apoptosis.[73,101]

From the results of flow cytometric assessment of Bcl-2 expression in DP16-1 and C88, it can be concluded that C88 cell line expresses Bcl-2 more strongly that DP16-1. This is in agreement with previous findings on DP16-1 cell lines.[68] The fact that DP16-1 has a lower Bcl-2 reactivity can explain why this cell line is more susceptible to apoptosis because it does not have as much protection from apoptosis by Bcl-2.

Aiello et al in 1992[105], conducted a flow cytometric detection of Bcl-2 in normal and neoplastic human lymphoid cells. Using formaldehyde fixation, he found that compared to ethanol fixation, formaldehyde offered better specific/background staining ratio. In my study using ethanol fixation and a permeablising agent Triton-X-100 at the concentration of 0.25% instead of a concentration of 0.1% used by Aiello, I have found no problems in detecting Bcl-2 in these two cell lines. It would appear that in adequately permeablised cells, the ethanol fixation does not significantly affect the protein configuration as to mask the Bcl-2 antigenic sites. The experiments I have performed (see chapters 2.3 and 3.3) on these cell lines have shown that C88 is more resistant to apoptosis than DP16-1. The results give further evidence for a protective role of Bcl-2 against apoptosis.

4.10 The Role of c-myc in Apoptosis in HL-60 and DP16-1 Cell Lines as Investigated by a Flow Cytometric Technique.

Previous workers have shown that c-myc plays a role in apoptosis.[4,72,73] They have also shown that c-myc is down regulated during apoptosis. This has been further confirmed in my experiment on the two cell lines. C-myc oncogene is one gene that can facilitate cell proliferation on one hand or cell death on the other. This has been particularly shown for RAT-1 fibroblasts carrying c-myc, which can move from G_0 to the G_1 part of the cell cycle in presence of serum, or undergo apoptosis in absence of serum. C-myc is acting as a negative regulator of cell proliferation. The current thinking is that c-myc acts as a fail-safe mechanism to delete cells which harbour mutations, such cells with activated c-myc can promote inappropriate cell cycle progression.[106]

c-myc acts through a transcriptional target gene called ornithine decarboxylase (ODC).[113] The ODC gene harbours two conserved consensus c-myc binding sites (CASGTG). ODC catalyses the first and rate limiting enzyme for polyamine biosynthesis. Enforced ODC expression like c-myc expression is sufficient to induce apoptosis in the interleukin-3 (IL-3) dependent cells and an inhibitor of ODC enzyme activity L-difluoromethyl ornithine (DMFO) effectively blocks c-myc induced apoptosis.[106]

Engelhard H.H. et al,[107] in 1991 in his work on HL-60 cell line, demonstrated that that there was a decline in the c-myc levels following treatment with Cycloheximide 2·5μg. This drug is known to induce apoptosis at that dose in this cell line. He found that the flow cytometric measurements of the c-myc proteins correlated well with the biochemical measurements of the same protein. My results show similar findings. Inappropriate expression of one of the members of the c-myc gene family is one of the most common events occurring in tumours.[107] c-myc co-operates with Ha-ras to transform normal cells. The transformation of low grade follicular lymphomas to a high grade diffuse lymphomas are often associated with either c-myc over expression or p53 expression. On the other hand inappropriate expression of c-myc leads to apoptosis. In 1994 James Ryan et al, found that co-expression of c-myc and Bcl-2 in DP16-1 cell line overcame p53 induced apoptosis.[107] My results indicate a downward regulation of c-myc protein during apoptosis using a flow cytometric technique and are in agreement with his findings. It can be concluded that c-myc participates in apoptosis.

4.11 Flow Cytometric Analysis of p53 in Murine Leukaemia Cell Lines DP16-1 and C88.

From these results it can be concluded that the C88 cell line has a mutated p53 and a dominant epitope. This is clearly in keeping with the fact that this cell line is resistant to apoptosis. It has been demonstrated by Benchmol et al using western blotting technique that DP16-1 cell line has no detectable anti-p53.[108]

In 1993, James Ryan et al[75] transfected the DP16-1 cell line using electroporation with temperature sensitive mutant of p53.[Val-135] The cells with mutated p53 grew normally at

37°C while those carrying wild p53 when grown at 32°C, showed G_1 arrest died rapidly. DNA cleavage was demonstrated as ladders on gel electrophoresis. It appears that by flow cytometry p53 is detectable in DP16-1, but most probably both alleles are inactive or deleted. The deletion of this p53 oncogene also fits the theory of carcinogenesis, because mutated p53 encourages transformation.[18,76]

The wild p53 is supposed to act as a policeman in the cell cycle at the G_1S interphase allowing cells with DNA damage enough time to repair or die by apoptosis.[33,98] It is wild type p53 that plays a role in apoptosis and not the mutated type.[33,75] Cells with wild type p53 are more susceptible to apoptosis. Myeloid leukaemia cell line M1 lacking wild p53 died by apoptosis when forced to express p53.[4] Oligonucleosomal fragments could be demonstrated by DNA ladders. In the detection of p53, adequate permeablisation of the cells is essential with a permeablising agent like Triton-X-100 to get good detection. In 1993 Ian Brotheric et al did a flow cytometric determination of p53 using three different antibodies. He found that the different antibodies could different results.[109] Since different antibodies recognise different epitopes when a tumour is negative for p53 with one antibody, it is better to use a panel of antibodies.

The most widely used techniques for p3 detection are immunocytochemistry and polymerase chain reaction (PCR).[109,110,111] The reduction in the staining pattern (nuclear and cytoplasmic) can cause problems in the determination of positivity by immunocytochemistry. The PCR product is usually less than that demonstrated by immunocytochemistry. This may reflect substrate loss during PCR amplification. Flow cytometry offers the quickest way of measuring and even quantifying p53.

4.12 Effects of X-ray Irradiation on p53 Expression on Leukaemia Cell Lines, DP16-1 and C88.

The results clearly indicate that there is no rise in p53 in C88 cell line in response to irradiation. It can be concluded that this cell line has a mutated p53. On the other hand the DP16-1 cell line shows some increase in p53 levels in response to irradiation. This indicates that probably the deletion of the wild p53 epitope in this cell line is not total. It is the wild type p53 which protects cells from irradiation. It is not surprising that there is

no response to irradiation in the C88 cell line as I have shown it to carry mutated p53. A more accurate and quantitative method of measuring changes in p53 expression could be achieved if standardised fluorescent beads are used as previously shown by Brotherick et al.[109] Previous reports have demonstrated the role of p53 in response to irradiation.[78,79] Patients with low levels of or with no p53, such as Ataxia telengiectasia are very sensitive to irradiation and are unable to repair DNA damage.[16] It is the wild type of p53 that has a role in response to DNA damage.[16]

Various workers have shown that wild type p53 causes a G_1 arrest in response to DNA damage.[35] If the damage is severe, cells respond by going into apoptosis.[16,78] G_1 arrest is supposed to help cells to repair DNA damage otherwise cells proceed to S and mitotic phase with damaged DNA which would lead to malignancy.[16] Individual cells vary in their response to DNA damage. For instance, cells with mutated p53 are more radiation resistant. This has been demonstrated in my experiments on the Raji cell line and C88 cell line.

A lymphoid cell line 9LY-R with mutated p53 is very resistant to irradiation.[78] Cells with wild type of p53 on the other hand, are very sensitive to irradiation. Fukunaga-Johnson N. et al. in 1995 transfected a wild p53 into DP16-1 cell line and showed that the cells died by apoptosis in response to irradiation whereas those with no p53 did not.[68] When forced to express Bcl-2 DP16-1 cells were resistant to irradiation. My work on p53 expression on these cell lines has contributed further to the literature on p53 levels in response to irradiation in those two cell lines.

4.13 Cell Differentiation in Murine Leukaemia Cell Line C88. The Role Played by c-myc and p53 and the Link with Apoptosis as Confirmed by DNA Ladders.

Cell death by apoptosis is a recognised phenomenon. During erythropoiesis it acts as a regulatory mechanism against cell proliferation.[73] Cell differentiation is a multistage process in which among other factors oncogenes play a prominent role.[113] Use of inducers of cell differentiation like DMSO and HMBA in murine leukaemia cell lines mimics *in vivo* erythrocyte differentiation.[82,84] For years it had been known that a relationship exists between cell proliferation and differentiation. Cancer is thought to

arise when the relationships between these two are upset. For instance, loss of cellular differentiation in favour of cell proliferation leads to development of malignancy.

Reports on 3T3 fibroblasts have shown that growth arrest precedes cell differentiation and terminal differentiation leads to loss of proliferative potency.[113] Linkages in the control of cell proliferation and differentiation are most evident at the growth arrest (PGA) state and the non terminal differentiation state (NTD). Cells at both stages are quiescent. The cells at the PGA state can either exit the PGA and return to normal cell cycle and proliferate or differentiate.

Table 9.

States of differentiation of various cell types and their proliferation potential

Multistep Differentiation Process	Abbreviation	Highly Differentiated Phenotype	Proliferative Potential	Examples *in vivo* Counterpart
Rapid growth ↓ ↑	RG	-	+	Regenerative Hepatocytes
Reversible Pre-Differentiation growth arrest	PGA	-	+	Quiescent Stem cells
Reversible non terminal ↓ ↓ ↑	NTD	+	+	Lymphocytes and Hepatocyte
Irreversible terminal differentiation	TD	+	-	Neurons and muscle cells

The NTD stage serves as a link in the control of cell proliferation because, at this stage the cells can return to the cell cycle and proliferate or undergo terminal differentiation with loss of proliferative potential. 3T3 fibroblast experiments in which fibroblasts were induced to differentiate, allowed to go back in the cell cycle, and then induced to differentiate again, showed that these fibroblasts lost their transformed neoplastic potential.[113] This also opens a way to chemotherapeutic manoeuvres. Recent reports have shown that transformed cells including those containing the SV40 T gene produce

autocrine anti-TD factors that block terminal differentiation. This knowledge could be exploited for new therapeutic strategies against cancer.

Using neutralising antibodies or antisense oligonucleotides to TD factors for instance would make cells terminally differentiate and lose their malignant character. Movement from NTD to TD in humans is due to a protein called Aproliferin.[113] It is a protein of Mol. wt. 45kD. 6hrs. of exposure to this protein leads to TD. Also progression from NTD to TD is associated with loss of proteins in the cells called P2Ps (proliferation potential). The P2Ps are nuclear polypeptides Mol. wt. 34-38kD and are thought to represent a subset of nuclear hnRNP.

An experimental model in which a multistep process can lead to carcinogenesis is exemplified by the effects of transforming virus SV40 T antigen. The SV40 T antigen antagonises the ability of cells to undergo pre differentiation growth arrest.[113] It makes cells extremely sensitive to mitogens including those that are not usually complete mitogens for non transformed cells i.e. insulin. SV40 T antigen transformed cells do not require the c-fos, c-myc or Pkc activation or polyamine induction to undergo DNA synthesis which non transformed cells require. Instead, they require c-jun and B-jun. Using stringent culture conditions which are anti proliferative, it is possible to force SV40 virus transformed cells to undergo PGA and NTD.

The role of p53 in cell differentiation

Wild type of p53 has been shown to control a differentiation pathway of B-cell lineage.[114] When 70z/3, a pre B cell line which expresses wild type p53 was treated with differentiation inducer Lipo polysaccharide LPS'. It was seen that increased levels of p53 mRNA preceded specific changes in Kappa (K) immunoglobulin expression. Induction of cell differentiation involves transcription of the p53 gene.[114] Using flow cytometry mutated p53 levels have also been shown to decline during HMBA or DMSO induced differentiation in erythroleukaemia cell lines.[112]

C-myc

C-myc encodes for nuclear protein with transcriptional motifs which are involved in neoplastic transformation.[72] Previous reports show that c-myc oncogenes are expressed in several cell lines at the early phase of differentiation leading to a decrease in c-myc levels. HL-60 cells induced to differentiate using DMSO or retinoic acid and mouse murine leukaemia cell lines induced to differentiate with HMBA are good examples.[87,113] The role of c-myc has also been proven further by showing that over expression of c-myc in murine leukaemia cell line U-937 inhibits cell differentiation.[120] Interferon tends to abrogate the c-myc induced differentiation block in U-937 cell line, indicating that interferon induces a signal that circumvents c-myc. From these experiments it can be concluded that there is a reduction in c-myc levels during differentiation and this is in agreement with previous findings.[81] C-myc levels are also down regulated during apoptosis. c-myc apoptosis has been demonstrated by DNA ladders in this study. C-myc oncogene appears to provide a link between cell differentiation, cell proliferation and apoptosis. Apoptosis has been shown to occur during differentiation in the keratinocyte skin model. Apoptotic keratinocytes were shown to be present in the basal layers and keratinocytes were shown to produce a Ca^{++} dependent endonuclease that cleaves DNA into oligonucleosomal fragments. This enzyme is only activated when cells migrate to the suprabasal layer.

4.14 Experiment to Induce and Demonstrate Cellular Differentiation of a Murine Leukaemia Cell Line C88 Using DMSO and Cytospin Preparations

Various reports have indicated the ability of DMSO to induce cell differentiation in murine leukaemia cell lines.[115,116] This has also been demonstrated using other agents like HMBA (Hexamethylene bisacetamide). Haematopoiesis is a process through which the organism acts to renew and replace cells and formed elements of blood.[119] This is a complex and vital process which involves interactions of various growth factors and progenitor cells which lead to formation of mature erythrocytes (see fig 72).[117]

The early progenitor cells (BFU-E) are characterised by their response to growth factor Interleukin 3 (IL3) and erythropoietin. Erythropoietin is produced primarily by the kidney and it is a polypeptide Mol. wt. of 34-38kDa.[116] Erythropoietin induced signal

transduction mechanism is poorly understood. Proliferation and Erythropoietin induced changes involve phospholipases A2 and C, including lipoxygenase metabolites of arachidonic acid.[117] Calcium ions (Ca^{++}) are also required. There is evidence for the opening of calcium channels through the cell membrane as a result of Erythropoietin action. Their growth requires those growth factors.

Maturation of BFU-E is associated with an increase in Erythropoietin receptors.[116] The next stage in maturation of CDF-U is heavily dependent on Erythropoietin. CFU-E are committed erythrocyte precursors which respond to Erythropoietin by undergoing several rounds of proliferation followed by terminal differentiation and loss of proliferative capacity. The more mature erythrocyte precursors proerythroblast series are committed to erythroid differentiation and no longer require Erythropoietin.

Cytospin Preparation

The Cytospin uses a centrifugal force to deposit a monolayer of cells in a defined spot on the slide. It offers advantages in terms of specimen retention, standardisation and ease of evaluation. It makes morphological evaluation of cells easier. This is better than direct smears which produce cell layers of varying thickness. There is a likelihood that cells of a different size would be in different areas of a smear.

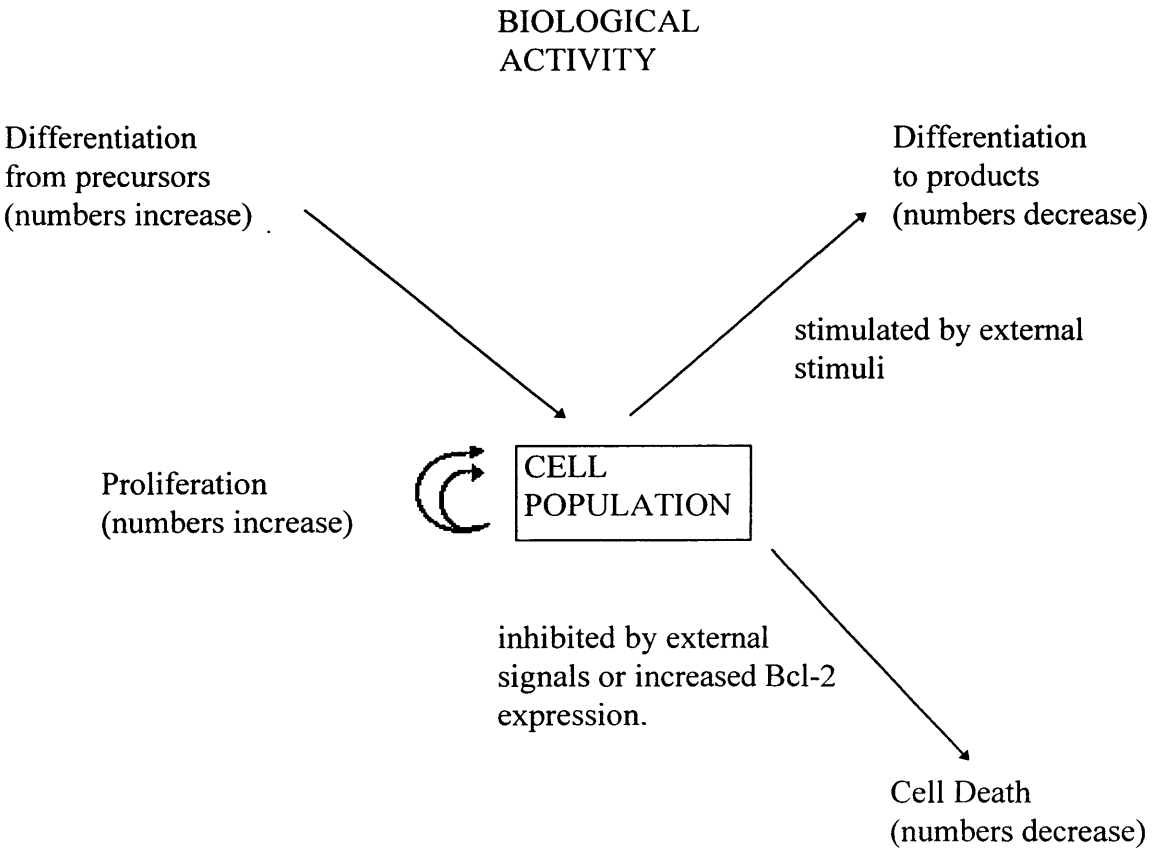
The cytospin technique also ensures maximum containment of the potentially hazardous specimen and minimises the risk to the operator. The induction of cellular differentiation using DMSO has been clearly illustrated in this experiment.

4.15 General Discussion

This study was set out to test the hypothesis that cell death by apoptosis acts as a modulating force to cell proliferation. I also set out to investigate the role of oncogenes in those two processes. Another objective was to establish a link between cell death by apoptosis and cell differentiation. The concept that apoptosis acts as an opposite force to cell proliferation was first proposed by Kerr in 1972.[19] Under physiological conditions it acts as an opposing factor to cell proliferation to maintain homeostasis in a variety of tissues[24] This is seen for instance, in the haemopoietic system where the immature

haemopoietic cells produced in excess die by apoptosis. In the development of the immune system, only the B cells producing useful high affinity receptors are selected for while others die by apoptosis. Another parallel exists in the nervous system where the excess neurons produced during development die by apoptosis.[24] The size of any particular population of can be controlled by changes in the rate of cell death as well as cell proliferation or cell differentiation.

The relationships between these three states are represented in the diagram below.



Adapted from Gwyn Williams (see ref. no.28.).

One experimental model that tests the hypothesis is the skin model. According to this model the epidermal homeostasis is achieved by an interplay between apoptotic and mitotic rates (proliferation rates) as well as differentiation. The skin keratinocytes undergo mitotic divisions in the basal layer. As they migrate to the top layers some die by apoptosis but most differentiate into keratinised cells which are dead and desquamate.[41] Most cytotoxic drugs in current use have been shown to induce cell death

by apoptosis. The fact that disparate agents which interact with different targets to induce cell death with common features i.e. endonucleolytic cleavage of DNA and chromatin condensation, suggests that cytotoxicity is determined by the drugs ability to engage a cell in programmed death[3]. The mechanisms which couple a stimulus (drug target interaction) to its response (cell death) remains unknown but the modulation of this coupling tends to affect the outcome of treatment.

The modulating agents which have been widely investigated are genes like wild type p53 or c-myc, bax and bcl-x_s which allow apoptosis to occur and Bcl-2, bcl-x_L or the Epstein-Barr virus gene BHRF1 which opposes apoptosis.[28,41] Recently other agents like Fas/Apo antigen and TNF known to facilitate apoptosis have been characterised.[41] The results from histological and paraffin embedded lymphoma material have demonstrated a clear relationship between apoptotic rates, as assessed by End labelling technique (ISEL), proliferation rate as assessed by mRNA histone and grade of malignancy.

It could then be inferred that in the high grade tumours the high proliferation rates are being countered by the high apoptotic rates. The role of oncogenes, in this case Bcl-2 has been demonstrated by the fact that more of the low grade tumours stained for Bcl-2. It is the low grade tumours that had the lowest apoptotic rates implying that Bcl-2 modulated their apoptotic response. Bcl-2 is known to protect tumours from apoptosis. My results also show that not all low grade tumours are Bcl-2 positive, which indicates that these tumours have an alternative protective mechanism. Possession of mutated p53 or viral genes like BHRF1 would make cells resistant to apoptosis.

The results of both murine and human cell lines (DP16-1, C88 and HL-60 as well as Raji), have clearly shown a dose response relationship to apoptotic activity. This is shown on the histograms as taller apoptotic peaks and a great diminution in the proliferative compartment with increasing doses of the drugs. In all these cell lines, apoptotic activity has been confirmed by use of DNA laddering. The diminution in S-phase being paralleled by the increase in apoptotic activity, indeed supports the hypothesis that apoptosis counteracts cell proliferation.

The role of oncogenes

c-myc gene

The role of the c-myc oncogene in apoptosis has been clearly demonstrated in Rat-1 fibroblasts. These cells expressing high levels of c-myc undergo apoptosis on serum withdraw.[72] The Chinese hamster cells (CHO) overexpressing c-myc underwent apoptosis.[41] My work on the DP16-1 and HL60 cell lines has shown a reduction in the c-myc levels upon induction of apoptosis. This offers further support for the role of c-myc in apoptosis

P53.

The evidence that p53 participates in apoptosis comes from several quarters. Myeloid leukaemia cell lines transfected with wild type of p53 easily underwent apoptosis.[28] DP16-1 cells carrying the wild type when grown at 32°C underwent apoptosis.[75] Philip Coates et al. using an End labelling technique and confocal microscopy was able to show increased levels of p53 at the sites of DNA damage.[118]. Transgenic mice lacking wild type p53 are unable to under go apoptosis induced by ionising radiation.[18] As regards p53, my work has shown that cell lines C88 and Raji which have mutated p53 are resistant to apoptosis.

Bcl-2 gene

The protective role of Bcl-2 against apoptosis has been demonstrated in various cell lines. Toshiyuki and J.Reed demonstrated this elegantly using a pre-B cell line 697 transfected with a recombinant Bcl-2 containing retrovirus and a control from the same cell line not having the Bcl-2. The Bcl-2 carrying cell line showed reduced apoptosis and prolonged survival.[101] Lam M et al. when they transfected Bcl-2 into a mouse leukaemia cell line WH17.2 there was protection from apoptosis but the same cell line without Bcl-2 was not protected.[57] The interactive role of the three genes (c-myc, p53 and Bcl-2) was neatly demonstrated by James Ryan et al.[104]

When MEL cell lines were transfected with these genes in various combinations, co-expression of Bcl-2 and p53 prolonged survival of the murine leukaemia cell lines. This was associated with loss of G_1 mediated p53 arrest. Co-expression of c-myc with Bcl-2

overcame p53 induced apoptosis. My work has shown that C88 cell line which expresses Bcl-2 more strongly than the DP16-1, is more resistant to apoptosis than the DP16-1 cell line even though these are two related virus transformed cell lines.

Cellular differentiation

Co-ordination between diffirentiation and proliferation is an essential feature of the successful development and replenishment of tissues, but it is not entirely clear how a decline in one is related to the other. Oncogenes are known to play a crucial role in these two processes. Oncogenes have been shown to overcome the limited proliferative capacity of normal mammalian cells in culture, enforcing them to an immortalised phenotype. This in turn may act as a primary step in carcinogenesis.

Examples these oncogenes are: adenovirus E1A, SV40 and polyoma large T antigens and human papilloma virus E6 and E7. The cellular oncogenes which show similar behaviour are: c-myc, fos, jun and mutant p53 Oncogenes, which cause cellular immortalisation tend to have nuclear localisation while those that cause transformation have cytoplasmic localisation. Oncogenes from both families interfere with differentiation process. There is some evidence that some of these oncogenes act as regulators of normal cell differentiation and when immortalisation occurs this is a process of blocking a cell from achieving a differentiated state.

The concept that cell differentiation plays a modulating role in cell proliferation is well illustrated in the mammalian skin model.[3,119] The proliferating cells in the basal layer give rise to differentiated and keratinised cells at the top which are shed off. McCall C.A and Cohen J. have shown in the skin model, a link between cell death by apoptosis and terminal cellular differentiation. They showed that apoptotic cells in differentiated cells fragmented their DNA internucleosomal fragments which was demonstrable by DNA ladders on gel electrophoresis.

My work on the DP 16-1 cell line has shown that c-myc levels fall during apoptosis and differentiation in C88 cell line. This provides evidence of its role in those two processes.

The demonstration of DNA ladders in C88 cells during differentiation is further proof for the link between differentiation and apoptosis.

In summary the main conclusions of my work are:

1) There is a positive correlation between apoptosis, and cell proliferation in lymphomas.
2) There are on the whole higher apoptotic rates and proliferation rates in the high grade lymphomas reflecting a high turn over rate in the tumours, compared to the low grade ones.
3) Bcl-2 offers some protection against apoptosis.
4) There is evidence of death by apoptosis during cellular differentiation.

CHAPTER 5

RECOMMENDATIONS FOR FURTHER STUDIES

The study on 20 cases in each category of high and low grade non Hodgkin's lymphomas could be expanded to include more cases. A follow up period could be extended for at least 10 years in order to get more meaningful prognostic information, particularly as the low grade non-Hodgkin's lymphomas tend to follow a long indolent course. Immunocytochemical demonstration of their p53 or c-myc status could be done to relate these to proliferative or apoptotic activity and see if they offered prognostic information.

The *in vitro* experiments on leukaemia cell lines showing drug response to chemotherapeutic agents by apoptotic activity, could be extended to live patients. After administering chemotherapeutic agents, the apoptotic activity could be assessed for instance measuring an End labelling activity before and after apoptosis. Reports about this technique have already been published.[6] The resistance to chemotherapeutic agents could be related to Bcl-2 expression or mutated p53 expression. Prognostic information could also be sought according to Bcl-2 expression.

For the leukaemia cell lines, more direct kinetic information could be obtained by using mitogens to stimulate cell proliferation i.e. Concanavalin-A, Phytohaemaglutinin or growth factors like TGFβ1 to stimulate cell proliferation and then use chemotherapeutic agents to induce apoptotic activity. By simultaneously administering mitogens and apoptosis inducing drugs on one hand, and comparing with the controls in which those had been administered separately, it would be possible to directly evaluate more directly the role of apoptosis modulating cell proliferation. The role of oncogenes like p53 or Bcl-2 or c-myc in cell proliferation or apoptosis, could be evaluated more directly, by using anti-sense oligonucleotides against those genes which could achieve the effect of neutralising their roles.

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APPENDIX

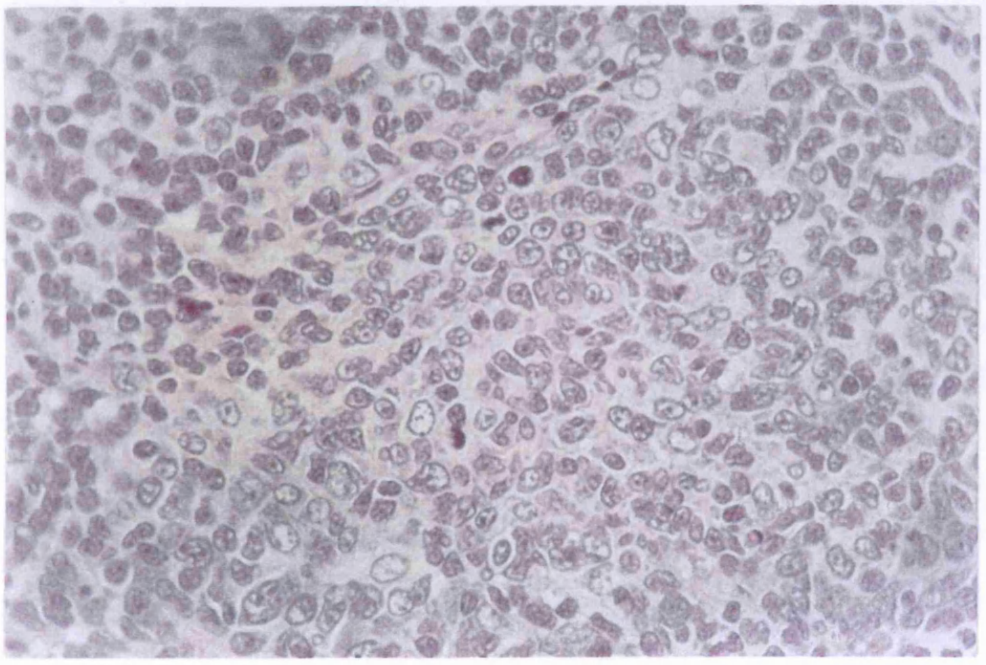

Plate I

x400 Mag. HE. (Hematoxylin Eosin) section of a low grade non Hodgkin's lymphoma. The tumour is composed of predominantly centrocytes with a few centroblasts

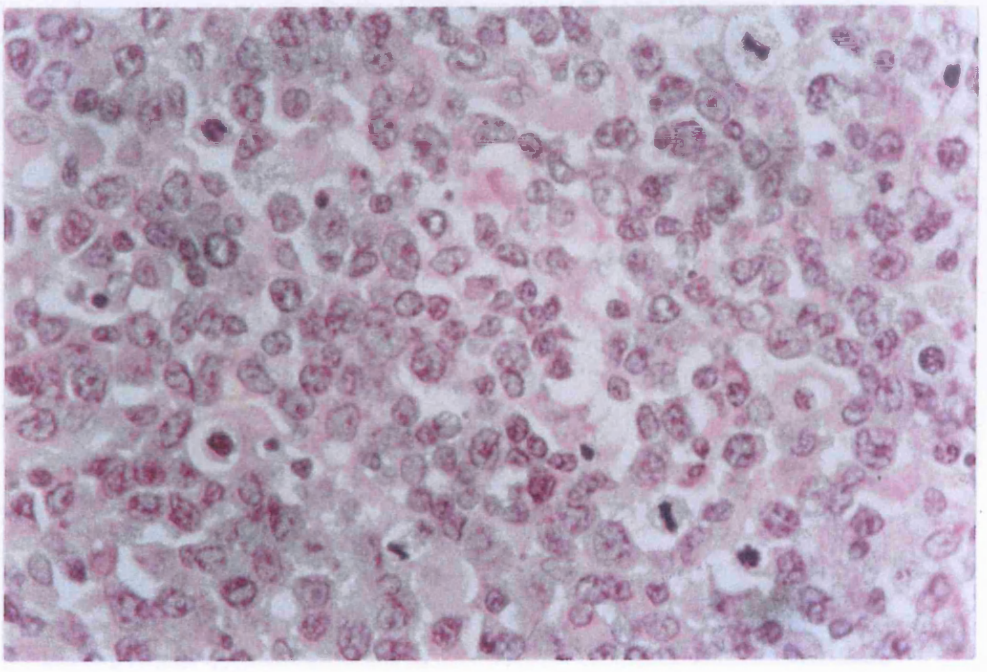

Plate II

x400 Mag. HE (Heamatoxylin Eosin) section of a high grade non Hodgkin's Lymphoma. The tumour is composed of predominantly centroblasts and a few controcytes

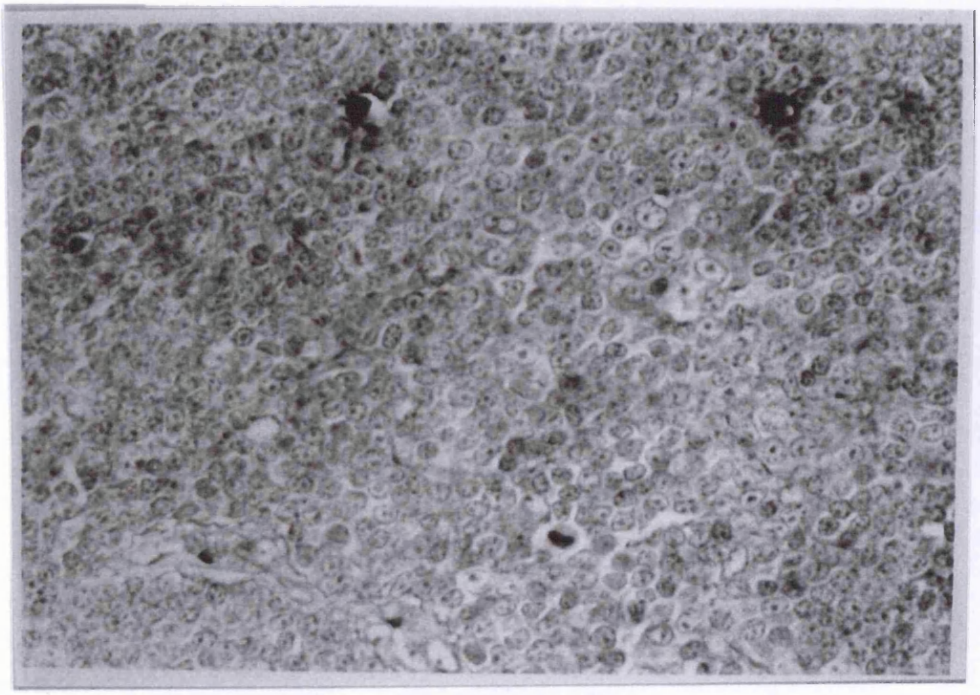

Plate III

x400 Mag.(H.P). End labelling technique (ISEL). Low grade non Hodgkin's lymphoma. Apoptotic sites are displayed by the black staining of the nuclei.

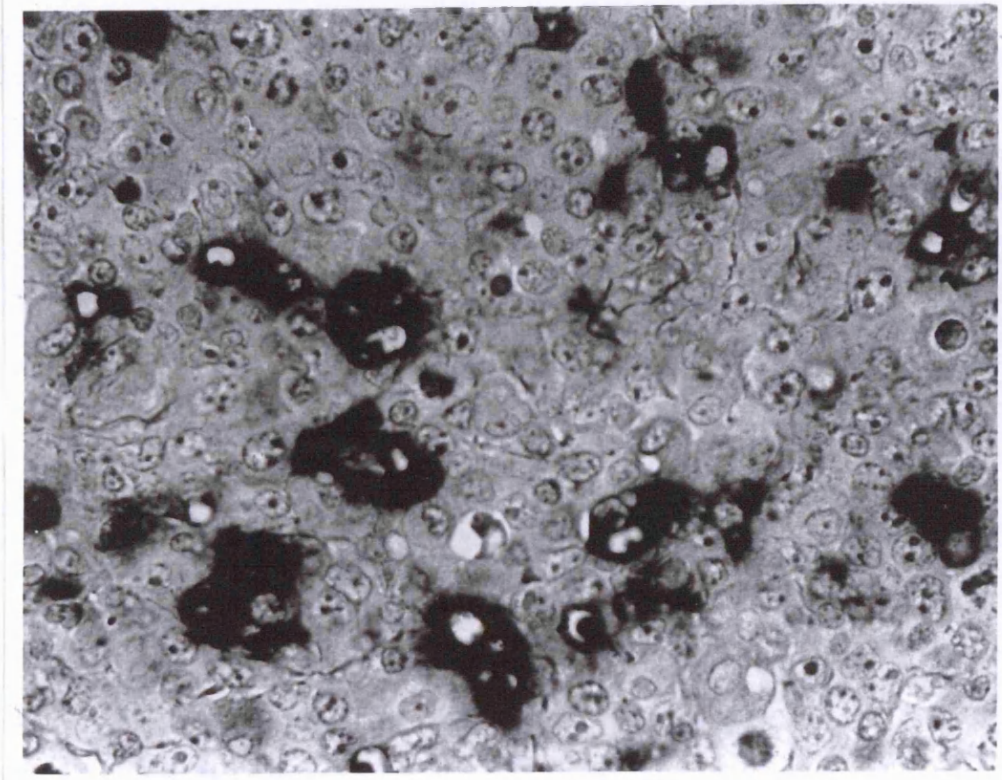

Plate IV

x400 End labelling technique (ISEL) .A high grade non Hodgkin's lymphoma with apoptotic activity displayed by black staining of the nuclei and also some macrophages.

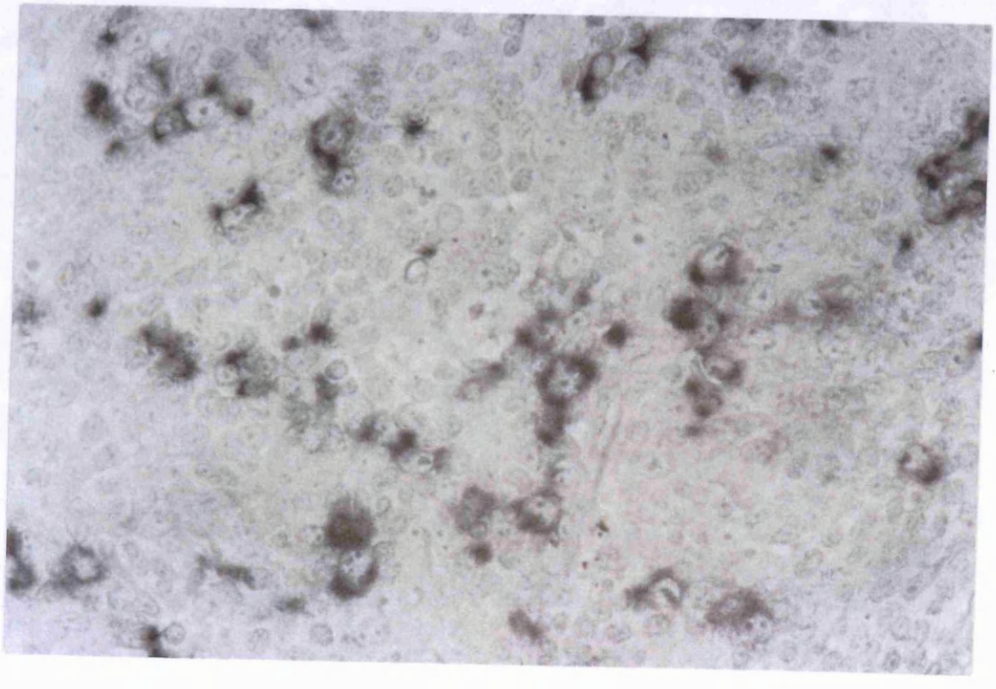

Plate V

x400 (HP) mRNA-Histone.
Low grade non Hodgkin's lymphoma. Black staining of the cytoplasm in the peri nuclear regions is evident.

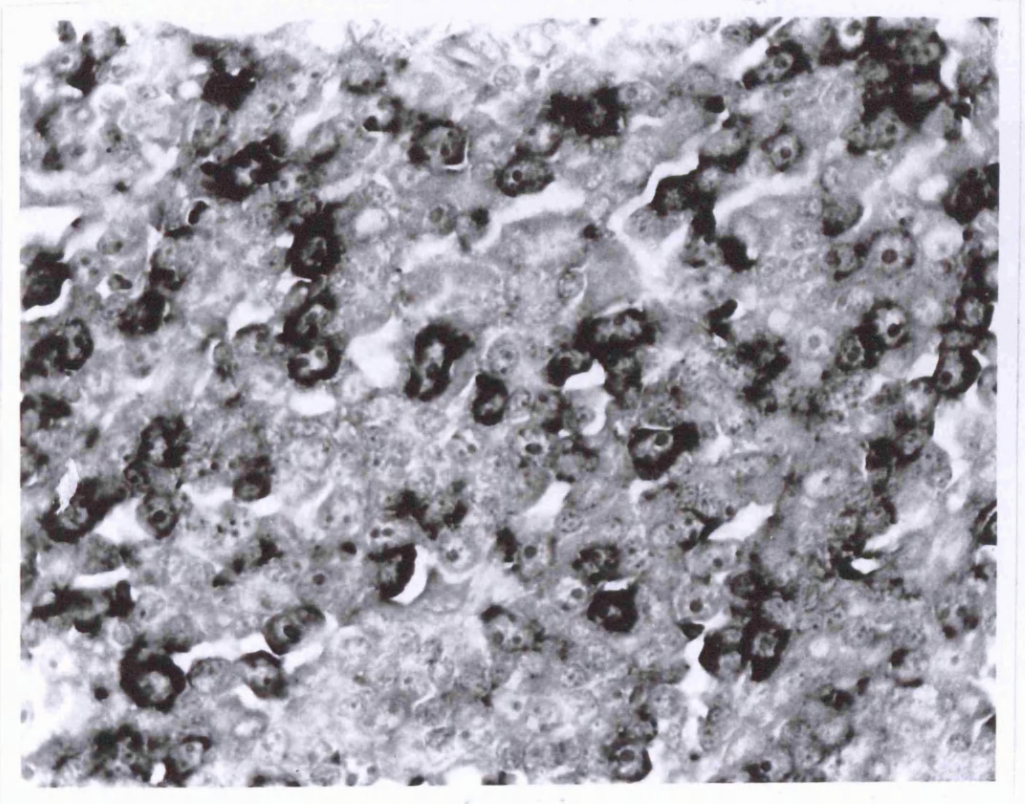

Plate VI

x400 (HP) mRNA Histone.
High grade non Hodgkin's lymphoma. Black staining of the cytoplasm in the perinuclear region is evident.

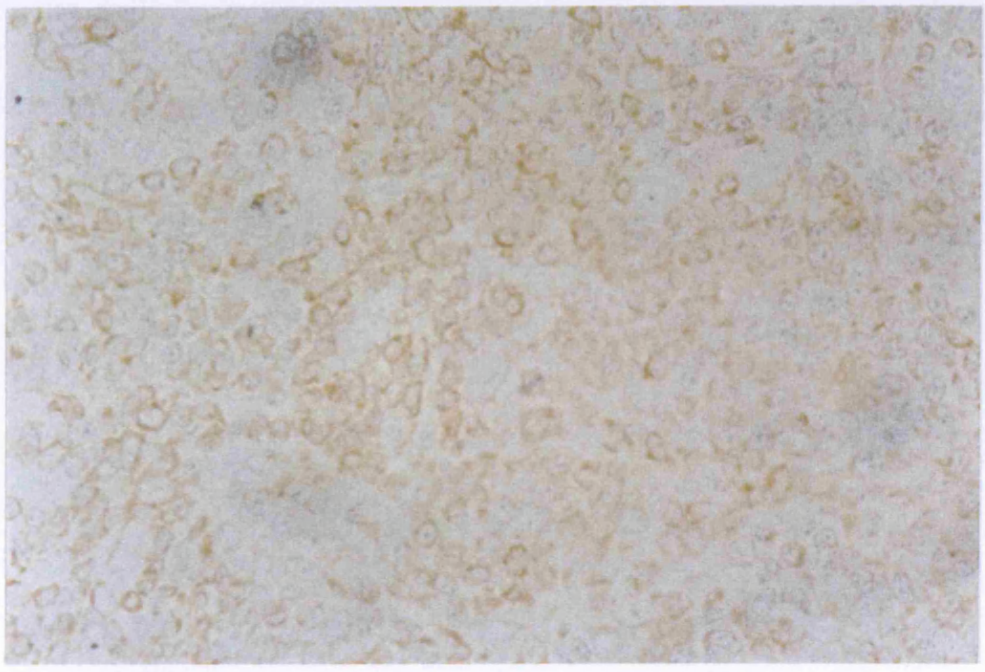

Plate VII

x400 (HP) Immunoperoxidase Bcl-2 staining .
Low grade non Hodgkin's lymphoma.
There is a brown staining of the cytoplasm around the nuclei.

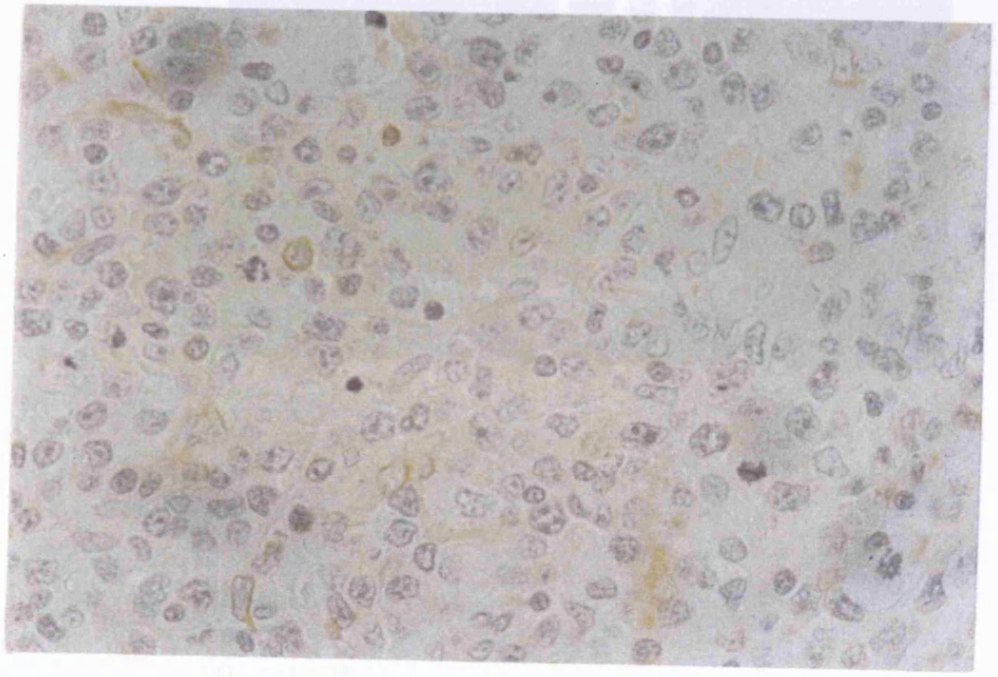

Plate VIII

x400 (HP) Immunoperoxidase Bcl-2 staining.
High grade non Hodgkin's lymphoma.
There is faint brown staining of the cytoplasm around the nuclei.

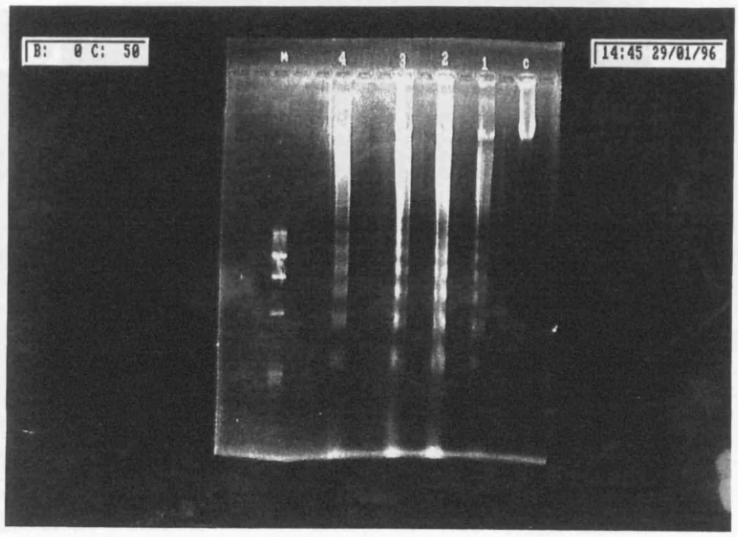

Plate XX

HL-60 (PCNA. expt.) treated with camphotecin
C - control DNA
Lane - 1 - 0.15nM - 2hr.
Lane - 2 - 0.15nM - 3hrs.
Lane - 3 - 0.3nM - 2hrs
Lane - 4 - 0.3nM - 3hrs.
M - Marker DNA (Physex/Hind III)

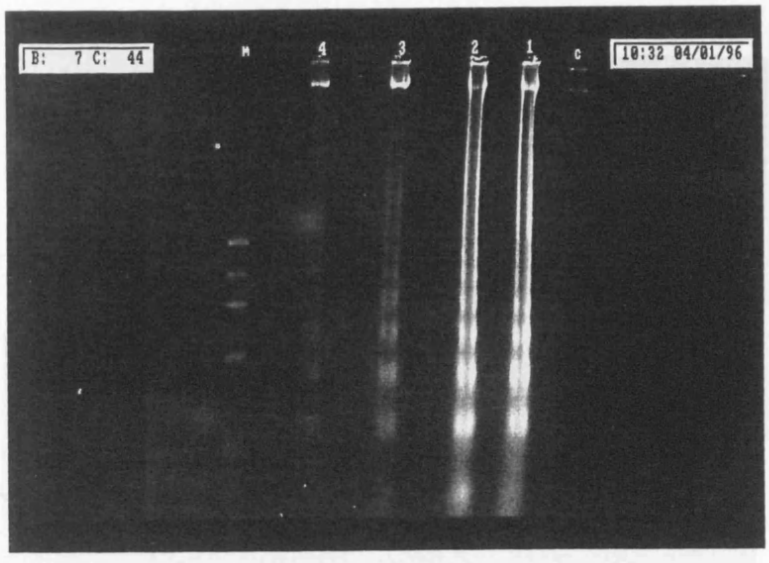

Plate XXI

Gel electrophoresis
DP-16-1 cell line (BrdU. expt.)treated with
30µgms Thapsigargin.
C - Control DNA
Lane - 1 -24hr. DNA
Lane -2 -8hr. DNA
Lane -3 - 6hr. DNA
Lane - 4 - 2hr. DNA.
M - Marker DNA (Physex/Hind III)

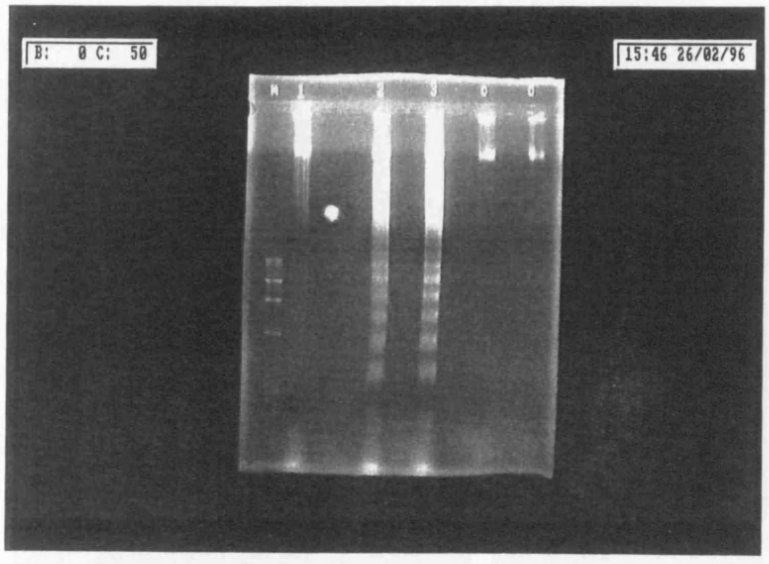

Plate XXII

Gel electrophoresis
HL-60 (BrdU.expt.) treated with 0.3µM Camphotecin .
C - Control DNA
Lane - 1 - 1hr. DNA
Lane - 2 - 2hr. DNA
Lane - 3 - 3hr. DNA
M - Marker DNA (Physex/Hind III)

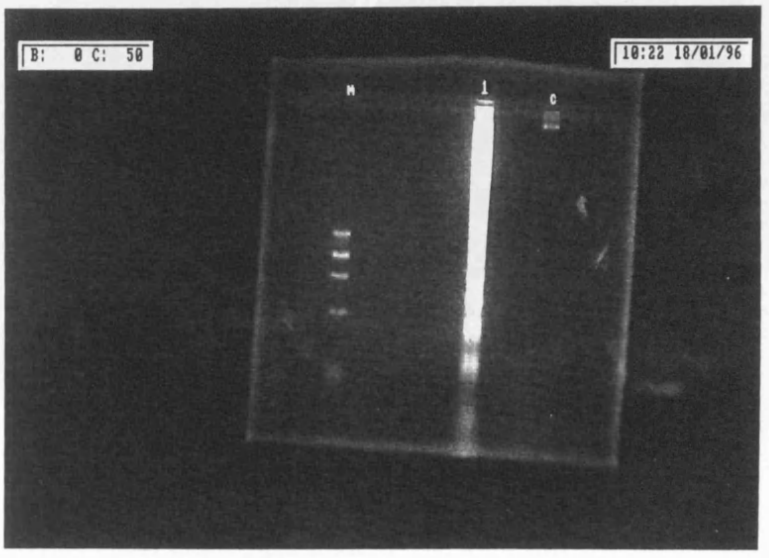

Plate XXIII

Gel electrophoresis
HL-60 cell line (c-myc. expt.)treated with Camphotecin
0.3µM - 3hrs .
C - Control DNA
Lane - 1 - treated DNA
M - Marker DNA(Physex/Hind III)

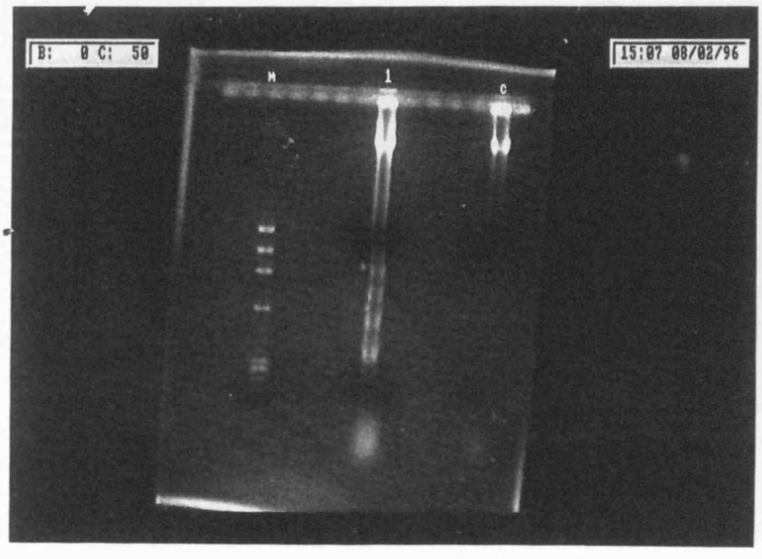

Plate XXIV

Gel electrophoresis
DP16-1 (c-myc. expt.) treated with 30µgms - Thapsigargin.
C - Control DNA
Lane -1 - treated sample DNA
M - Marker DNA (Physex/Hind III)

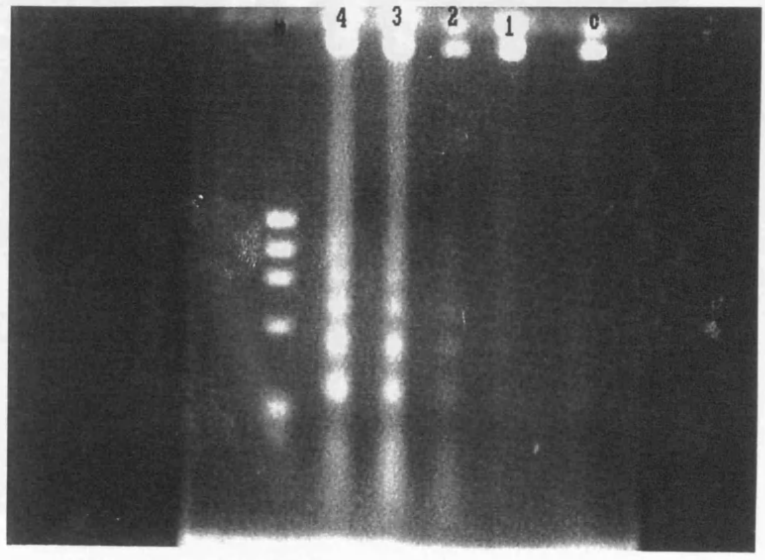

Plate XXV

Gel electrophoresis
C88- Cell line
C - Control DNA
Lane -1- 100 Rads- DNA
Lane -2- 200 Rads- DNA
Lane -3- 500 Rads-DNA
Lane -4- 1000 Rads -DNA
M - Marker DNA (Physex/Hind III)

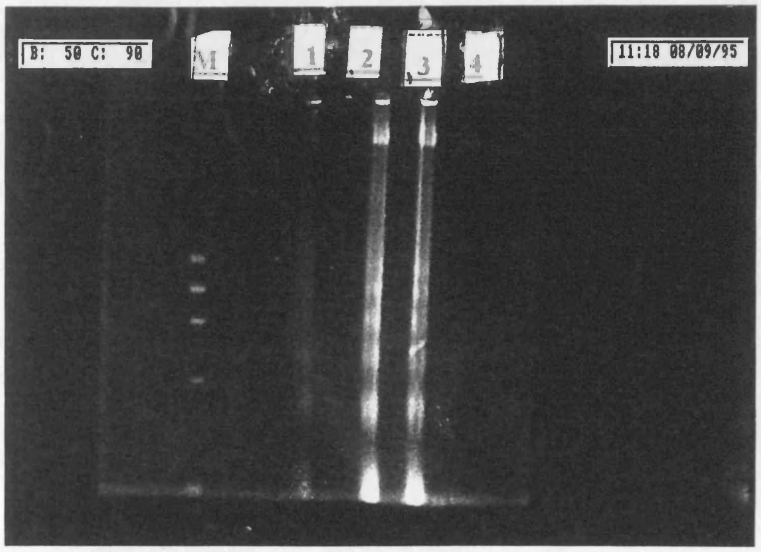

Plate XXVI

Gel electrophoresis
C88 cell line treated with 2% DMSO
for 24hrs. ,48hrs. and 72hrs.
C- Control - DNA
Lane -3- DNA - 24hrs.
Lane -2- DNA - 48hrs.
Lane -1- DNA - 72hrs.

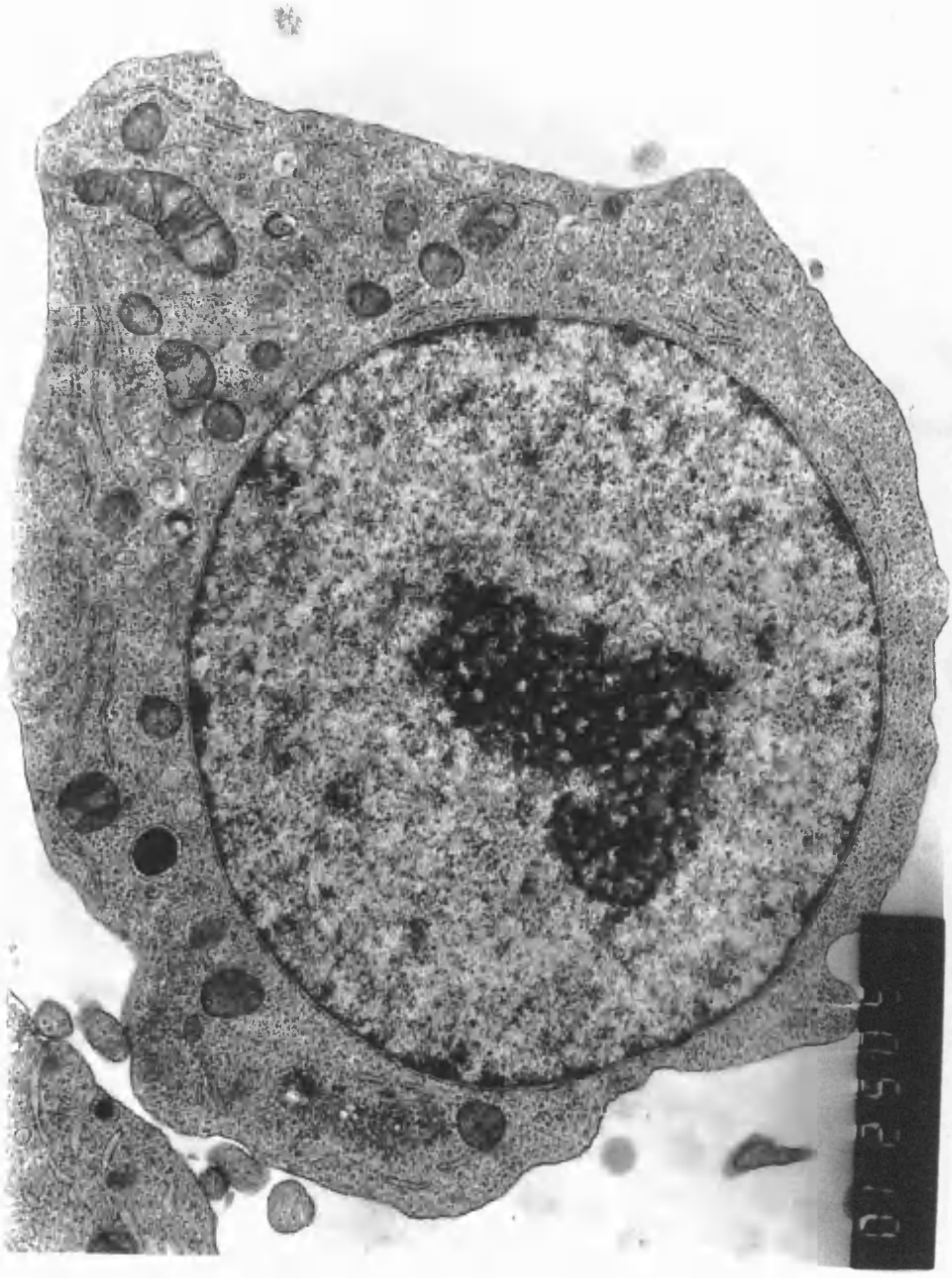

Plate XXVII

E.M. Photomicrograph Mag. x10,000.
HL60 cell line normal control.
The cell has a big nucleus and prominent nucleolus surrounded by cytoplasm containing many mitochondria.

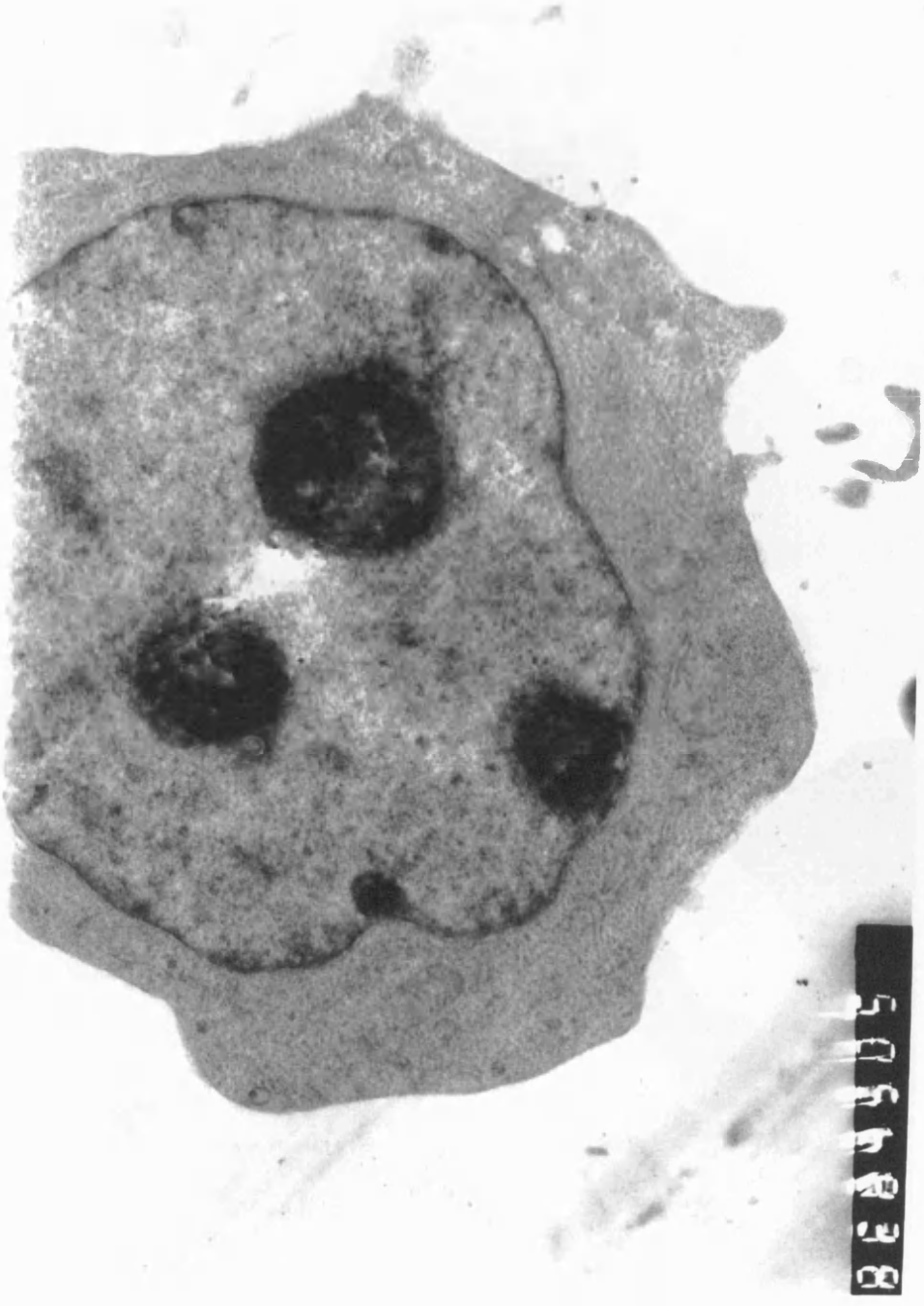

Plate XXVIII

E.M. Photomicrograph. Mag. x10,000.
HL-60 treated with 0.15μM Camphotecin- 3hrs.
There is fragmentation and condensation of nuclear chromatin.

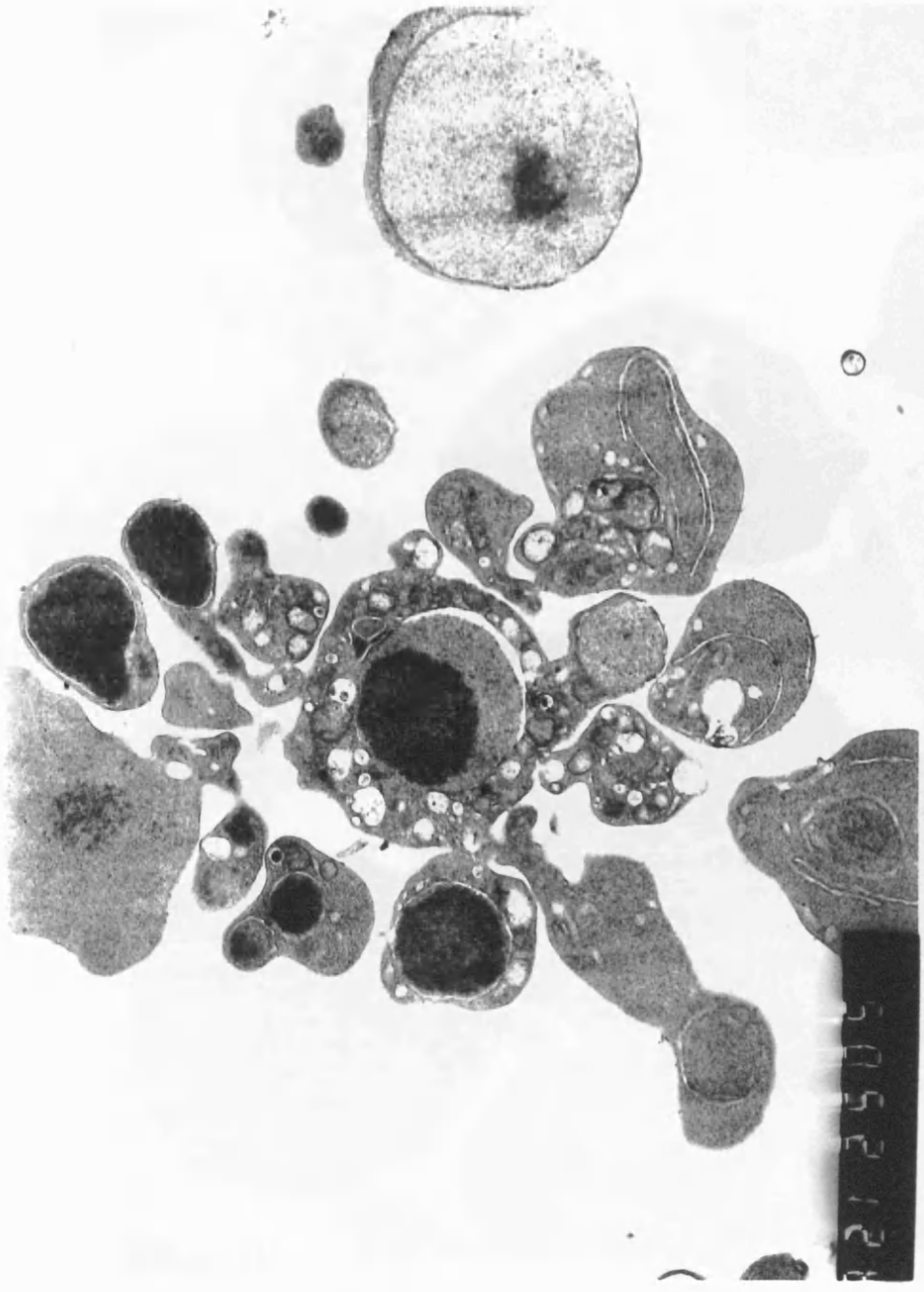

Plate XXIX

E.M. Photomicrograph. Mag. x10,000.
HL-60 treated with 0.15µM Camphotecin- 3hrs.
The late stages of apoptosis are displayed.
There is fragmentation of the nucleus into multiple apoptotic bodies surrounded by thin rims of cytoplasm.

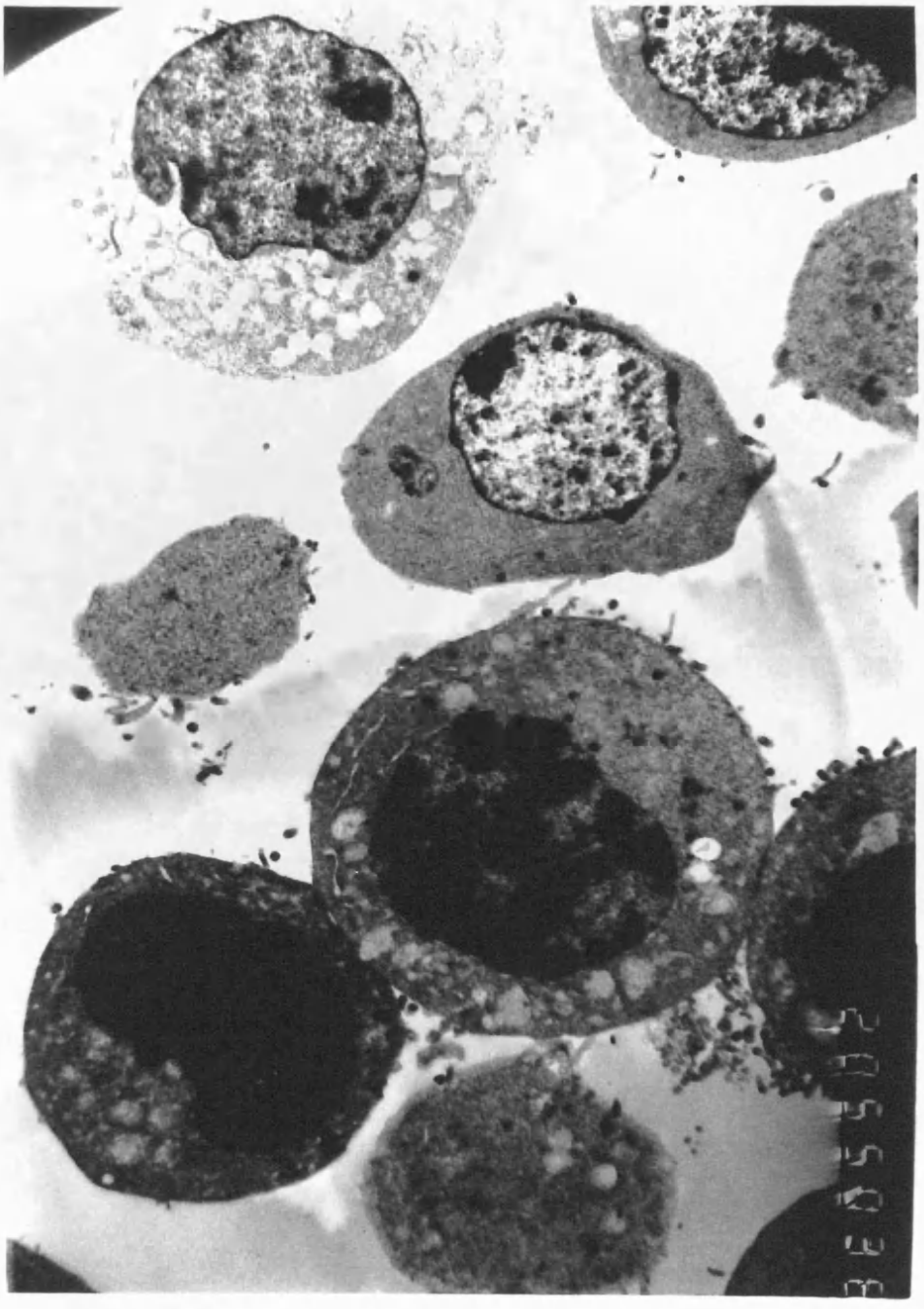

Plate XXX

E.M. Photomicrograph. Mag. x4,000.
Raji cell line- normal control.
The cells have indented nuclei with coarse chromatin surrounded by moderates amounts of cytoplasm.

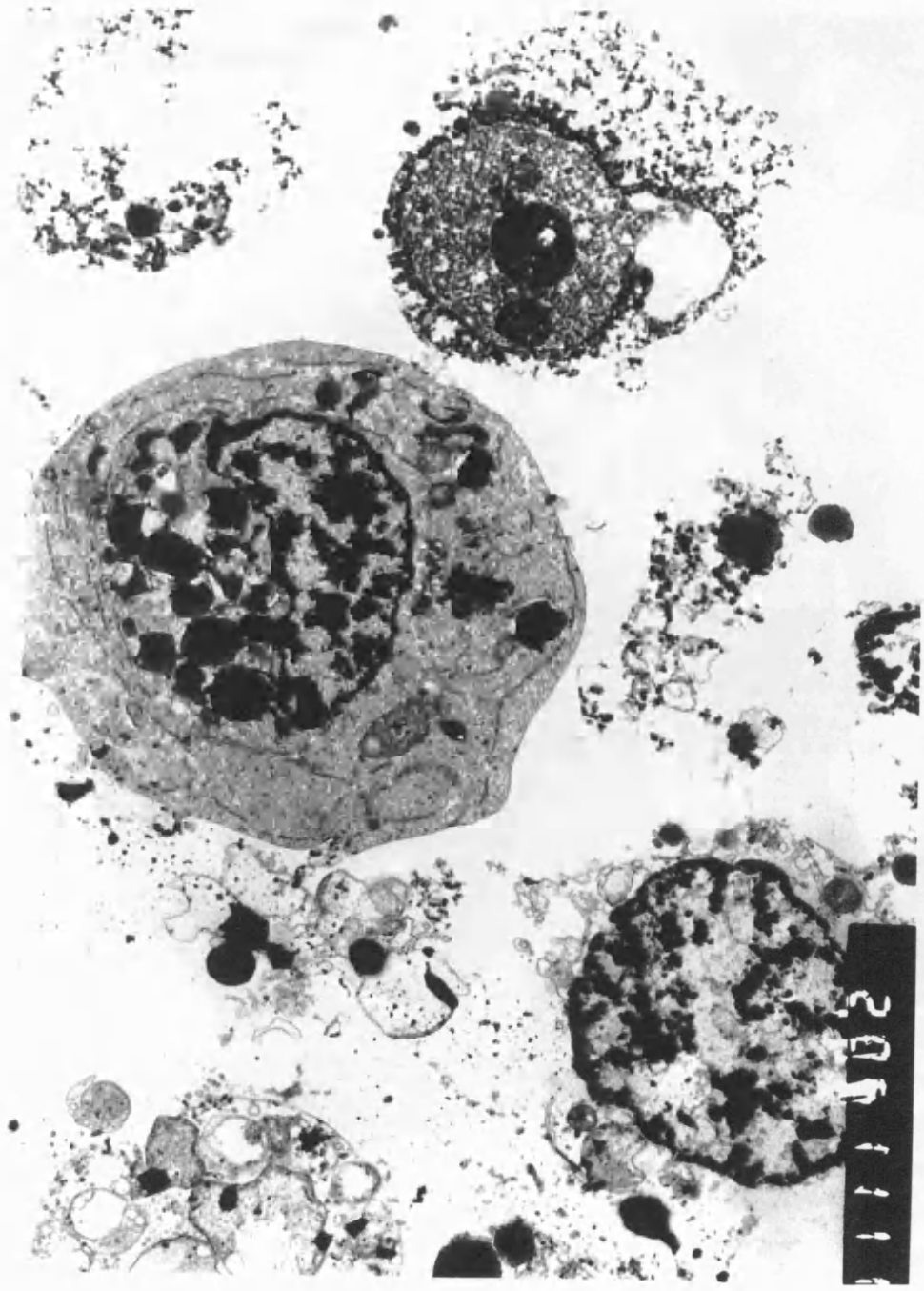

Plate XXXI

E.M. Photo micrograph. Mag. x4,000.
Raji treated with 250µgms Cycloheximide- 72hrs.
There is fragmentation and condensation of nuclear chromatin .Some cells show degeneration of cytoplasm.
Isolated apoptotic bodies are also evident.

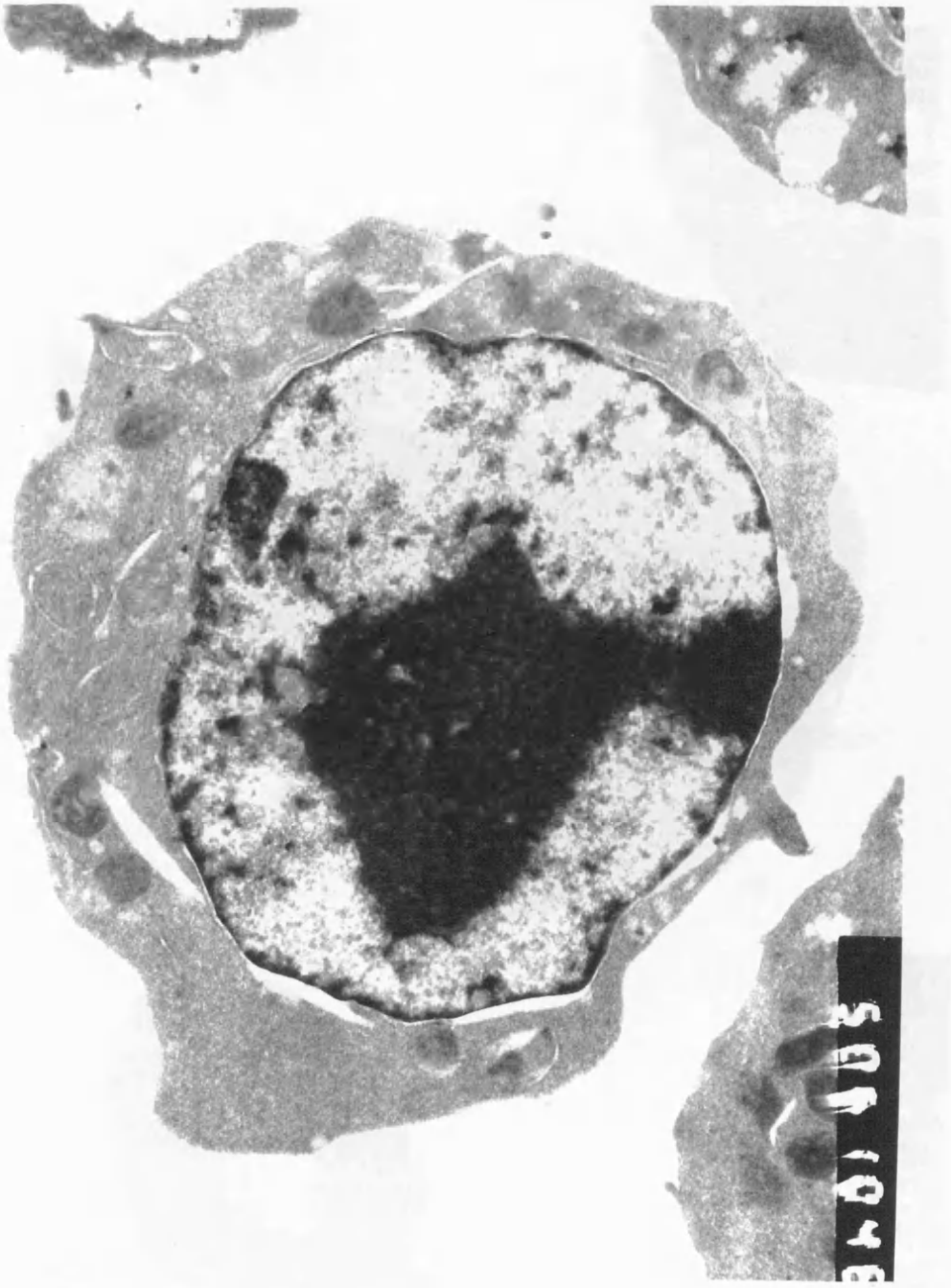

Plate XXXII

E.M. Photomicrograph. Mag. x10.000.
C88 cell line - normal control.
The cell has a big nucleus with a prominent nucleolus surrounded by small amounts of cytoplasm and containing many mitochondria.

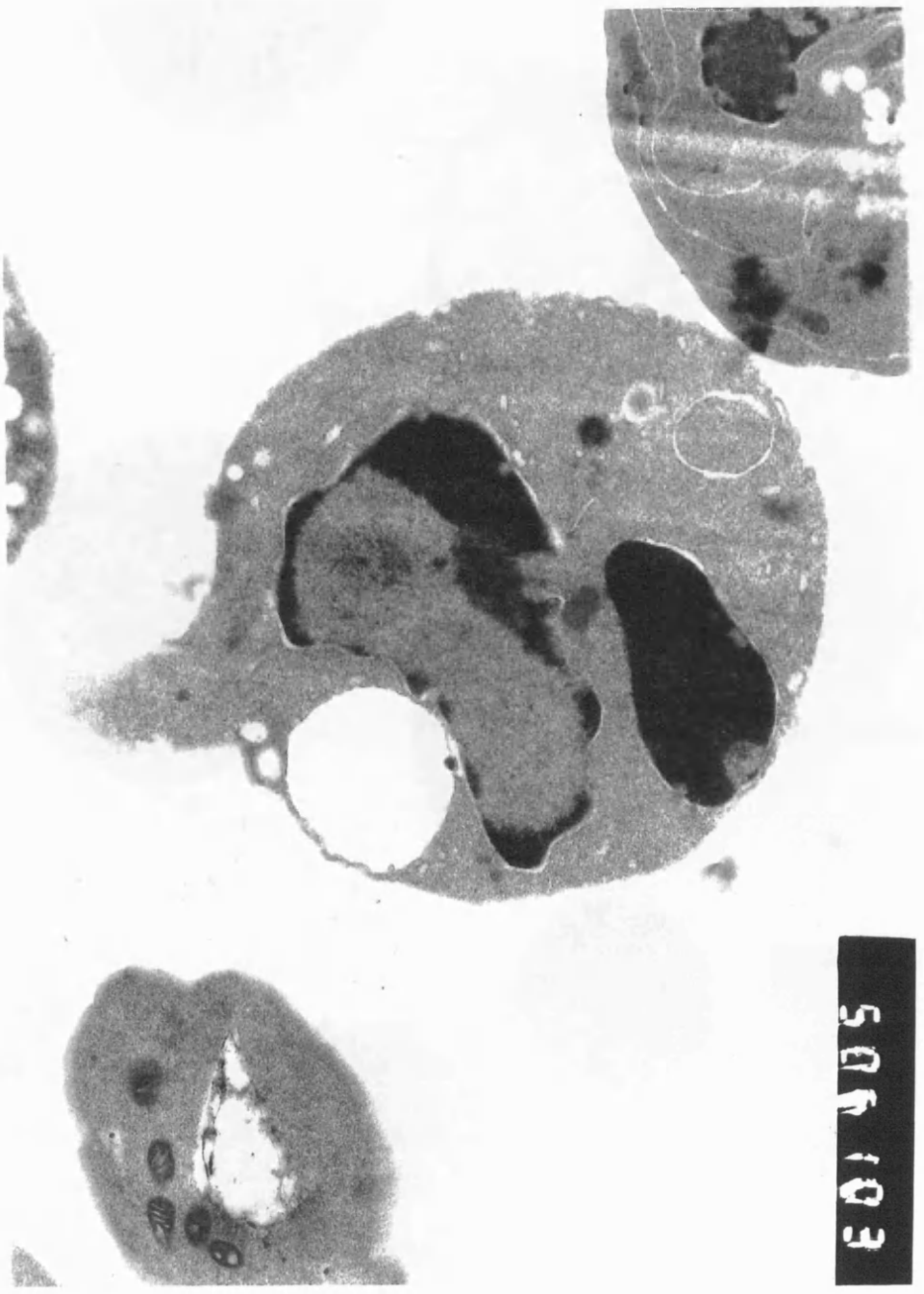

Plate XXXIII

E.M. Photomicrograph. Mag. x4,000.
C88 cell line treated with 30µgms Thapsigargin- 24hrs.
There is fragmentation and condensation of nuclear chromatin towards the perinuclear membrane.

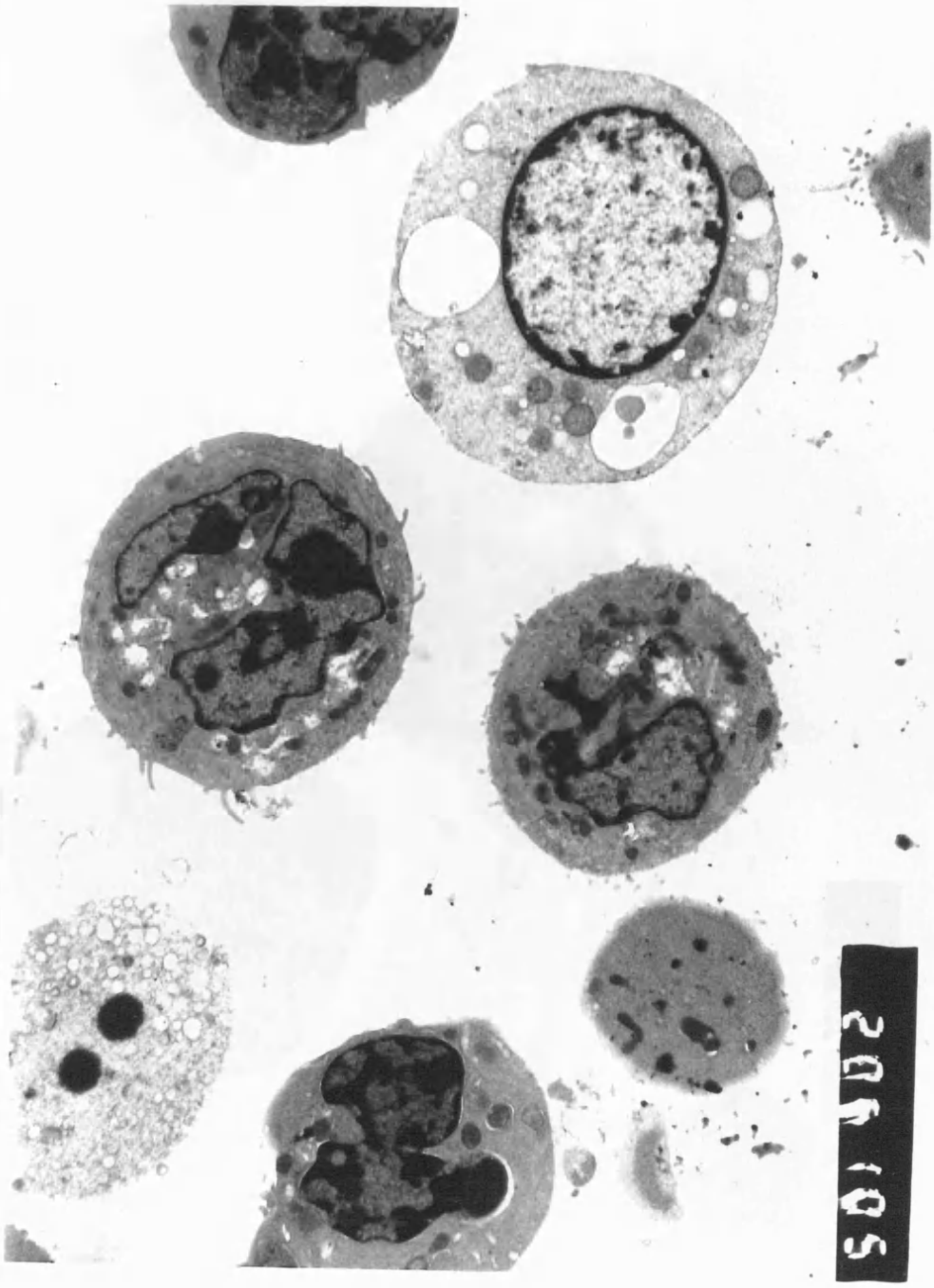

Plate XXXIV

E.M. Photomicrograph. Mag. x. 4,000.
DP16-1 cell line normal control.
cells show an open and in some cases lobed nuclei
by moderate amounts of cytoplasm. In places
the cytoplasm is vacuolated.

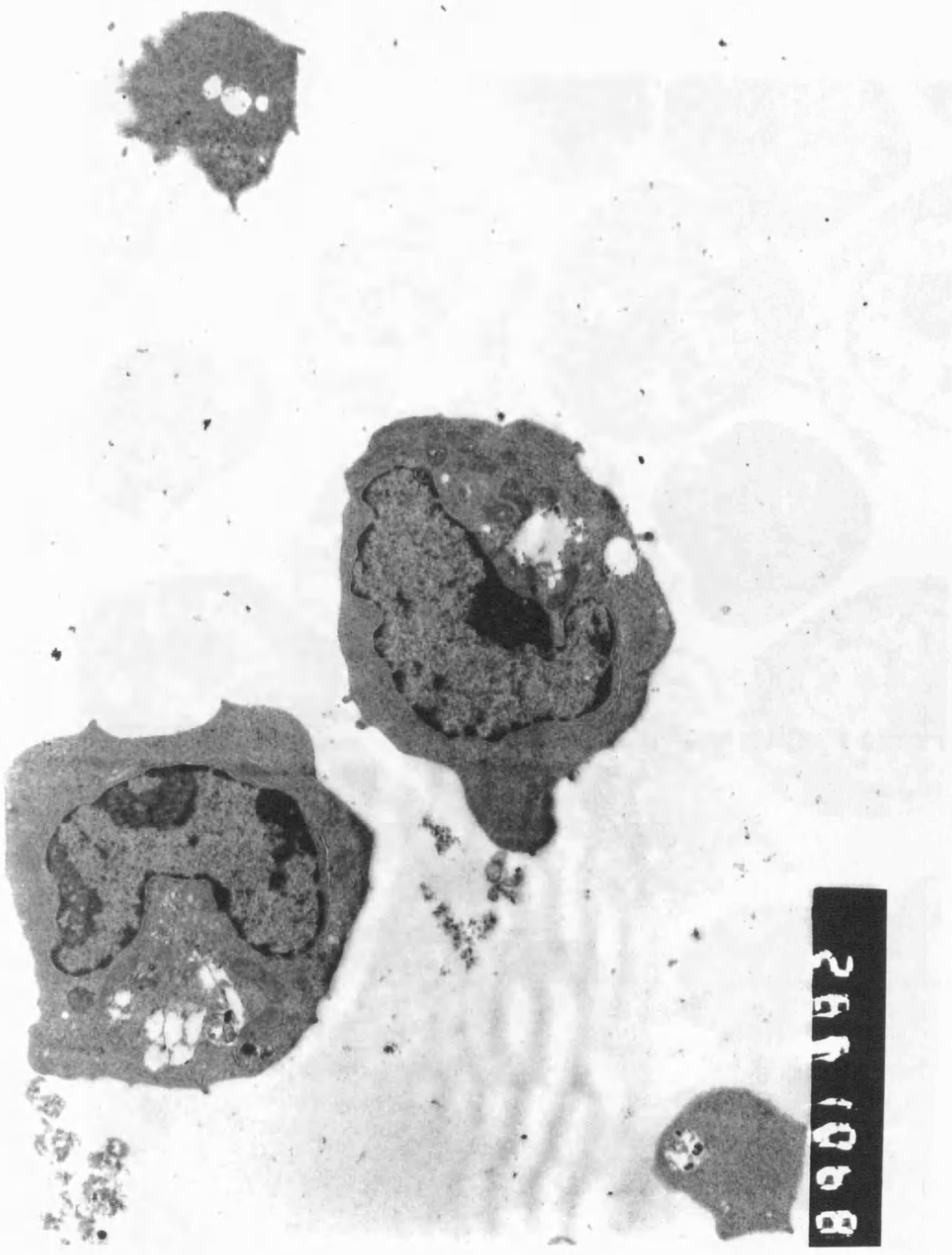

Plate XXXV

E.M. Photomicrograph. Mag. x5,200.
DP16-1 treated with 30µM Thapsigargin- 6hrs.
The nuclei show fragmentation and condensation of nuclear chromatin towards the peri nuclear membrane

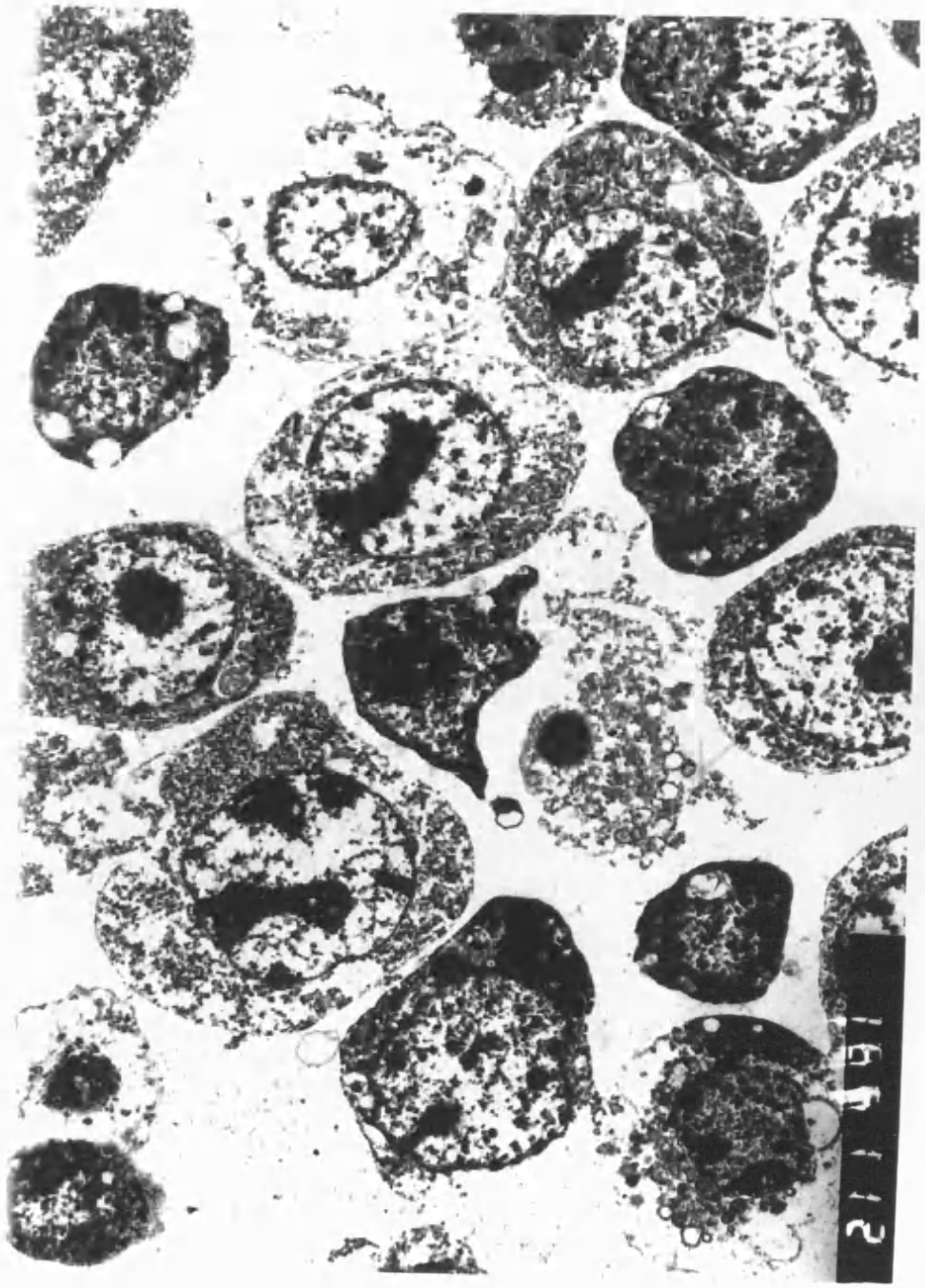

Plate XXXVI

E.M. Photomicrograph. Mag. x 3,200
C88 cell line exposed to 1000 Rads.-X-rays
There is widespread fragmentation and condensation of nuclear chromatin. There is also extensive vacuolation of the cytoplasm.

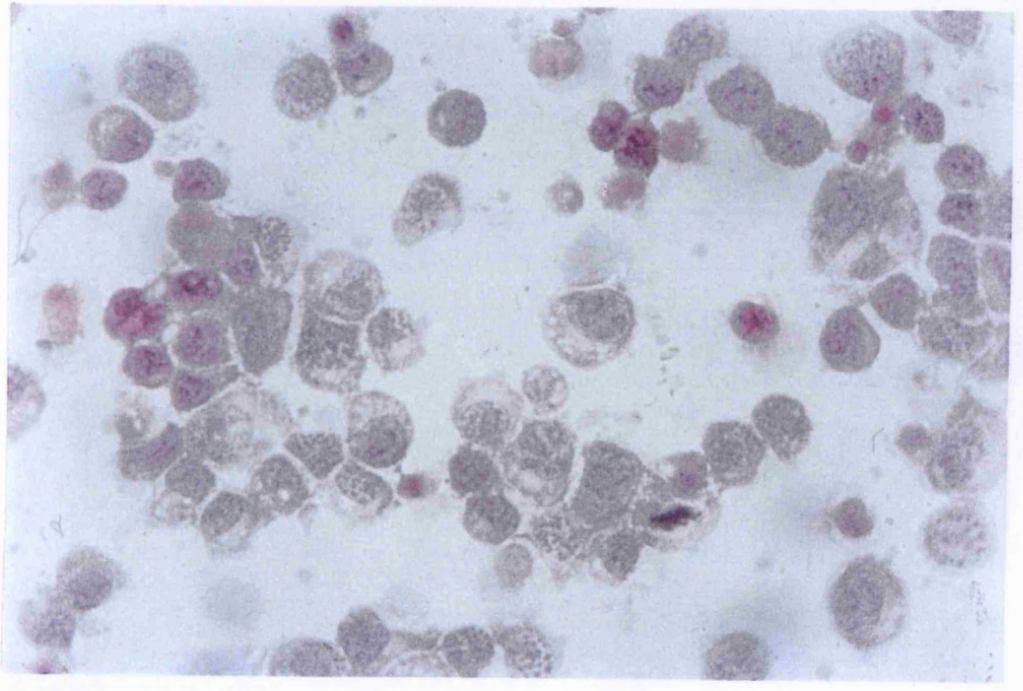

Plate XXXVII

Photomicrograph.(HE.) Mg. x400.
C88 cell line with cells having big nuclei
coarse chromatin and small amounts of cytoplasm.
an occasional mitotic figure is present.

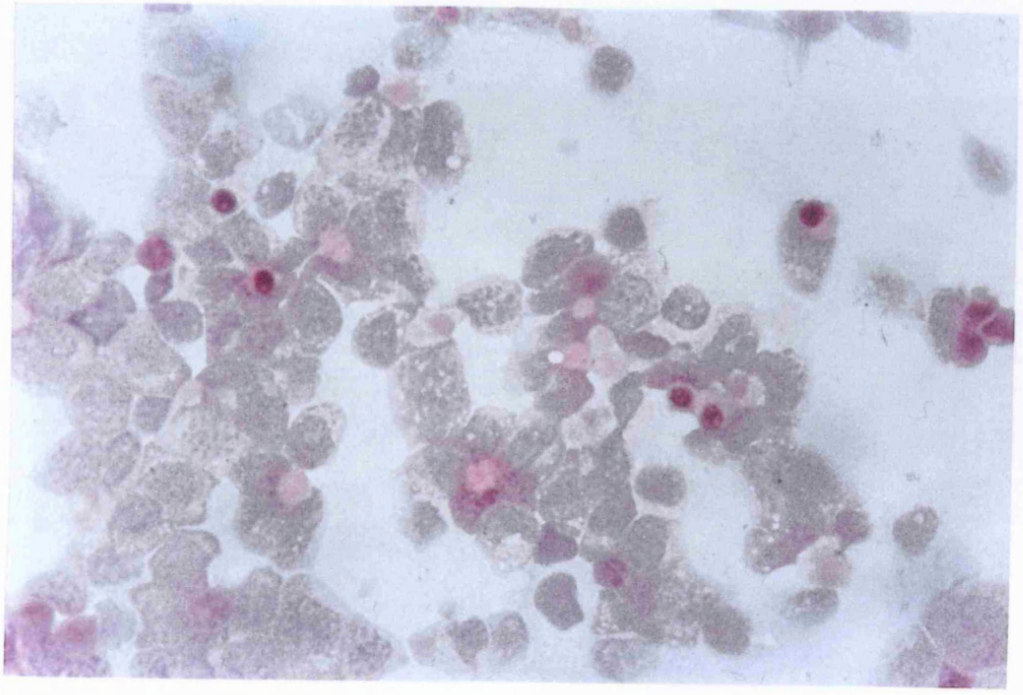

Plate XXXVIII

Photomicrograph. (HE.) Mag .x400.
C88 cell line treated with 2% DMSO -24hrs.
There is some differentiation towards the erythrocyte stage. some nucleated red blood cells are evident.

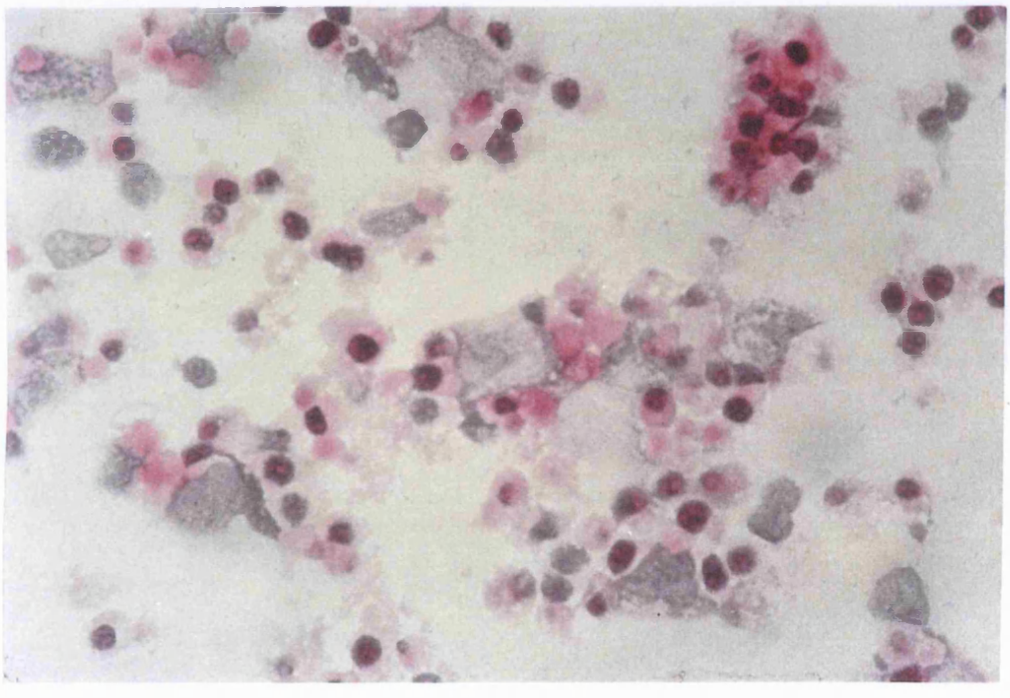

Plate XXXIX

Photomicrograph .(HE.) Mag. x400.
C88 cell line treated with 5% DMSO- 24hrs.
There is extensive differentiation with very many nucleated red blood cells.