HEAT SHOCK PROTEINS IN LEUKAEMIA CELL DIFFERENTIATION AND CELL DEATH

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SUMMARY

When HL60 cells were induced to differentiate to granulocyte-like cells with the agents N-methylformamide and tunicamycin at concentrations marginally below those which were cytotoxic, there was a decrease in the synthesis of the glucose-regulated proteins which preceded the expression of markers of a differentiated phenotype. There was a transient increase in the amount of hsp70 after 36 hours in NMF treated cells but in differentiated cells negligable amounts were detected. Inducers which were known to modulate hsp70 such as azetadine carboxylic acid did not induce differentiation suggesting early changes in the endoplasmic reticulum maybe involved in the commitment to terminal differentiation of HL60 cells. These changes in grp synthesis were not observed when K562 human chronic myelogenous leukemia cells were induced to differentiate to erythroid-like cells but there was a comparable increase in amounts of hsp70.

When cells were treated with concentrations of drugs which brought about a loss in cell viability there was an early increase in the amount of hsp70 protein in the absence of any increase in synthesis. HL60 cells were treated with NMF (225mM), Adriamycin (1μM), or CB3717 (5μM) and there was an increase in the amounts of hsp70, in the absence of any new synthesis, which preceded any loss of membrane integrity and any significant changes in cell cycle but was concomitant with a later loss in viability of >50% and a loss in proliferative potential. The amounts of hsp70 in the cell after treatment with any of the drugs was comparable to that obtained after a heat shock.

Following a heat shock hsp70 was translocated from the cytoplasm to the nucleus, but treatment with toxic concentrations of drug caused hsp70 to remain localised in the cytoplasm. Changes in hsp70 turn-over was observed after a heat shock compared to NMF-treated cells. Morphological studies suggested that cells that had been treated with NMF and CB3717 were undergoing necrosis whereas the Adriamycin cells showed characteristics that were indicative of apoptosis. The data supports the hypothesis that an increase in amounts of hsp70 is an early marker of cell death.

Key words: cell death, differentiation, HL60, K562, stress proteins.

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LIST OF ABBREVIATIONS.

AMPS	Ammonium persulphate
ara-C	Cytosine arabinoside
ATP	Adenosine triphosphate
BFU	Burst-forming unit
Bis	Bisacrylamide
CFU	Colony-forming unit
CSF	Colony stimulating factor
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene-diamine-tetra-acetic acid
FACS	Fluorescence-activated cell sorting
GM-CSF	Granulocyte macrophage colony stimulaing factor
grp	Glucose-regulated protein
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonate
HPLC	High performance liquid chromatography
HSE	Heat shock element
hsp	Heat shock protein
HSTF	Heat shock transcription factor
IL-2	Interleukin-2
IL-3	Interleukin-3
MEL	Mouse erythroleukemia cells
mRNA	Messenger ribonucleic acid
Mw	Molecular weight
NBT	Nitroblue tetrazolium
NMF	N-methylformamide

PBS	Phosphate-buffered saline
PPO	2,5 diphenyloxazole
RPMI	Roswell Park Memorial Institute medium
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
TPA	12-O-tetradecanoylphorbol-13-acetate
Tris	Tris(hydroxymethyl)aminomethane base

CHAPTER ONE

INTRODUCTION

1.1 General introduction

Classically, many anticancer drugs rely on mitotic cell death of proliferating cells as a means of removing malignant cells from the body; the excessive nuclear damage caused by the cytotoxic agent prevents the cells from dividing further and they will go on to die by apoptosis or necrosis (see figure 1.1). But often, due to the inability of drugs to distinguish between normal and tumourous cells, normal cells are damaged by the cytotoxic chemotherapy. In particular the gastrointestinal, epidermal and myelopoietic tissues are affected and the dose-limiting consideration for chemotherapy is often haemopoietic stem cell toxicity. Long term complications of chemotherapy may also include skeletal alterations, testicular and ovarian suppression and the development of new tumours as many of the highly cytotoxic drugs used are mutagenic and carcinogenic themselves.

In attempts to overcome these and other problems associated with chemotherapy new strategies are required (Berger 1986). One such strategy has been the development of agents which, rather than being cytotoxic to cells, induce terminal differentiation. This results in mature cells which are no longer able to proliferate (Sartorelli 1985). It is thought that this form of therapy will reduce many of the problems of toxicity to normal cells.

This thesis aims to investigate some of the factors involved in cell differentiation and cytotoxicity. In order

Figure 1.1 Abolition of mitotic capacity in a clonogenic cell

(from Allen et al 1989)
A clonogenic cell can be eliminated by mitotic cell death, or by differentiation. Both these processes will eventually result in either apoptosis or necrosis.
1. mitotic death
2. differentiation
3. apoptosis
4. necrosis

to design new drugs (and to understand the mechanism of action of some existing drugs) which may be able to induce differentiation and ultimately cause tumour cells to die, more needs to be known about the processes that operate during differentiation and cell death (necrosis and apoptosis).

1.2 Cancer is a disease of cell differentiation

Differentiation is the process by which cells in a developing organism achieve their specific set of structural and functional characteristics. Under normal circumstances a subset of cells in a tissue differentiate into the functional cells of that tissue and cannot normally divide again. A limited number of stem cells will proliferate in a controlled manner, some of the progeny remaining in the stem cell pool, while others undergo further divisions resulting in more highly differentiated cells. Usually this balance between self-renewal and differentiation is controlled so that a tissue remains in a steady-state balance with sufficient newly differentiated cells being produced to replace those mature cells that are eventually lost through senescence (Sachs 1986). In 1970 Pierce proposed that the origin of malignant tumours were normal tissue stem cells that possess the ability to replicate. Cells become altered by the action of, for example, a carcinogen such that the genes controlling cell proliferation are expressed and the genes controlling

differentiation are either not expressed or are expressed imperfectly; the result is a partially differentiated cell with unlimited proliferative potential. This abnormality is not however an irreversible or complete blockage of differentiation. Evidence to support this idea of a cancer cell originating from a stem cell was provided in 1978 by Graf et al. When chicken bone marrow was transformed with avian erythroblastosis virus (ABV) rapidly growing cells with the characteristics of early progenitors of the erythroid lineage were produced. These cells, burst-forming units-erythroid, differentiated into colony-forming units-erythroid, but then arrested in the differentiation pathway and did not go on to form mature red blood cells.

Potter (1969) explained the generation of cancer by the phrase "oncogenecy is blocked ontogeny". In other words malignancy arises due to a blockage in the normal pathway of development and differentiation. Blockages in differentiation could occur at one or more steps in the differentiation pathway. Once a cell has differentiated it will have a limited life span and then die by apoptosis or necrosis (figure 1.1).

1.3 Differentiation

The haemopoietic system provides a good experimental model as the differentiation of both normal and malignant cells can be studied.

1.3.1 The haemopoietic system

In adults, the process of haemopoiesis occurs largely in the bone marrow. The mature cells of the haemopoietic system show a wide diversity of function with all of the different cell types being derived from several types of progenitor cell. All the progenitor cells are derived from the same pluripotent stem cell (figure 1.2). The lymphocytes, erythrocytes, neutrophils, eosinophils, basophils and platelets, the mature cells of the haemopoietic system have a limited life span and only the lymphocytes and monocytes are capable of proliferation.

In 1979 Schofield demonstrated that if the haemopoietic system of an animal was impaired by radiation or chemicals, normal functioning could be re-established if the animal was infused with normal haemopoeitic cells from a bone marrow. Further experiments went on to demonstrate that relatively few cells (approximately 0.01% of the total) were required to fully reconstitute the haemopoietic system. In order to prove that these mature cells in the radiation chimeras were derived from stem cells Abramson et al (1979) used unique radiation induced chromosome markers and showed that the precursor cells from several lineages carried the same karyotype marker. It was however as early as 1961 that Till and MuCulloch had recognised these pluripotent stem cells when they showed that when haemopoietic cells were injected into potentially lethally irradiated mice some of the cells

Figure 1.2 Stem and functional cells of the haemopoietic system

(From Ruddon, 1987 p151)
CFU-L-M: colony-forming unit-lymphoid-myeloid
CFU-S: colony-forming unit-spleen; CFU-M: colony-forming unit- magakaryocyte; CFU-GM: colony-forming unit-granulocyte and monocytic; BFU-E: burst-forming unit-erythroid; CFU-E colony-forming unit-erythroid

migrated to the spleen where they proliferated to form discrete nodules. These nodules were clonal, that is derived from a single cell, and contained multiple cell lineages; they became known as the spleen colony forming cells (CFU-S) (Moore et al 1972). The progenitor cells can also be recognised by their ability to undergo proliferation in soft agar to produce colonies of mature cells of the appropriate lineage. The nomenclature for the progenitor cells is based upon the mature cells which they produce.

The stem cells, which are known as lymphoid and myeloid colony-forming units (CFU-L-M), give rise to CFU-S and the lymphoid precursors. The CFU-S in turn can differentiate to produce CFU-C, also known as CFU-GM (granulocytic and monocytic), CFU-M (megakaryocytic), and BFU-E (erythroid). The BFU-E divides to produce CFU-E cells which proliferate and differentiate to mature erythroid cells. The monocytes are derived from the precursor cells which are known as the myeloblasts, but this process is as yet undefined. The monocytic cells are then released into the blood stream and subsequently they enter the tissues as macrophages. The maturation for granulocytes is much more clear with the myeloblasts dividing to produce promyelocytes which then divide to produce myelocytes which subsequently mature and pass into the blood stream as granulocytes (either neutrophils, eosinophils or basophils). Once in the blood stream the mature cells circulate for different amounts of time depending on the cell type, before being eliminated

(Bessis 1973); a recent paper by Savill et al (1989) has shown that aging neutrophils undergo apoptosis and are eventually recognised as intact cells by macrophages in vitro (see section 1.4).

The maintenance of haemopoiesis is a balance between proliferation and development. There is evidence to suggest that the direction of differentiation of stem cells is both stochastic and under the influence of such factors as growth regulators and the extracellular matrix.

Studies by Metcalf et al. (1983) have indicated that the direction of differentiation is influenced by the presence and concentration of specific haemopoietic growth factors. Growth factors are required throughout the development of stem cells in vitro, allowing for their survival, proliferation and differentiation (Dexter et al 1987). Since the methods for growing blood cells in culture (Bradley et al 1966, and Pluznick et al 1986) four "granulocyte-macrophage" colony stimulating factors (CSFs) have been isolated and many have been molecularly cloned and so purified to homogenicity; these four growth factors are progressively restricted in their activities and in their target cells. The first factor to be identified stimulated the production of granulocytes and macrophages from murine spleen and marrow cells, and it was named granulocyte-macrophage inducer or CSF (Metcalf et al 1967). Soon after the discovery of this molecule a second factor was identified that stimulated the production of macrophage cells in

preference to granulocyte colonies. Since this factor was also a colony stimulating factor a different nomenclature had to be decided upon; the first molecule was renamed GM-CSF and the second M-CSF (Cline et al 1979). The third factor was isolated by two groups, from the conditioned medium from human placenta (Nichola et al 1979) and from the serum of mice treated with endotoxin (Burgess et al 1980a); this factor was found to be specific for granulocytic cells and was hence designated G-CSF (Nichola et al 1983). The fourth haemopoietic growth factor is also capable of stimulating the production of granulocytes and macrophages and it was identified by several laboratories who were working on the different properties of the conditioned media of lectin stimulated murine spleen cells (Burgess et al 1980b) and the conditioned medium from a myeloid cell line WEH13B(D-) (Bazill et al 1983). Unlike the previously described factors, this factor, termed interleukin 3 (IL-3), was demonstrated to be lineage independent in its action promoting growth and differentiation of all the different myeloid progenitor cells. It was also found to be active against the pluripotent CFU-S, and the multipotent CFC-Mix stem cells facilitating the self-renewal of both. It must be made clear however that the precise roles of IL-3, GM-CSF, M-CSF and G-CSF in in vivo myelopoiesis is in doubt since there is not enough information about their producer cells and the systems controlling their production.

Erythropoietin, a glycoprotein that acts at an

intermediate stem cell BFU-E stimulates the proliferation and differentiation of erythroid cells (Goldwasser 1976). In contrast to the CSFs, the major sites of production have been identified, with the majority being produced in the kidney. Production of this growth factor occurs in response to tissue hypoxia and this is detected by the kidney in a feedback loop control system.

A variety of other factors that are responsible for the growth and development of eosinophils and megakaryocytes have also been described, but only the growth factor IL-4, which results in the differentiation of eosinophils has been defined (O'Garra et al 1986). There is also a set of proteins that exert their effects on myeloid cells - the differentiation factors or MGI-2 (Sachs and Lotem 1984). Cells cultured with CSF endoeneously produce MGI-2 so that cells stimulated with CSF will subsequently differentiate in response to MGI-2. Cells express receptors for different growth factors at different stages of differentiation and some factors may, at certain stages, render cells responsive or unresponsive to other factors (Caracciolo et al 1989).

A couple of recent publications have suggested that growth factors may promote cell survival by preventing haemopoitic cells from undergoing apoptosis (section 1.4). Withdrawal of IL-3, GM-CSF or G-CSF from haemopoietic precursor cells results in their death by apoptosis (as determined by double-stranded cleavage of DNA into

internucleosomal fragments and on morphological grounds) (Williams et al 1990). Erythropoietin-dependent erythroid progenitor cells will also undergo apoptosis if deprived of erythropoietin; DNA cleavage was observed 2 to 4 hours after withdrawal and by 16 hours the cells had begun to die (Koury et al 1990).

An inhibitory haemopoietic factor has been found in medium conditioned by marrow stromal cells. This factor was first identified on the basis of its ability to decrease the population of CFU-S cells that were undergoing DNA synthesis and it was subsequently shown to be able to prevent the entry into S phase of CFU-S cells that had been stimulated with cell-cycle activators (Lord et al 1977). The effects of this factor have been shown to be non-toxic, reversible and dose-dependent; its inhibitory effects are however ineffective against the lineage-restricted progenitor cells.

The local environment is also important in determining which pathway of differentiation the progeny of the pluripotent stem cell will take. Observations that the majority of the erythroid cells that were present in the spleen of an irradiated mouse after injection of donor marrow cells were in the red pulp whereas the granulocytic colonies were isolated along the trabecular or in the subcapsular part of the spleen, and megakaryocytic colonies were usually below the spleenic capsule led to the conclusion that the location of the colony-forming cells does, to some extent, determine their pathway of differentiation. In terms of in vitro

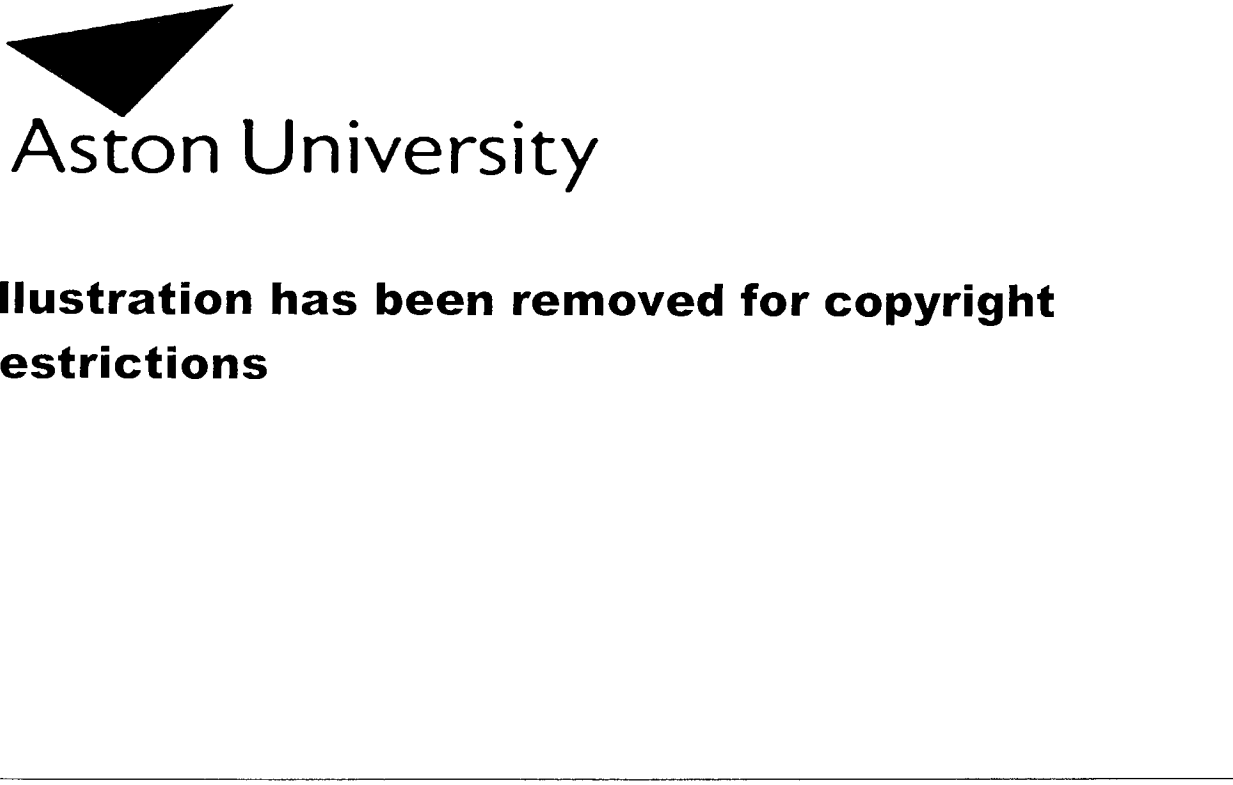
techniques that have been used to demonstrate the role of the stromal cell network both long term (where haemopoiesis can be maintained for several months) and short term marrow cultures have proved useful (Dexter et al 1977). These systems have been used to demonstrate that the marrow stromal cells provide the necessary extracellular matrix and growth factors essential for the establishment of haemopoiesis since isolated cells grown in soft-agar systems will die without the addition of growth factors. It is also apparent that the production of growth factors is under strict control since a balance is maintained between stem cell self-renewal/differentiation and growth and development of the committed progenitor cells for several months. Other points of note that have arisen from long-term marrow studies are that the haemopoietic cells must be in direct contact with the stromal cells for their survival (it is thought that the growth factors may be surface bound molecules) and that discrete stromal cells show characteristic interactions with the different maturing myeloid cells and with each other.

1.3.2 Haemopoiesis and leukemia

The process of leukemia results from the interruption of normal bone marrow cell differentiation (figure 1.3). The immature cells fail to differentiate and continue to proliferate resulting in immature blast cells in the circulating blood.

Figure 1.3 Myeloid differentiation demonstrating how leukaemia may arise

(from Ruddon 1987,p172)
Interaction of leukaemogenic agent with an immature cell leads to the production of an altered cell that fails to undergo a normal pattern of differentiation and retains its proliferative capacity.



Leukemias can be classified as acute, which evolve and progress very quickly or chronic where the progression is much slower; cell differentiation is not as seriously impaired in chronic leukemias as in acute cases. Leukemias are also futher classified according to their cell type, the most common being lymphoid and myeloid (Ruddon 1987).

Many cell lines have been derived from leukemias and these will proliferate *in vitro*, and many can be induced to terminally differentiate *in vitro* so that the cells take on the characteristics of mature, non-proliferating peripheral blood cells.

1.3.3 The HL60 cell line

The HL60 cell line was derived in 1977 from the peripheral blood leukocytes of a female patient diagnosed as having acute promyelocytic leukemia (FAB class M3) (Collins *et al* 1977), although a recent re-evaluation of the original patient specimens has indicated that FAB class M2 (acute myeloblastic leukemia with differentiation) would have been a more accurate description of the state of the disease (Dalton *et al* 1988). The culture was established using conditioned medium that had been taken from six different monolayer cultures of various first trimester and early second trimester foetal organs and this resulted in the development of a growth factor independent immortal cell line with distinct characteristics (Gallagher *et al* 1979). In response

to certain chemicals and pharmacological agents HL60s will cease proliferation and undergo virtually complete differentiation along the granulocyte/monocyte/macrophage pathway. HL60 cells undergo continuous proliferation in suspension culture with a doubling time of between 20 and 45 hours, depending on the subline.

HL60 cells have the morphology of myeloblastic/promyelocytic cells: large blast-like cells with characteristically large, rounded nuclei containing 2-4 distinct nucleoli, and a basophilic cytoplasm with azurophilic granules. In most sublines greater than 90% of the population display this morphology with the remainder resembling more mature myeloid cell types. Cytogenetic analysis has shown the occurrence of many karyotypic abnormalities including monosomy, trisomy and tetrasomy, and a variety of chromosomal translocations (Wolman et al 1985); the p53 gene has been largely deleted (Wolf et al 1985), and one allele of the GM-CSF gene on chromosome 5q21-q23 is rearranged and partially deleted (Hubner et al 1985). It is thought that it is the combination of abnormalities in the oncogenes N-ras, which is mutated in codon 61, and c-myc which is over-expressed due to between 4- and 30- fold amplification depending on the subline, that have played an important role in the establishment of the HL60 cell line (Land et al 1983).

It is thought that HL60 cells produce certain CSF-like compounds which act as autostimulators of their growth, and

several compounds which exhibit such "autocrine" activity have been isolated from HL60 conditioned medium. A compound of molecular weight 13 000 has been isolated which stimulated HL60 growth but which had no CSF activity on normal mouse bone marrow cells (Brennan et al 1981). Perkins et al in 1984 described a 25 000 molecular weight product which has similar autostimulatory activity, exhibits CSF activity in normal bone marrow and it also shares antigenic determinants with certain CSFs; these products have yet to be cloned.

The characteristic of HL60 cells that has created the most research interest (more than 700 papers in the last 10 years) is their capacity to differentiate in vitro. When incubated with agents such as DMSO (Collins et al 1978), retinoic acid (Britman et al 1980), chemotherapeutic drugs including 5-azacytidine (Christman et al 1983) and anthracyclines (Schwartz et al 1982), and inhibitors of glycoprotein synthesis such as tunicamycin (Nakayasu et al 1980), HL60 cells differentiate into mature functional neutrophils. The differentiated cells can reduce nitroblue tetrazolium dye as the result of superoxide production, respond to chemotactic stimuli and kill bacteria. Agents such as TPA (Rovera et al 1979), 1,25-dihydroxyvitamin D_3 (Tanaka et al 1983) or cytosine arabinoside (Griffin et al 1982) cause differentiation into monocyte or macrophage-like cells; these differentiated cells can phagocytose complement-coated yeast particles, adhere to plastic and express antigens specific for the monocyte pathway of

development. It has however been reported by several authors that the granulocytic differentiation of HL60 cells is incomplete and defective. For example, the granulocyte-induced HL60 cells lack lactoferrin, suggesting that they are deficient in secondary granules (Olsson et al 1981) and the LDH isoenzyme profile of granulocyte-induced cells differs quantitatively from the LDH isoenzymes of normal granulocytes, consistent with incomplete differentiation (Pantazis et al 1981). Deficiencies have also been described in the myeloperoxidase/peroxide/halide killing system in HL60 granulocytes (Pullen et al 1985). However some agents that induce terminal differentiation of HL60 cells do show some activity in vitro against a subset of patients with acute non-lymphocytic leukemias despite the fact that most fresh leukemia cells do not exhibit differentiation when incubated with granulocyte-inducing agents in short-term culture. A recent publication has suggested that once differentiated HL60 cells will eventually undergo apoptosis after several days in culture (Martin et al 1990). This parallels the work by Savill et al in studies of normal neutrophils (section 1.3.1)

It is at present uncertain as to whether the commitment to differentiate is initiated during a specific phase of the cell cycle. HL60 cells have been synchronised at different stages of the cell-cycle and then exposed to various inducers and the proportion of differentiated progeny measured. Unfortunately, some of the agents that have been used to

synchronise cells such as thymidine and ara-C appear to have some inducing effects of their own and the possibility that some drugs may be taken up in a cell-cycle specific manner does make interpretation of results difficult (Yen et al 1984). However from the work carried out using DMSO (Tarella et al 1982) it seems that cell-cycle does not play an important role in commitment.

The HL60 cell line provides a convenient means of studying the control mechanisms of differentiation.

1.3.4 The K562 cell line

The K562 cell line is a multipotential leukemic cell line that was established from the bone marrow and peripheral blood of individuals with chronic myelogenous leukemia (Lozzio et al 1975). It is a more primitive cell than the HL60 and it can be induced by a variety of chemicals to differentiate into erythroid-like cells, megakaryocytes, and also myeloid cells (Tabilio et al 1983).

1.4 Necrosis and apoptosis: types of cell death

Necrosis is viewed as being a gross departure from physiological conditions and results in acute inflammation and degeneration usually in groups of contiguous cells. Historically this was the first mode of cell death to be recognised and occurs as a result of complement attack,

severe hypoxia, hyperthermia, lytic viral infection or exposure to a variety of toxins and respiratory poisons. Apoptosis, initially termed shrinkage necrosis, in contrast to necrosis, affects single cells and is thought to involve active cellular self-destruction, not degeneration, and is dependent upon protein synthesis. It is observed when death is part of organised tissue reactions as in embryogenesis, metamorphosis, endocrine-dependent tissue atrophy and in the control of normal tissue turnover. It is also seen in regressing tumours. These two forms of cell death are morphologically and biochemically distinct (Searle et al 1982).

1.4.1 Morphology of necrosis and apoptosis

When cells undergo necrosis there is first a series of reversible phases as the cell undergos a mild degree of swelling; these phases include dilation of the endoplasmic reticulum, a change in cell shape with "blebbing" of the surface, an increase in mitochondrial density as the inner membrane shrinks away from the outer membrane and the marginal clumping of loosely textured nuclear chromatin (pyknosis). This reversible phase is followed by irreversible changes. The mitochondria undergo high amplitude swelling with dilation of both inner and outer compartments and the appearance within them of densely lipid-rich aggregates, probably of matrix liposomes.

Subsequently the nuclear, organelle and plasma membranes rupture. As the nucleus swells and the membrane ruptures the marginated chromatin masses form small discrete masses (karyorrhexis); all basophilia (the ability of basic stains such as methylene blue to stain the nucleus) is lost leaving a faintly stained nuclear "ghost".

The hall-mark of apoptotic cells, and so far one of the earliest stages recognised, is the aggregation of the chromatin in large compact granular masses that border on the nuclear membrane. The nuclear outline is often abnormally convoluted and at a slightly later stage becomes grossly indented. Concomitant with the early nuclear changes, the cytoplasm shrinks as the cell rounds up and loses any microvilli; this may also be accompanied by the development of translucent cytoplasmic vacuoles. The organelles become very crowded but retain their integrity. The nucleus breaks up into a number of discrete fragments and the condensed chromatin becomes arranged in cresentic caps. Finally the plasma membrane becomes convoluted so that the cell separates into membrane bound segments, "apoptotic bodies" which are spherical or ovoid in shape and often contain morphologically normal organelles and nuclear fragments. The membrane-bound apoptotic bodies are rapidly phagocytosed; the estimated half-life is between 2 and 4 hours (Potten 1987) when phagocytosed by macrophages or epithelial cells. In tumours the apoptotic bodies are ingested by adjacent tumour cells. In suspension cultures the free-floating bodies undergo

spontaneous degradation with swelling and membrane rupture. This secondary necrosis of apoptotic bodies is morphologically identical with necrosis but the two can be distinguished by the size of the cell fragments. Lysosome rupture plays no part in the first phase of apoptosis but after phagocytosis lysosomal digestion occurs with the digesting enzymes coming from the phagocyte. Apoptotic cells that are committed to die are able to exclude vital dyes such as trypan blue until secondary necrosis occurs.

The above observations have been made in a number of different cell types (reviewed by Wyllie et al 1980).

1.4.2 Biochemistry of necrosis and apoptosis

In necrosis the morphological changes are a consequence of a loss in homeostatic ion control due to increased membrane permeability; this permeability may result from alterations in membrane structure e.g. by complement or by failure of cationic ion membrane pumps e.g. hypoxia. Na^+, K^+, Ca^{2+} and Mg^{2+} move down concentration gradients with concomitant uptake and cellular redistribution of water. The correct internal ion concentrations and ATP levels cannot be maintained and macromolecular synthesis ceases. The rise in intracellular calcium precedes the loss in cell viability, as determined by trypan blue.

Increases in intracellular calcium have also been observed in cells undergoing apoptosis and indeed, appear to

be pivotal in the regulation of the process (Orrenius et al 1989). In immature thymocytes treated with the glucocorticoid hormone methylprednisolone calcium rises were observed in a dose dependent manner (McConkey et al 1989). ATP levels are maintained in apoptotic cells.

A characteristic of some cell lines undergoing apoptosis is the cleavage of DNA at internucleosomal sites, reportedly by a calcium dependent endonuclease which is thought to be of nuclear origin (Wyllie 1984). Agarose gel electrophoresis of DNA from apoptotic cells shows "ladders" with DNA fragments of approximately 180 base pairs but this cleavage can be inhibited by inhibitors of protein synthesis. However the significance of endonucleocytic cleavages in cell death has been recently questioned. Treatment of HL60 cells with a wide variety of cytotoxic agents induced DNA fragmentation typical of endonucleolytic cleavage (Kaufman 1989); this occurred in the presence of inhibitors of protein and RNA synthesis. Kaufman proposed that the enzyme was an endogenous cellular protein and was probably lysosomal in origin since he was unable to induce DNA fragmentation in isolated nuclei. Extensive degradation of many other nuclear proteins was observed concomitant with drug-induced DNA degradation. In necrosis, endonuclease and lysosomal proteins are activated leading to general dissolution of DNA.

A number of investigators have reported that specific gene expression was enhanced in response to injury. Buttyan et al (1988) noted a "reactive cascade" of gene activity

during prostatic cell death. A transient increase in c-fos RNA after 36 hours was followed by an increase in c-myc transcripts after 72 hours and hsp70 transcipts after 96 hours. This cascade of gene activity was concomitant with cell death although paradoxically, this activity mimics that which occurs during proliferation (see also section 1.6.1.1).

There is a wide range of sensitivities of different cell types to necrosis and apoptosis. For example tumour necrosis factor has been reported as being able to induce both apoptotic and necrotic forms of cell death in different cell types (Laster et al 1988) whereas in other systems it is the severity of the assault that determines the type of cell death. Increasing evidence suggests that apoptosis, mitotic capacity and differentiation share some intracellular control mechanisms (Allan et al 1989).

1.5 Differentiation and cytotoxicity

The majority of agents that induce differentiation are cytotoxic. The relationship between cytotoxicity and differentiation has been noted by several groups and it is apparent that many drugs induce optimal differentiation at concentrations that are marginally below those which are cytotoxic (Sartorelli 1985). The optimal concentration for differentiation was taken to be that which induced the maximum percentage of cells expressing markers of a more mature phenotype whilst maintaining viability at >70%.

In 1987 Langdon and Hickman studied the induction of differentiation in HL60 human promyelocytic leukemia cells by a variety of alkylformamides, alkylacetamides, alkylureas and alcohols. There were no structural requirements for the induction of differentiation but for each molecule the concentration which induced differentiation was found to be marginally below that which was cytotoxic. Such a relationship has been shown using DMSO (Bunce et al 1983), HMBA and analogues (Haces et al 1987), ara-A (Munroe et al 1984) and tunicamycin (Nakayasu et al 1980) as inducers in the same cell line.

In mouse erythroleukemia cells (MEL) two groups have reported similar findings. Supino et al in 1986 induced differentiation with poly-L-lysine molecules of different sizes and showed that toxicity occurred when concentrations were used that were greater than that which induced differentiation and Reuben et al (1987) used polar solvents to demonstrate the same relationship.

Much of the evidence therefore would seem to suggest that some form of sub-toxic threat or stress is involved in the induction of differentiation in HL60 and MEL cell lines, although it should be pointed out that not all agents will induce differentiation when used at sub-toxic concentrations. For example, Adriamycin does not induce differentiation of HL60 cells (Schwartz et al 1982). Chow and Ross (1987) suggested it was the response of a cell to the damage imposed by the drug that was important in cytotoxicity.

Factors which were expressed during S phase were necessary for cell death. It follows therefore that the response of a cell to differentiating agents may also be critical to the commitment to differentiate.

The idea that it is the imposition of a non-specific stress by a drug and the way in which a cell responds to that stress, that induces differentiation is an attractive one. It would help to explain why so many disparate agents with a multitude of cellular targets can induce differentiation and why cells are unable to differentiate in response to certain agents; for example, retinoic acid is unable to induce differentiation in MEL cells (Breitman et al 1980). The concept of cells responding to a stress by the production of proteinaceous factors is not new.

1.6 The heat shock proteins

The heat shock proteins, or stress proteins, are a family of highly evolutionarily conserved proteins that are induced when cultured cells or whole organisms are exposed to elevated temperatures; these proteins are also produced in response to many other types of stress such as exposure to ethanol, heavy metals and a wide variety of other chemicals. Induction of these stress proteins has been observed in bacteria, yeast, soybeans, maize and in cells of many higher eukaryotes (reviewed by Lindquist et al 1988).

The stress response was first observed in Drosophilia

melanogaster where it can be observed with the aid of a light microscope. When the fruit fly is shifted from its physiological temperature of 25°c to 37°c and is treated with dinitrophenol or sodium salicylate, several new puffs can be observed in the salivary gland polytene chromosomes (Ritossa 1962). Over the following years it became apparent that these puffs were the sites of vigourous RNA transcription and that a number of these RNAs were translated into the heat shock proteins.

Most mammalian species produce heat shock proteins when incubated at temperatures of approximately 43°c (6°c above physiological temperatures). The major proteins produced have molecular weights of around 28, 32, 70, 72, 90 and 110kD with the two most abundant being hsps 70 and 90. These, and proteins closely related to them (the glucose regulated proteins) are also present in unstressed cells. Ubiquitin is also a heat shock inducible transcript (Fornance et al 1989b).

When cells are heat shocked there is preferential synthesis of the heat shock proteins to the virtual exclusion of all other proteins. Lindquist's group in 1982 quantified the amount of hsp70 produced in Drosophilia in response to an increase in temperature and demonstrated that there is a general trend of increased synthesis with increased stress (DiDomenico et al 1982). Hsp70 transcription is maximal under conditions of a heat shock and degradation of the hsp mRNA is inhibited following a heat shock; this is

mediated by the untranslated leader at the 5' end of the hsp70 mRNA (Petersen et al 1988). Transcription to produce other mRNA is not inhibited per se but processing is (Yost et al 1988); electron microscopy reveals granules of unprocessed mRNA in the nucleus after a heat shock (Welch et al 1985). Once synthesised, the hsps translocate to the nucleolus and bind to the matrix in a hydrophobic manner (see section 1.6.4). Other cellular changes that have been observed following a heat shock include a drop in pH (Drummond et al 1986), an increase in intracellular calcium which is accompanied by a rapid breakdown of polyphosphoinositides (Stevenson et al 1986) and an increased level of K^+ and a corresponding decrease in Na^+ inside the cell due to a compromise in Na^+/K^+ ATPase activity (Burdon et al 1982). Cytoskeletal rearrangements have also been noted (Welch et al 1985). The signal to release the repression of the genes active prior to the heat shock is thought to be provided by the accumulation of hsps (DiDomenico et al 1982).

The heat shock response is thought to be involved in the protection and/or enhanced survival of the cell; a heat shock is lethal to cells that have been microinjected with antibodies raised against hsp70 (Riabowol et al 1988). It has been demonstrated that mammalian cells exposed to a mild, sublethal temperature elevation acquire resistance to a subsequent lethal heat shock challenge; this phenomena has been termed thermotolerance and has been correlated with the expression, accumulation, and relative half-lives of the

major stress proteins hsp70 and p72 (Mizzen et al 1988). Thermotolerance can also be induced by organic solvents when used at cytotoxic concentrations (Hahn et al 1985).

The heat shock proteins can be grouped into families of proteins with comparable molecular weights. The members of the 70, 90 and 110kD families will be discussed in more detail.

1.6.1 Hsp70 and related proteins

In human cells there are at least five distinct protein members of the hsp70 gene family that exhibit constitutive and inducible regulation, share biochemical and antigenic properties but have different intracellular localisations (see section 1.6.4). These proteins are hsp70, hsp72, p72, grp75 and grp78 (using the nomenclature defined by Watowich and Morimoto 1988). Grp78, p72 and hsp70 are approximately 75-85% related. The human genome contains at least ten hsp70 gene-related sequences and Southern blot analysis reveals at least two genes each for grp78 and p72 genes and a minimum of five hsp70 genes (Morimoto and Milarski 1990). The human hsp70 gene is transcribed as an uninterrupted primary transcript of 2440 nucleotides comprising a 5' noncoding leader sequence of 212 nucleotides, a 3' noncoding region of 242 nucleotides and a continuous open reading frame of 1986 nucleotides (Hunt and Morimoto 1985) whereas the human grp78 gene consists of eight exons (Ting et al 1988). Grp78 is

identical to the immunoglobulin binding protein BiP (Munro et al 1986).

1.6.1.1 Expression of hsp70 and related proteins

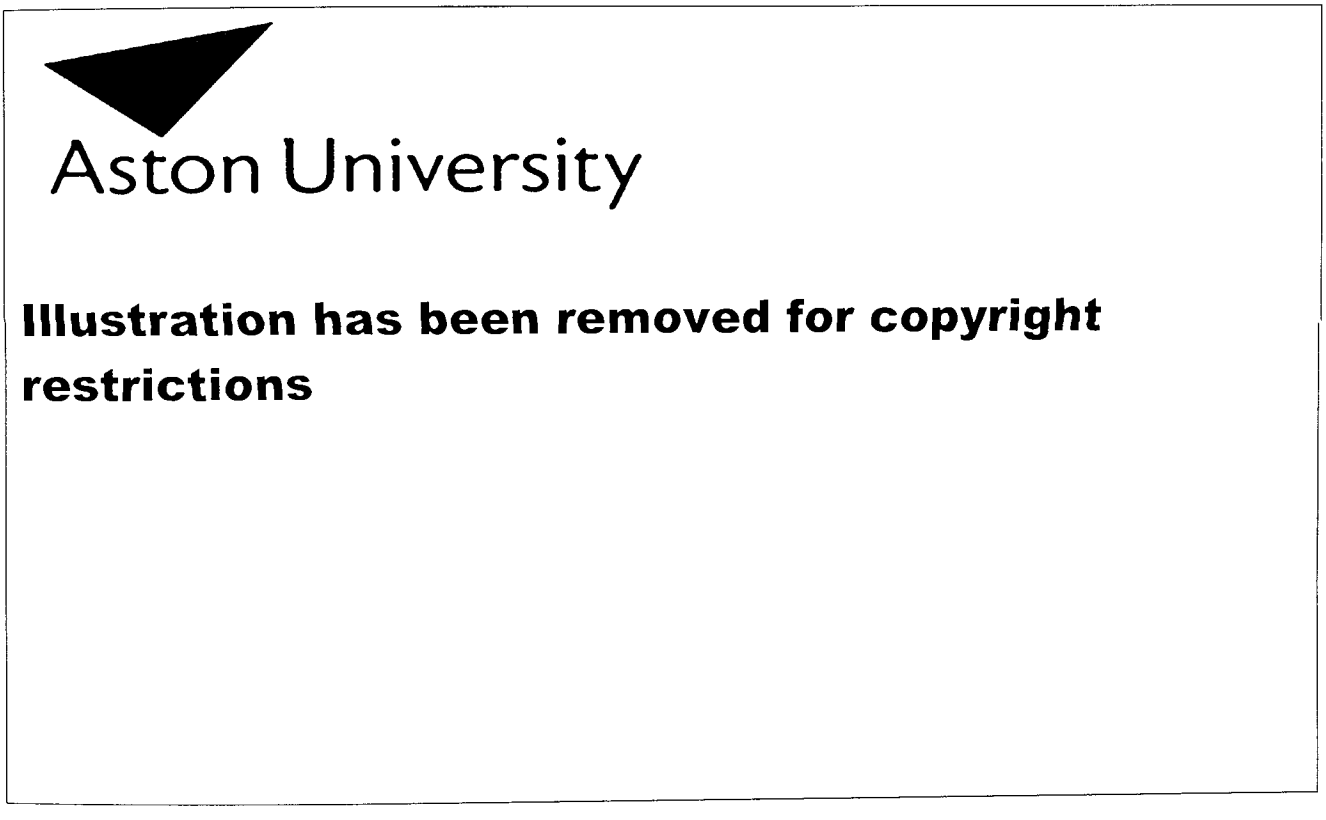
The genes that encode hsp70-related proteins exhibit distinct patterns of regulation. Although the genes are induced in response to various forms of physiological stress, the conditions that induce grp78 are often distinct from the conditions that activate hsp70. P72 is constitutively expressed and responds only slightly to many of the conditions that induce hsp70 and grp78. Hsp72 is purely heat inducible (Pelham 1986). Evidence suggests that these stress proteins bind to denatured cellular proteins with hydrophobic surfaces, such as are produced following a heat shock, and promote disaggregation and re-folding by using the energy of ATP hydrolysis (Pelham 1988). Hsp70 also binds to the damaged nucleoli of cells (Pelham 1984). In this way it can be seen how hsps can aid in the recovery from heat shock and help prevent any further damage caused by any subsequent exposure to stress. Evidence for the activation of hsps by denatured cellular protein was provided by Ananthan et al (1986). Purified bovine serum albumin was co-injected with a Drosophilia hsp70-B-galactosidase hybrid gene into frog oocytes. Activation of the hsp70 gene was only observed if the proteins were denatured by reductive carboxymethylation prior to injection. Grp78 binds to immature immunoglobulin

and aberrant proteins such as the unglycosylated proteins found in glucose deprived cells.

Several recent publications have also implied a role for hsp70 in protein folding under normal physiological conditions. Beckman et al (1990) have demonstrated that hsp70 and p72 interact with nascent polypeptides to facilitate proper folding. This is consistent with other findings which show that p72 facilitates disassembly of clatherin-coated vesicles (Chapell et al 1986) and grp78 interacts with proteins that are translocated into and assembled within the lumen of the endoplasmic reticulum (Munro et al 1986). Hsp70 has also been implicated in the transport of proteins into mitochondria (Murakami et al 1988). Grp 75 is thought to be involved in the assembly of mitochondrial proteins (Mizzen et al 1989). These interactions are all transient and are dependent on ATP hydrolysis although in some cases hsp70 and p72 may further stabilise proteins while they are translocated to organelles (the "chaperone" concept, Ellis and Hemmingsten 1989). Once a protein has assumed its final configuration it would no longer be a substrate for the hsps. Sequences in the amino terminus of hsp70 have been identified which are responsible for ATP-binding (Milarski and Morimoto 1989a). Subjecting cells to a stress interferes with normal protein maturation. Figure 1.4 shows the scheme proposed by Beckman et al (1990) for hsp70 interactions.

Figure 1.4 Possible model for hsp70 interactions in the folding and translocation of newly synthesised proteins

(from Beckman et al 1990)
1. Folding of newly synthesised proteins
2. Translocation of proteins from the cytosol to organelles
3. Subsequent folding of proteins within the organelle



A role in the lysosomal degradation of intracellular proteins has also been proposed for hsp70 (Chiang et al 1989). It binds to peptide regions that target intracellular proteins for lysosomal proteolysis and alters the conformation of these proteins into a transport competent state, analogous to the proposed role of hsp70 as a chaperone, in the presence of ATP and $MgCl_2$.

Hsp70 is also regulated during growth. Serum deprivation of HeLa or 293 cells resulted in reduced hsp70 transcription and mRNA levels but subsequent serum stimulation induced approximately 20-fold increases in hsp70 mRNA (Wu et al 1985). Hsp70 is also expressed following stimulation of resting T lymphocytes with the non-specific mitogen phytohaemagglutinin and the polypeptide growth factor IL-2 (Ferris et al 1988). Work by Wu and Morimoto (1985) has suggested that DNA synthesis and hsp70 expression are linked. Changes in hsp70 mRNA and protein levels have also been observed during the cell cycle. Levels of hsp70 mRNA increase 10- to 15- fold when synchronised HeLa cells enter S phase with maximum expression at the peak of DNA synthesis (Milarski et al 1986) then decline rapidly; these results are consistent with those obtained by Wu et al (1985). Further studies by Milarski et al (1989b) have suggested that hsp70 may be acting specifically with other proteins in a cell cycle-dependent manner. Growing evidence suggests that hsp70 is a member of the growth-regulated gene family that includes c-myc c-myb and c-fos. Growth related expression of hsp70

does decrease in aged cells (Faassen et al 1989).

The elevated expression of members of the hsp70 family during early stages of embryonic development and differentiation, in the absence of stress, has been observed amongst diverse species. The expression of these proteins in differentiation will be discussed in detail in section 1.6.6.

1.6.2 Hsp90 and related proteins

In human cells there are two 90kD stress proteins with roles comparable to those previously described for hsp70. The hsp90 gene consists of 12 exons and 11 introns (Hickey et al 1989 and Rebbe et al 1989) and is abundant at normal temperatures where it constitutes approximately 0.1-2% of all cytosoluble proteins (Farrelly et al 1984). It has a hydrophobic, negatively charged amino acid stretch which is thought to mimic a negatively charged DNA-like structure. It is also induced by heat. Grp94 (also known as 100kD) shares about 50% sequence homology with hsp90 but the two are not necessarily co-ordinately regulated and have distinct intracellular localisations (section 1.6.4).

1.6.2.1 Expression of hsp90 and grp94

Hsp90 has been shown to be associated with various different types of proteins. The first protein with which

hsp90 was shown to have a specific association was the transforming protein of the Rous Sarcoma Virus, $pp60^{src}$ (Opperman et al 1981). Associations of the hsps with oncogenes will be discussed in section 1.6.6. Hsp90 does associate with other cellular kinases such as heme-controlled eIF2-alpha kinase (Rose et al 1989). The kinase is stimulated by hsp90 thereby increasing phosphorylation of eIF-2 and inhibiting protein synthesis in reticulocyte lysates; this implicates hsp90 in the regulation of protein synthesis. The same workers have also demonstrated that caesein kinase II is also associated with hsp90 and that hsp90 is a substrate for in vitro phosphorylation by the kinase. Walker et al (1985) showed that various members of the hsp90 family were phosphorylated in vitro by a DNA-dependent mechanism but the significance of this was considered to be uncertain.

It has been established that a dimeric form of the 90kD heat shock protein is associated with the glucocorticoid receptor (Mendel et al 1988) and with unactivated steroid receptors (Baulieu 1987). It has been proposed from its sequence that hsp90 is a cap for the receptors and binds to their DNA binding sites. Complexes would form just after the receptors have been synthesised and this would preclude any interaction of the receptor with DNA under physiological conditions until the hormone were present.

Hsp90 and grp94 have been shown to associate with actin in vivo in a manner that is dependent on calcium and

regulated by calmodulin (Nishida et al 1986). This actin association may provide a mechanism for transport of hsp90.

So far no functional activity has been ascribed to grp94 but it may complement the role of grp78 in protein folding. Grp94 was found to be a low-affinity, high-capacity Ca^{2+} binding protein and may serve as an agent for intracellular calcium sequestration. Upon physiological stimulation, the calcium would be released to signal cellular responses (Koch et al 1986).

The expression of hsp90 and grp94 during differentiation will be discussed in section 1.6.6.

1.6.3 Hsp110

Most eukaryotes produce proteins of greater than 100kD in response to a heat shock. In human cell lines a protein of 110kD is synthesised which is present in the nucleolus of cells at the site of nucleolar chromatin. This protein associates with RNA or with a complex of proteins that bind RNA (Welch and Suhan 1986). The hsp110 gene has not yet been cloned but it may be related to the yeast protein hsp104 which has recently been shown to be required for thermotolerance (Sanchez et al 1990).

1.6.4 Intracellular localisation of hsps.

The identification of hsp related proteins in distinct

subcellular compartments allows for similar functions to be carried out by the different proteins but within their own intracellular locale; members of the hsp family contain protein sequences which localise them to particular intracellular locations. In response to a physiological stress they may redistribute to other compartments. This is illustrated by hsp70 and p72 which are typically found in the cytoplasm of the cells in the G_1 phase of the cell cycle but following heat shock (or other forms of stress) they redistribute to the nucleus and nucleolus (Milarski et al 1986). Nuclear import of proteins occurs by rapid binding of the protein to the nuclear pore complex followed by a slower energy-dependent translocation into the nuclear compartment. Specific nuclear protein amino acid sequences containing basic amino acids can target proteins into the cell nucleus; such a target has been located in the human hsp70 protein (Dang et al 1989). It is thought that further sequences may specifically target hsp70 to specific subnuclear compartments. Deletion mutations of hsp70 have determined that this nucleolar localisation sequence is at the carboxy terminus (Milarski et al 1989). Translocation of hsp70 and p72 would be dependent upon the accessibility of the nuclear localisation sequence. Grp78 and grp94 contain the sequences lys-asp-glu-leu at the carboxy terminus which retain them in the endoplasmic reticulum (Munro et al 1987). Hsp90 is predominantly cytoplasmic although some relocalisation to the nucleus does occur following a heat shock.

The different localisations may explain why different stresses induce different hsps. Stresses that result in the appearance of damaged or altered proteins in the cytosol or nucleoli induce hsp70 whereas damaged or altered proteins in the endoplasmic reticulum would result in the synthesis of grps. Accumulation of hsp70 in the nucleoli does require the appearance of damaged substrates within the organelle. Treatment of cells with amino acid analogues, such as azetadine carboxylic acid induces both nucleolar damage and the appearance of altered proteins in the endoplasmic reticulum due to the incorporation of azetadine into proteins, and co-ordinately induces both hsp70 and grp78.

1.6.5 Expression and association of hsps with oncogenes

The synthesis of heat shock proteins following a heat shock is often accompanied by an increase in oncogene mRNA levels. In HeLa cells, heat shock induces the elevated expression of the c-*fos* proto-oncogene (Andrews *et al* 1987) and in B16 murine melanoma cells c-*myc* transcripts increase in heat shocked cells (Cajone *et al* 1988). In human lymphoid cells the expression of c-*fos*, c-*jun*, and c-*myc* genes is thought to be regulated by a heat shock at the level of transcription (Bukh *et al* 1990). A heat shock stabilised the oncogene mRNA and increased the half life.

This idea of a heat shock stabilising oncogenes by increasing half-lives is not a new one. Luscher and Eisenman

in 1988 demonstrated that c-myc and c-myb protein turnover was decreased in cells that had been heat shocked. Finlay et al (1988) demonstrated that a mutated p53 protein was associated with p72 and that its half-life was extended under these circumstances. The authors went on to suggest that this complex may contribute towards transformation. Stabilisation of mutated p53 and c-myc mRNA has also been demonstrated in undifferentiated F9 mouse teratocarcinoma cells but what was particularly interesting was that on differentiation of the cells a destabilisation of the mRNA was observed (Dony et al 1985).

Grp78 is induced when cells are transformed by Rous Sarcoma virus and this induction occurs independently of glucose deprivation (Stoeckle et al 1988). From the time course of induction of grp78 the authors concluded that the increased expression was a response to metabolic changes that occur in a transformed cell rather than being a primary effect of $p60^{v\text{-}src}$ transfection; a transformation defective virus did not induce increased synthesis of grp78.

High levels of hsps have been reported in chemically transformed cell lines (Chang et al 1986) although this may be due to the toxic effects of the chemical (see section 1.6.7). In a transformed cell oncogenes are present as abnormal proteins by virtue of their over-expression or a mutation; since stress proteins bind to abnormal proteins and are expressed in proliferating cells, a role for them in maintaining the transformed state is possible.

1.6.6 Hsps and differentiation

Evidence for a role for hsps in development and differentiation is accumulating. A link between hsp expression and differentiation was first proposed in 1982 when Ireland and Berger established that the hormone ecdysterone increased the synthesis of low molecular weight hsps (from 23 to 27 kD) in embryonic Drosophilia cells when used at physiological concentrations. Bensaude et al (1983) showed that in mouse embryonic cells the 89kD, 70kD and 59kD hsps are spontaneously expressed in high amounts and this synthesis precedes the activation of the genome at the late two-cell stage. These cells were not able to synthesis hsps over constitutive levels when heat shocked. In the same paper the authors also described how levels of hsp decrease during the in vitro differentiation of F9 cells treated with the inducer retinoic acid. These cells are thought to be similar to the pluripotent cells of the early mouse embryo and they express high constitutive levels of several heat shock proteins.

A decrease in a constitutive hsp90 protein has been observed by Yufu et al (1989) after DMSO induced differentiation of HL60. This protein termed hsc90 was closely related to hsp90 in respect of its molecular weight, isoelectric point, peptide map and immunoreactivity but the authors were unable to separate the two by one-dimensional gel electrophoresis. Work by Richards et al (1988) also

noted an early decrease in a 90kD protein, which was not heat inducible, during NMF induced differentiation of HL60 cells. As well as the decrease in the 90kD protein, possibly grp94, a decrease in hsp70 protein synthesis was observed between 20 and 30 hours. Similar results were reported in 1988 by Hensold et al in MEL cells; two-dimensional electrophoresis and Northern blotting revealed rapid decreases in hsp70 protein synthesis and mRNA. These decreases in synthesis preceded the development of markers of differentiation.

The decrease in hsp70 observed in F9, HL60, and MEL cells is however in contrast to results obtained with erythroid cell lines. During differentiation of avian erythroid cells there is an initial burst of hsp70 expression (Banerji et al 1987). As the cells mature, the levels of hsp70 expression decreases but high levels of hsp70 are maintained throughout the differentiated state and are accumulated in the adult erythroid cells. In K562 human leukemic cells induced to differentiate with haemin, hsp70 expression is elevated after 48 hours (Singh et al 1983).

1.6.7 Induction of hsps by toxic drugs

The degree of induction of hsps by chemical agents may well be dependent upon the concentration of drug used and as to whether the drug is toxic to the cell. Recently, several publications have suggested that hsps are expressed after injury and that their expression does not necessarily confer

a protective role on cells. It may be that different members of the hsp family are expressed after incubation with different inducers depending on their site of action (see section 1.6.4).

Observations by Richards et al (1988) on protein synthesised after HL60 cells were treated with 1M NMF (a highly toxic concentration) showed that both hsp70 and hsp90 synthesis was elevated. Prostaglandins that suppressed the proliferation of K562 cells induced the synthesis of a 74kD (p74) protein and this induction of synthesis was dose-dependent; the p74 protein cross-reacted with a monoclonal antibody raised against hsp70 (Santoro et al 1989). The hsp70 protein was localised in the soluble and membrane fraction of the cells after treatment with PGA_1, but after PGJ_2 treatment which inhibited the synthesis of several nuclear proteins, some hsp70 was localised in the nucleus. The induction of hsp70 synthesis was reversed upon removal of the drug indicating that synthesis was not necessarily associated with cytotoxicity. Prostaglandins that did not inhibit cell proliferation did not induce p74 synthesis. Gonzalez et al (1989) have demonstrated that hsp70 immunocytochemistry may be used as a marker of neural injury. However Schaefer et al (1988), investigating the induction of hsp70 by anticancer drugs that covalently modify DNA and other cellular macromolecules, concluded that induction of hsp70 was neither a direct consequence of DNA crosslinks nor of cytocidal drugs. Work by Fornance et al (1989a) also

concluded that the heat shock response was not induced in response to genotoxic stress, although this study only investigated mRNA levels and not hsp protein synthesis or amounts.

1.6.8 Regulation of hsps

The regulation of the heat shock response is complex. Regulation of hsp70 induction by chemicals is thought to be through transcriptional activation whereas chemicals that repress hsp70 act through posttranscriptional regulation (Watowich et al 1988). Transcriptional activation of the heat shock response requires interaction between a specific heat shock promoter element (HSE) and a heat shock transcription factor (HSTF). The HSE has been identified by deletion mapping and site specific mutagenesis and it appears to exist as multiple arrays of the consensus sequence C--GAA--TTC--G which are located at varying distances from the transcription initiation site (Pelham 1982). From various studies on Drosophilia, yeast and in human cells it appears that different mechanisms exist to activate this HSE-protein complex under different conditions. For the purpose of this introduction only the mechanisms in human cells will be discussed.

The studies in human cells have mainly involved the investigation of transcriptional regulation in HeLa cells. Following a heat shock of 43^{o}c, transcription increases

approximatly 10-fold from promoter sequences that extend from positions -68 to -107 (Wu et al 1986). Within this region is a highly conserved HSE at position -100 which has 8 out of 8 positions matching to the consensus sequence and activation of this single sequence has been found to be sufficient for the stress-induced transcription of the human hsp70 gene (Mosser et al 1988). The HSE is able to confer heat shock responsiveness when fused to a heterologous gene and transfected, or microinjected into Xenopus oocytes (Pelham et al 1982). The sequence at -100 overlaps a weak HSE with only 4 out of 8 positions matching the consensus sequence, and flanks adjacent weak HSEs at -68 and -110 with 5 of 8 positions matching the consensus sequence (Hunt et al 1985 and Wu et al 1986). Expression of the hsp70 gene under conditions of normal cell growth, differentiation and regulation by adenovirus type E1A, is thought to be under the influence of a promoter that extends to position -68.

Along with increased transcription from the heat shock promoter after heat shock is the increased (5- to 7- fold) level of a protein factor (Kingston et al 1987); the presence of this factor in heat shocked cells is unaffected by cycloheximide although the rate of transcription repression is more rapid in these cells. Since this factor, the heat shock transcription factor which is an RNA polymerase II transcription factor, must be present in control cells (as indicated by the cycloheximide data and the immediate transcription of hsp70 after a heat shock) there must be some

form of activation of the HSTF. In HeLa cells the control form of HSTF represents a transcriptionally inactive state while stress-induced HSTF corresponds to the active state. This gives rise to the interpretation that basal expression of the human hsp70 gene does not require the HSE at position -100 and is primarily dependent on the proximal promoter elements extending to the position at region -68 where it undergoes weak binding. Suprisingly however the haemin-induced transcriptional activation of hsp70 in K562 cells induced to differentiate with haemin, is due to binding of the stress-induced form of HSTF to the heat shock element (Theodorakis et al 1989).

There is some controversy surrounding the binding abilities of the HSTF in control and heat shocked cells. The studies by Larson et al 1988 indicate that the HSTF remains unbound to DNA and is activated by an ATP independent heat-induced alteration where on it is able to bind to the promoter suituated at position -100. From ultraviolet cross-linking studies the apparent size of the factor is 90kD in unshocked cells and 92kD in heat shocked cells. Treatment with calf intestinal phosphatase indicates that the HSTF isolated from intact heated cells is more extensively phosphorylated than that from control cells. The studies by Mosser et al (1988) and Sorger et al (1987) however have been unable to detect phosphorylation in heat shocked HeLa cells but they have demonstrated binding to a promoter at -68 in control cells. In MEL cells, which are unable to respond to

a heat shock in respect of their ability to synthesise hsps, the HSTF is unable to undergo the secondary modifications, such as phosphorylation, required for heat shock activation (Hensold et al 1990).

The regulation of grps is thought to be through transcriptional regulation but is distinct from that of hsp70 (Watowich et al 1988). The transcriptional regulation is sensitive to cycloheximide and the most potent inducers of grp78 do not induce hsp70 (Resendez et al 1988). Grp78 and grp94 are thought to be co-ordinately regulated through common trans-acting factors which bind to a conserved 28 base pair region (Chang et al 1989). Grp78 does not bind to the HSE (Theodorakis et al 1989).

1.7 The aim of these studies

It has been proposed that differentiation of HL60 cells is induced at drug concentrations that are marginally below those which are cytotoxic and that this differentiation process involves the regulation of members of the stress protein family. Under conditions of cytotoxicity there is synthesis of hsp70 and hsp90 so it has been proposed that stress proteins may also be modulated under these conditions but with different members of the stress protein family being regulated during differentiation and cytotoxicity. Although many genes have been reported to be activated or repressed during the processes of differentiation and cytotoxicity only

the heat shock proteins have a well defined function.

The aim of these studies was to further characterise the ability of a heat shock to induce terminal differentiation in HL60 cells. Following on from this the synthesis and amounts of the 70kD and 90kD heat shock protein families during NMF induced differentiation of HL60 was to be studied.

To develop the hypothesis that it is the modulation of the glucose-regulated proteins that is involved in HL60 differentiation and modulators of hsp70 that are associated with cytotoxicity it was proposed that cells would be treated with known modulators of grp78 and hsp70 and the effects of these agents on differentiation and cytotoxicity investigated. The differentiation of a second human leukemia cell line was also to be studied and the synthesis and amounts of members of the hsp family determined to assess the generality of the results with respect to other cell lines and inducers.

To determine whether changes in hsp70 could act as a marker of cell death, HL60 cells would be treated with different agents and the synthesis and amounts of hsp70 and hsp90 related proteins determined. These results could then be correlated with markers of cell viability.

CHAPTER TWO

SOURCES AND SOLUTIONS

2.1 SOURCES

2.1.1 Purchased from Sigma Chemical Company Ltd., Poole, Dorset.

Adriamycin

agarose (electrophoresis grade)

alpha-naphthyl acetate

ammonium persulphate

azetadine carboxylic acid

bromophenol blue

4-chloro-1-napthol tablets

coomassie brilliant blue

dithreiothritol

ethidium bromide

ethylene glycol monomethyl ether

glycerol

glycine

hydrogen peroxide

iodoacetamide

low melting point agarose

methotrexate

methyl green

nitroblue tetrazoluim

pararosaniline

phenylmethylsulphonylfluoride

pre-stained molecular weight markers

propidium iodide

proteinase K

ribonuclease A

sodium azide

sodium lauryl sarkosinate

sodium nitrate

tetramethyl-benzidine

12-O-tetradecanoylphorbol-13-acetate

N,N,N',N'-tetramethylethylenediamine

toludine blue

tris(hydroxymethyl)aminomethane base

triton X-100

tween-20

tunicamycin

2.1.2 Purchased from Fisons Scientific Equipment, Loughborough, Leics.

acetic acid

calcium chloride

dimethylsulphoxide

hydrochloric acid

formaldehyde solution (40%)

Optiphase

orthophosphoric acid

potassium chloride

sodium hydroxide

trichloroacetic acid

2.1.3 Purchased from BDH Chemicals Ltd., Poole, Dorset.

acetone

acrylamide

carboxymethylcellulose

DNA 123 base pair ladder

2,5-diphenyloxazole

ethanol

magnesium chloride

B-mercaptoethanol

methanol

N,N'-methylenebisacrylamide

potassium dihydrogen orthophosphate

sarkosyl N30

sodium chloride

sodium dodecylsulphate

trypan blue (Gurr)

2.1.4 Purchased from Amersham International, Amersham, Surrey

anti-actin monoclonal antibody

anti-hsp72 monoclonal antibody

biotinylated-protein A

goat anti-mouse IgM-biotinylated

Hibond nitrocellulose

horseradish peroxidase-streptavidin

L-[^{35}S]-methionine

^{14}C-methylated molecular weight markers

sheep anti-mouse IgG-biotinylated

2.1.5 Other purchases

Agar Scientific, Stanstead, Essex.

50x50 copper mesh grids

dental wax

E.M. grade gluteraldehyde

Epon 812

Aldrich Chemical Company Ltd., Gillingham, Dorset

dimethyl sulphoxide (HPLC grade)

disodium hydrogen orthophosphate

N-methylformamide

propylene oxide

Becton-Dickinson Labware Oxnard, CA., USA

Falcon 2054 plastic tubes with lids

Bio-Rad Laboratories Ltd., Watford, Herts

agarose protein A beads

cellophane drying membrane

mini-protean II electrophoresis and electroblotting apparatus

Protean II gel electrophoresis apparatus

Trans blot apparatus

trans-blot membrane - 0.45um nitrocellulose

Coulter Electronics Ltd., Luton, Beds.

Isoton balanced electrolyte solution

Ernest F. Fullham, Latham, N.Y., USA.

araldite 506

dibutyl phthalate

dodecenyl succinic anhydride

epon 812

tris-dimethylaminomethyl phenol

Gibco Ltd., Paisley, Scotland

dialysed foetal calf serum

foetal calf serum

penicillin, streptomycin solution

RPMI-1640 tissue culture medium with L-glutamine and 25mM HEPES

methionine-free RPMI-1640

Nunc cryotubes

Nunc 25ml plastic tissue culture flasks

Nunc 250ml plastic tissue culture flasks

Flow Laboratories,Rickmansworth, Herts

mycoplasma detection kit

Kodak Ltd., Hemel Hempstead, Herts.

GBX developer and fix

Technical Pan film

X-omat AR film

New England Nuclear, Dupont (UK) Ltd., Southampton

Protosol

Oxoid Ltd., Basingstoke, Hants.

complement fixation buffer tablets

phosphate buffered saline tablets

Sterilin Ltd., Feltham

25ml plastic universal tubes

7ml plastic bijoux bottles

sterile plastic pipettes

Whatman Labsales Ltd., Maidstone, Kent

14MM chromatography paper

GF/C glass filters

Immobilon nitrocellulose membranes

2.1.6 Gifts

Anti-hsp90 antibody was donated by Dr. D.O. Toft, Mayo Clinic, Rochester, Minnisota, USA.

Anti-H2B antibody was donated by Dr. B. Turner, Department of Anatomy, University of Birmingham.

5A5-1 antibody was donated by Dr. R. Morimoto, Department of Molecular Biology, Cell Biology and Biochemistry, Northwestern University, Evanston, USA.

7.10 antibody was donated by Dr. S. Lindquist, Department Molecular Genetics and Cell Biology, University of Chicago, Chicago, USA.

CB3717 was donated by Dr. R. Griffen, Pharmaceutical Sciences Institute, Aston University, Birmingham.

Human plasma was kindly provided by the Blood Transfusion Centre, University of Birmingham.

2.2 SOLUTIONS

2.2.1 Phosphate buffered saline

Oxoid PBS tablets	10
distilled water	to 1000ml

This gave a solution equivalent to 0.8g of sodium chloride, 0.2g potassium chloride, 1.15g disodium hydrogen orthophosphate dihydrate, 0.2g potassium dihydrogen orthophosphate per litre, pH 7.4.

2.2.2 Complement fixation buffer

Oxoid CFB tablets	10
distilled water	to 1000ml

This gave a solution equivalent to 0.575g barbitone, 0.185g soluble barbitone, 8.5g sodium chloride, 0.168g magnesium chloride, 0.028g calcium chloride, per litre, pH 7.4.

2.2.3 Formal acetone fixative

potassium dihydrogen orthophosphate	0.1g
disodium hydrogen orthophosphate	0.02g

distilled water	30ml
acetone	45ml
formaldehyde (40%)	25ml

2.2.4 Sodium dodecylsulphate sample buffer

tris(hydroxymethyl)aminomethane base	1.514g
hydrochloric acid, dropwise	to pH 7.0
sodium dodecylsulphate	4.0g
glycerol	20ml
bromophenol blue	0.002g
distilled water	to 100ml

just prior to use:

dithieothritol	0.025g/ml

for a 2 or 3 x strength buffer the volume of water was adjusted accordingly

2.2.5 Acrylamide solution for one-dimensional SDS-PAGE

acrylamide	150g
distilled water	to 500ml

the solution was filtered and stored in the dark at $4^{o}c$

2.2.6 Bisacrylamide solution for one-dimensional SDS-PAGE

bisacrylamide	5g
distilled water	to 500ml

the solution was filtered and stored in the dark at $4^{o}c$

2.2.7 Resolving gel buffer for electrophoresis

tris(hydroxymethyl)aminomethane base	90.825g
hydrochloric acid, dropwise	to pH 8.9
distilled water	to 500ml

2.2.8 Stacking gel buffer for electrophoresis

tris(hydroxymethyl)aminomethane base	60.55g
hydrochloric acid, dropwise	to pH 6.8
distilled water	to 500ml

2.2.9 Electrolyte buffer

tris(hydroxymethyl)aminomethane base	15.14g
glycine	72.07g
sodium dodecylsulphate	5g
distilled water	to 5l

2.2.10 Gel stain

Coomaissie blue R250 stain	0.25% w/v
methanol	500ml
glacial acetic acid	100ml
distilled water	400ml

2.2.11 Gel destain

methanol	430ml
glacial acetic acid	70ml
distilled water	500ml

2.2.12 Blotting transfer buffer

tris(hydroxymethyl)aminomethane base	9.68g
glycine	45.04g

methanol	800ml
distilled water	to 4000ml

2.2.13 Ponceau S stain

Ponceau S	5g
glacial acetic acid	0.5ml
distilled water	to 500ml

2.2.14 Tris buffer for peroxidase reaction

tris(hydroxymethyl)aminomethane base	12.11g
hydrochloric acid, dropwise	pH 7.4
distilled water	to 100ml

2.2.15 Neutrophil buffer

sodium chloride	4.09g
potassium chloride	0.19g
sodium hydrogen carbonate	0.12g
calcium chloride	0.75ml
magnesium chloride	0.1g
magnesium sulphate	0.007g
glucose	0.5g

HEPES	1.19g
distilled water	to 500ml

for calcium-free buffer the calcium chloride was ommitted and the volume made up with distilled water.

2.2.16 Carboxymethylcellulose stock

carboxymethylcellulose	2.07g
distilled water	to 100ml

this solution was autoclaved before use and stored at $4^{o}c$

2.2.17 Hypotonic lysis buffer

sodium chloride	1.5g
tris(hydroxymethyl)aminomethane base	0.3g
EDTA	1.2g
distilled water	to 100ml

2.2.18 Laemmli buffer, without dyes, for immunoprecipitation

tris(hydroxymethyl)aminomethane base	0.484g
hydrochloric acid, dropwise	to pH 7

20% sodium dodecylsulphate	5ml
distilled water	to 100ml

just prior to use:

B-mercaptoethanol	1ml
dithrieothritol	0.771g

2.2.19 RIPA buffer

tris(hydroxymethyl)aminomethane	0.605g
hydrochloric acid, dropwise	to pH7.5
sodium chloride	0.69g
nodidet P-40	1ml
sodium deoxycholate	0.5g
distilled water	to 100ml

for RIPA/SDS buffer 0.1g of sodium dodecyl sulphate was added

2.2.20 Tris buffered saline

tris(hydroxymethyl)aminomethane	0.605g
sodium chloride	0.45g
hydrochloric acid, dropwise	to pH7.4
distilled water	to 500ml

distilled water	to 500ml

2.2.21 Lysis buffer for DNA purification

EDTA	0.7448g
tris(hydroxymethyl)aminomethane	0.1211g
sodium lauryl sarkosinate	0.1g
distilled water	to 20ml

2.2.22 Loading buffer for DNA analysis

EDTA	0.0373g
sodium hydroxide	to pH 8
agarose	0.1g
bromophenol blue	0.125g
sucrose	4g
distilled water	to 10ml

2.2.23 Tris-phosphate buffer (10x stock)

tris(hydroxymethyl)aminomethane	108g
EDTA	4.85g
orthophosphoric acid (85%)	15.1ml
sodium hydroxide, dropwise	to pH 8

distilled water to	1000ml

just prior to use:

ethidium bromide	10ug

For a 1x concentrated solution, one volume of the above buffer was mixed with nine volumes of distilled water.

2.2.24 Phosphate buffered saline for preparation of samples for electron microscopy.

sodium dihydrogen orthophosphate	7.25g
sodium hydroxide	1.6g
hydrochloric acid	to pH7.2
distilled water	to 500ml

2.2.25 Fixative for electron microscopy

sodium dihydrogen orthophosphate	7.25g
sodium hydroxide	1.6g
E.M. grade gluteraldehyde	40ml
distilled water	to 500ml

2.2.26 Resin for embedding cells for transmission electron microscopy

epon 812	2.5ml
araldite 506	3.3ml
dodecenyl succinic acetate	4.2ml
dibutyl phylate	0.15ml
tris-dimethylaminomethyl phenol	0.25ml

CHAPTER THREE

METHODS

3.1 Assays of cell growth and differentiation

3.1.1 HL60 cell culture

HL60 human promyelocytic leukaemia cells were provided by Dr. G. Brown, Immunology Department, University of Birmingham and by Professor T.M. Dexter, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester. They were grown by serial subculture every 2 or 3 days in 50 or 250ml plastic tissue culture flasks with RPMI-1640 medium containing 2mM L-glutamine and 25mH HEPES, supplemented with 10% foetal calf serum (virus and mycoplasma screened), under aseptic conditions in a Flow Laboratories Gelaire BSB3 laminar flow cabinet. The cells were gassed with a mixture of 10% CO^2 in air and incubated at 37°C. The cells were maintained in logarithmic phase of growth between 1.0×10^5 and 1×10^6 per ml with the cell number determined using a Coulter Laboratories ZB1 electronic cell counter. At regular intervals the cells were screened for the presence of mycoplasma using a Flow Laboratories Mycoplasma Detection kit.

3.1.2 K562 cell culture

K562 human chronic myelogenous leukaemia cells were obtained from Dr. R. Hoffman, Charing Cross Hospital, London. They were grown using the conditions as described for HL-60

cells except for the maximum density to which they grown; K562 cells were maintained between 1 x 10^5 and 7 x 10^5 per ml.

3.1.3 Storage of cells in liquid nitrogen

Cells in logarithmic phase of growth were centrifuged for 5 minutes at 1000 rpm in a Heraeus Labofuge 6000, then resuspended at 1 x 10^6 per ml in medium containing 10% foetal calf serum plus 10% dimethylsulphoxide, before measuring 1 ml aliquots into cryotubes and freezing slowly at 1°C per minute on ring C of a Linde BF-5 biological freezer in liquid nitrogen. Cells were resurrected by thawing a cryotube at 37°C and washing the cells in 10ml of PBS (section 2.2.1) to remove the DMSO before resuspending them in 10ml fresh medium. After a 4 to 5 day period the cells were growing logarithmically. Cells were used for upto 40 passages following resurrection from the cell bank.

3.1.4 Induction of differentiation in HL-60 cells

The cells were diluted to a density of 1 x 10^5 per ml with fresh medium and the appropriate volume of drug added. A flask of cells was also set up as a control, and to this flask was added an equivalent volume of the drug solvent. After gassing the flasks were incubated at 37°C and assays of viability and differentiation were carried out daily or 96

hours after drug addition. All results are expressed as the mean of 3 experiments +/- the standard deviation.

3.1.5 Assay of cell viability by the exclusion of trypan blue

1×10^5 cells were centrifuged in a 1.5ml microcentrifuge tube for 30 seconds at 10,000 rpm using a Hereaus Labofuge. The supernatant was removed and to the cell pellet was added 5ul of a 0.1% solution of trypan blue dye in sterile PBS. The cells were resuspended before pipetting onto a microscope slide and viewing with a 10x eyepiece and 40x objective on a Zeiss microscope. Non-viable cells had taken up the dye and appeared blue in colour whereas viable cells were able to exclude the dye and remain colourless. A minimum of 300 cells were counted per sample, and the percentage of viable cells calculated.

3.1.6 Assay of HL-60 cell differentiation by the reduction of nitrobluetetrazolium

A solution of nitrobluetetrazolium (NBT) was made as follows:-

Approximately 10 mg of NBT was added to 10ml of PBS to give a saturated solution. To this was added an aliquot of 100ug of TPA (stored frozen in glass vials) dissolved in 100ul acetone.

1×10^5 cells were centrifuged in a 1.5ml microfuge tube

at 10 000 rpm for 30 seconds and the supernatant removed. 0.5 ml of the NBT solution was added to the cell pellet which was then mixed and incubated in shaking water bath at 37°C for 30 minutes. Following this the cells were centrifuged again and most of the supernatant removed before pipetting a drop onto a microscope slide and viewing with a 40x objective. Cells which had differentiated to either granulocytic or monocytic cells were stimulated by the TPA to produce an oxidative burst yielding superoxide which then reduced the NBT dye to blue/black formazan granules, causing the cells to appear purple. Undifferentiated cells remained colourless. A minimum of 300 cells were counted and the percentage of differentiated cells calculated.

3.1.7 Assay of HL60 differentiation by the capacity for phagocytosis

a) Preparation of complement-coated, red-dyed yeast particles

Yeast particles were prepared according to the method described by Shaala et al (1979) as follows:

3 grams of baker's yeast were washed twice with PBS and centrifuged at 5 000 rpm for 5 minutes using a Heraeus Labofuge 6000 and resuspended in 50 ml of PBS then autoclaved. Following this the yeast particles were washed 7 or 8 times with PBS until the supernatant remained clear.

and were then incubated at 37°C for 2 hours in 25 ml of PBS containing 0.17ml of B-mercaptoethanol. The yeast particles were washed once, alkylated by resuspension in 50 ml of 0.02M iodoacetamide in PBS (pH7.2), then incubated at room temperature for two hours checking that the pH remained at 7.2. The yeast were subsequently washed three times, autoclaved, and washed seven more times until the supernatant remained clear. The number of yeast particles per ml was determined using a Weber haemocytometer and they were resuspended at 2 x 10^9 per ml on 0.9% saline. To the yeast was added 120 ml of saline containing 260mg of procion rubine dye for a period of three hours at room temperature. The excess dye was removed by washing, and the red-dyed yeast resuspended at 2 x 10^9 per ml with 20 mg of sodium azide in 20ml of PBS, before storing at 4°C.

In order to be able to ensure efficient uptake into phagocytosing cells the alkylated, red-dyed yeast were then complement coated:-

5 x 10^9 of the yeast particles were added to 8ml of complement fixation buffer (section 2.2.2) and 2ml of rabbit serum and then incubated for 30 minutes at 37°C. After washing twice with RPMI-1640 medium and resuspending in a total of 20ml of medium, the yeast were aliquoted into 0.5ml portions and stored frozen at -20°C.

b) Assay for phagocytosis

1×10^5 HL-60 cells were centrifuged at 10 000 rpm for 30 seconds and the supernatant removed. The cell pellet was mixed with 50ul of a 1:4 mixture of RPMI-1640 medium and hepatitis-free AB+ human plasma, and 15ul of the red-dyed complement-coated yeast particles. After mixing thoroughly the cells were incubated at $37^{o}C$ for one hour, then centrifuged and the supernatant removed. 4ul of 0.1% toluidine blue in saline was added to counterstain the yeast and at least 300 cells counted by pipetting a drop of the yeast solution onto a microscope slide and viewing the slide using a 40x objective. Cells which had differentiated to either granulocytic or monocytic cells were able to take up the yeast by phagocytosis. The percentage of cells that had phagocytosed yeast particles was calculated.

3.1.8 Assay of HL60 monocytic differentiation by the production of non-specific esterases

This procedure was carried out essentially according to the method of Yam et al (1971). 2.5ml of a 2% solution of alpha-napthyl acetate in ethylene glycol monomethyl ether was added to 45 ml of PBS, followed by 3 ml of hexazotised pararosanaline (1:1 mixture of 4% sodium nitrate and 4% pararosanaline in 2M HCl). This solution was adjusted to pH 5.8 - 6.2 with 1M sodium hydroxide and filtered before use.

1×10^5 cells were centrifuged at 10 000 rpm and most of the supernatant removed. A drop of the remaining suspension was placed on a microscope slide smeared, and air dried. The cells were fixed with buffered formol acetone (section 2.2.3) for 30 seconds and washed with distilled water. The slide was air dried again then incubated in the freshly prepared staining solution for 2 hours at 37°C. After washing with distilled water the slide was counterstained with 1% methyl green in distilled water for 30 seconds, then washed again and dried before viewing with a 40x objective.

Cells which had differentiated to monocyte-like cells expressed non-specific esterases which were active between pH 5.8 - 6.2, and these were able to cleave the napthyl acetate to napthol, which then reacted with the hexazotised pararosanaline to give an azo dye, resulting in red staining of the cells. Cells which did not express the esterases stained green with the counterstain. At least 300 cells were counted and the percentage of esterase-positive cells was calculated.

3.1.9. Induction of differentiation in K562 cells

The cells were aliquoted following the same procedure as for HL-60 cells. Assays of differentiation were however carried out after 72 hours.

3.1.10 Assessment of differentiation in K562

K562 cells that have differentiated to erythrocytes contain haemoglobin and this can be detected by specific reaction with benzidine/hydrogen peroxide.

A solution of benzidine and hydrogen peroxide was made as follows:

2mg of tetramethyl-benzidine was dissolved in 1ml of 1% acetic acid and to this was added 20 ul of 30% hydrogen peroxide. 1 x 10^5 cells were harvested, centrifuged at 10 000 rpm for 30 seconds and the supernatant removed. 20ul of the benzidine hydrogen peroxide solution was added to the cells which were then incubated at room temperature for 5 minutes. The cell suspension was pipetted onto a glass slide and viewed under a 40x objective. Approximately 300 cells were scored for each sample.

The haemoglobin in the differentiated cells acts as a source of superoxide producing the Fe III form of haemoglobin methaemoglobin. This catalyses the oxidation of benzidine by hydrogen peroxide to give a blue product. Undifferentiated cells remained colourless.

3.11 Determination of the time course of commitment to differentiate

Cells were diluted to a density of 1 x 10^5 per ml with

fresh RPMI-1640 containing 10% foetal calf serum at least 24 hours before use; this ensured that the cells were in a logarithmic phase of growth at the start of the experiment. A minimum of 10ml of cells was prepared per flask and the optimum concentration of the particular drug under investigation, that is, the concentration required to bring about maximum differentiation whilst maintaining a viability of greater than 80%, was added to the flasks before they were gassed and incubated at 37^{o}C. One flask of cells was left untreated apart from the addition of the drug solvent; this was the control. At time points from 1 to 72 hours the drug removed from the medium by washing at least three times by centrifugation in a Heraeus Labofuge for 5 minutes at 1000 rpm. The cells were resuspended in 10ml of fresh drug-free medium to give a cell density of 1 x 10^{5} per ml. In the case of TPA six washes wee required to remove the drug from the cells (Hughes PhD. thesis 1987). Finally the cells were gassed and incubated at 37^{o}c. Seventy two hours from the start of the experiment all the samples were assayed for differentiation to determine the length of incubation with a particular drug required to irreversibly commit a significant proportion of the cells to differentiate.

3.2 Heat shock of cells

Cells in a logarithmic phase of growth were immersed in a Mercia Scientific water bath pre-warmed to the required

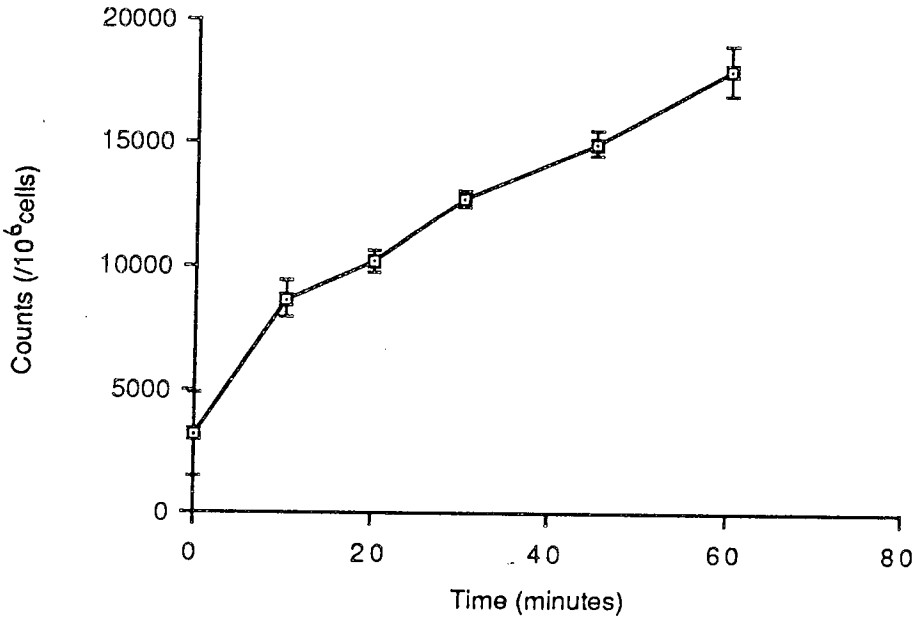
temperature and the cells left for 60 minutes. The time taken for the cells to reach the required temperature was approximately 7 minutes; this had been determined by placing a thermometer in the medium, sealing the top of the flask and taking temperature measurements every 60 seconds (data not shown). After 60 minutes the flasks were removed from the water bath and returned to 37^{o}C. Assays of viability and differentiation and measurements of DNA synthesis were taken at various times up to 96 hours after heat shock.

3.3 Measurement of DNA synthesis

DNA synthesis was measured by incorporation of [3^{H}-5-methyl] thymidine into acid insoluble material. 10^{6} cells were incubated with 0.5 uCi [^{3}H-5-methyl] thymidine (5 uCi/nmol) (Amersham, UK) for 30 minutes before filtering, in triplicate, onto 2.5 cm Whatman GF/C glass fibre filters. The filters were washed twice with 15 ml each of 0.9% saline, followed by 10% trichloroacetic acid, and then with PBS. To dry the filters they were washed with 10ml of methanol and then placed in an oven at approximately 40^{o}C for 5 minutes. 10 ml of Optiphase (Fisons, Loughborough, UK) was added to each filter and the radioactivity of measured using a Packard 2000 CA Tricarb scintillation counter. Preliminary experiments had shown that incorporation of thymidine into acid insoluble material was linear for 1 hour (figure 3.1).

Figure 3.1 Uptake of 3H-thymidine into HL60 cells

Each point represents the mean of triplicate values from three experiments.



3.4 Isolation and identification of cell proteins

3.4.1 Labelling of cell proteins with [^{35}S] methionine

To determine synthesis of proteins following heat shock or drug treatment cells were labelled with ^{35}S methionine.

Cells were treated with drug or heat shocked as required and 2 x 10^6 cells harvested from each treatment. The cells were washed twice with 10 ml of methionine-free RPMI-1640 which had been prewarmed to 37^{o}C before resuspending in 100 ul of methionine-free RPMI-1640 containing 10% dialysed foetal calf serum. To each sample was added 5 uCi of [L-^{35}S]-methionine (specific activity 1 mCi in 0.066 ml). The samples were gassed with 5% CO_2 in air and incubated in a shaking water bath at 37^{o}C for 2 hours. Following the two hour labelling period the samples were washed twice with methionine-free RPMI-1640 and resuspended in 100 ul PBS. A 10 ul aliquot was removed from each sample and mixed with 5 ml of Luma Gel scintillation fluid and counted on a Packard Tri Carb 2000CA scintillation counter. The cells were then centrifuged, the supernatant removed and the cells resuspended in 200ul sample buffer (section 2.2.4) containing dithiothreitol. The cell lysate was stored at -70^{o}C until required.

3.4.2 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

One dimensional SDS-PAGE enables polypeptides to be separated according to their size. SDS functions as the dissociating agent and it binds to the polypeptides in a constant weight ratio so that the SDS-polypeptide complexes have essentially identical charge densities. Analysis of polypeptides on polyacrylamide gels containing SDS was essentially performed according to the method initially described by Laemmli (1970).

Polyacrylamide gels were poured between 16 x 18cm glass plates, spaced 1.5mm apart, in a Bio-Rad Protean II gel system. Gel compostions were as described in table 3.1.

The protein samples were defrosted, reboiled for 5 minutes to denature the proteins and centrifuged at 13 000 rpm for 30 seconds before loading 2×10^5 cells (20 µl) onto the gel for radiolabelled samples and 7×10^5 cells for immunoblotting (see section 3.5) by means of a Hamilton syringe. 20µl of radioactive markers were loaded onto gels which were to be autoradiographed and prestained molecular weight markers for gels that were to be blotted; preparation of markers is described in sections 3.6.2 and 3.6.3.. The samples were overlayed with electrolyte buffer (section 2.2.9) and the apparatus immersed in a tank also containing electrolyte buffer. A current of 30mA per gel was applied to the system and the samples were electrophoresed for 4-5 hours

until the bromophenol dye front had migrated to the bottom of the gel. Following separation the polypeptides were stained (section 3.4.4) if the samples had been previously labelled with ^{35}S methionine, or otherwise immunoblotted (section 3.5).

Table 3.1 Gel compositions for SDS-PAGE

a) Resolving gel compostions

	7.5%	10%	15%
30% acrylamide (section 2.2.5)	10ml	13.4ml	20ml
1% bis (section 2.2.6)	7.73ml	5.2ml	3.5ml
1M tris pH 8.9 (section 2.2.7)	14.9ml	14.9ml	14.9ml
20% SDS	0.2ml	0.2ml	0.2ml
H_2O	7.10ml	6.2ml	1.3ml
TEMED	20ul	20ul	20ul
20% AMPS	0.2ml	0.2ml	0.2ml

b) Stacking gel composition

30% acrylamide (section 2.2.5)	1.67ml
1% bis (section 2.2.6)	1.3ml
1M tris pH 6.8 (section 2.2.8)	1.25ml
20% SDS	0.05ml
H_2O	5.2ml
TEMED	0.02ml
20% AMPS	0.2ml

3.4.3 Preparation of molecular weight markers

3.4.3.1 Radioactive molecular weight markers.

^{14}C-methylated polypeptides were purchased from Amersham International. 20ul of the polypeptide solution was added to 380ul sample buffer (section 2.2.4) containing dithreothrietol and placed in a boiling water bath for 5 minutes. This solution was stored at -20^{o}C. 20ul of this mixture was loaded onto the gel and this was sufficient to give bands after a 2 day exposure to film.
The molecular weights of the markers were as follows:

myosin	Mr 200 000
phosphorylase b	Mr 97 000
bovine serum albumin	Mr 69 000
ovalbumin	Mr 46 000
carbonic anhydrase	Mr 30 000
lysozyme	Mr 14 300

3.4.3.2 Prestained molecular weight markers

Prestained molecular weight markers were diluted in 0.4 ml SDS-loading buffer (section 2.2.4) without ditheiothreitol and 20 ul samples aliquoted into 1.5 ml microfuge tubes; the markers were stored at -20^{o}C until required.

Before use the markers were diluted with a further 20 ul of SDS-loading buffer (section 2.2.4), containing 50 mg/ml ditheiothreitol, and boiled for 5 minutes. The solution was allowed to cool to room temperature and 20 ul of ,marker solution loaded onto each gel.

The molecular weights of the markers were as follows:

macroglobulin	Mr 180 000
B-galactosidase	Mr 116 000
fructose-6-phosphokinase	Mr 84 000
pyruvate kinase	Mr 52 000
fumerase	Mr 48 000
lactic dehydrogenase	Mr 36 000
triose phosphoisomerase	Mr 26 000

3.4.4 Staining of polyacrylamide gels

Following electrophoresis the polypeptide bands on the gel were visualised by staining the gel with a solution of Coomassie brilliant blue (section 2.2.10) for at least 60 minutes.

The excess stain was removed from the gel by destaining in a solution of 15% methanol, 10% acetic acid (section 2.2.11). A length of pure wool was added to the destain solution to decrease the time of the destaining process.

3.4.5 Fluorography of labelled polyacrylamide gels

In order to improve the efficiency of the autoradiography process a scintillant was incorporated into the polyacrylamide gel. In 1975 Bonner et al reported that an increased detection efficiency of over 10-fold for ^{35}S could be obtained using the scintillator PPO (Bonner and Laskey 1974).

The stained polyacrylamide gel was washed twice in 20 volumes of HPLC grade DMSO for 30 minutes each wash and then soaked for 60 minutes in 4 volumes of a 22.5% solution of PPO in DMSO. The PPO was then precipitated by dropping the gel into cold water. Following this the excess DMSO was removed from the gel by washing for 2 hours in cold running water; the gel was then ready to be dried and autoradiographed as described in section 3.4.6.

3.4.6 Autoradiography of labelled polyacrylamide gels

The dried gel was placed on a sheet of Kodak X-Omat AR film in a Kodak cardboard exposure cassette and stored at $-70^{o}c$ for periods upto 7 days depending on whether the gel had been fluorographed or not.

Following exposure the film was developed in Kodak GBX developing solution for 5 minutes and fixed for 5 minutes in Kodak GBX fixative. Fluorographs were photographed using Kodak technical pan film.

3.5 Immunoblotting of proteins

Immunoblotting of proteins was used to determine the total amounts of a particular protein of interest in whole cell extracts and crude subcellular fractions.

3.5.1 Electrophoretic transfer of proteins onto nitrocellulose

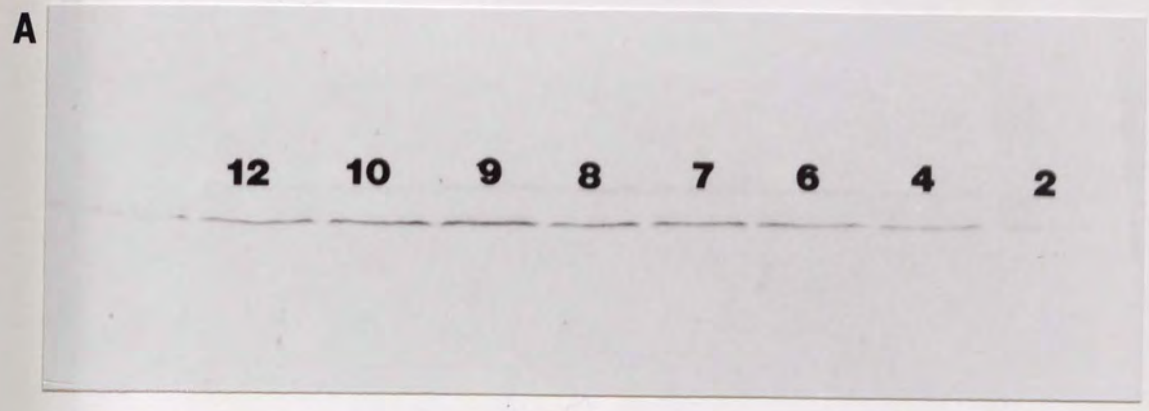
The method used for electrophoretic transfer was essentially that as described by Towbin et al. (1979).

Protein samples to be analysed by immunoblotting were first subjected to polyacrylamide gel electrophoresis under denaturing conditions (section 3.4.2). The number of cells that could be loaded per sample without exceeding the limits of either the electrophoretic transfer onto nitrocellulose, the sensitivity of the antibody or the staining procedure was determined before experimental samples were analysed (figures 3.3.2, 3.3.3 and 3.3.4). It was determined from these calibration blots that for the 72kD and AC88 antibodies $7x10^5$ cells should be loaded for analysis; this number of cells was used since it allowed for increases and decreases in hsp70 and hsp90 to be detected. For actin samples, $2x10^5$ cells were loaded as more cells gave non-specific binding. Following electrophoresis the gel was washed in blotting transfer buffer (section 2.2.12) under constant agitation for 30 minutes at room temperature to allow the gel to swell

(this was particularly important when a 15% gel had been used). The proteins were transferred to 0.45μ nitrocellulose membrane (Bio Rad) using a Bio Rad Transblot apparatus; a current of 150 mA was applied to the apparatus for 4 hours. After transfer the nitrocellulose membrane was blocked by washing in a solution of 1% Marvel in PBS for at least 30 minutes with constant agitation. The membrane was rinsed twice with PBS and was either dried between 2 sheets of Whatman 3MM paper and stored at room temperature or immediately used for incubation with an antibody against a particular protein of interest (section 3.5.2). The polyacrylamide gel was stained with Coommasie blue (section 3.4.4) after transfer to ensure that equal amounts of cells had been loaded in each lane and that even transfer had taken place.

Figure 3.2 Calibration blot for 72kD antibody

Increasing numbers of HL60 cells separated by SDS-polyacrylamide gel electrophoresis then electroblotted. The nitrocellulose blot was probed with 72kD antibody (A). Cell numbers are $x10^5$.
Laser densitometry of calibration blot to determine the number of cells to be used (B).



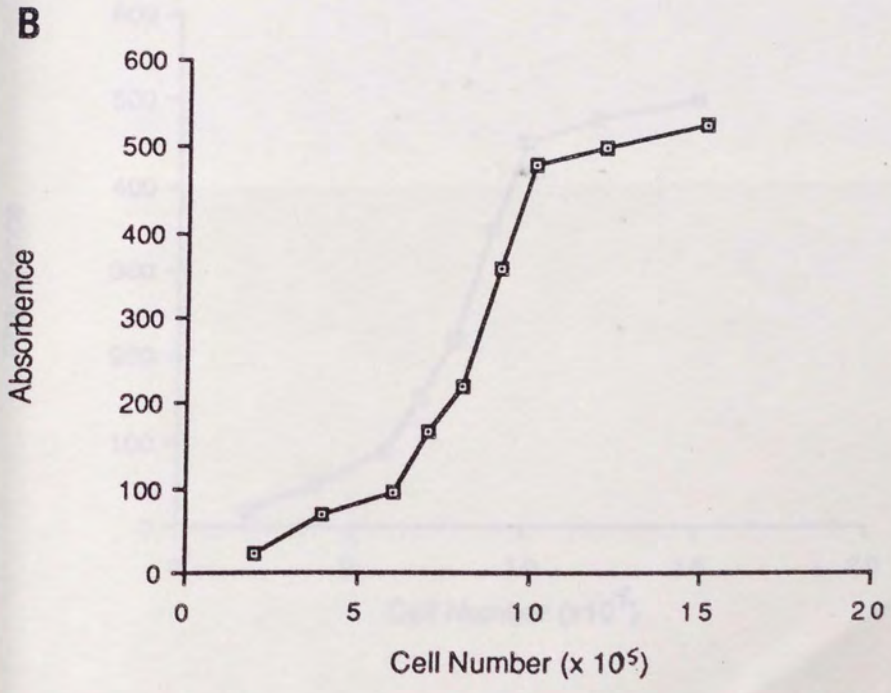
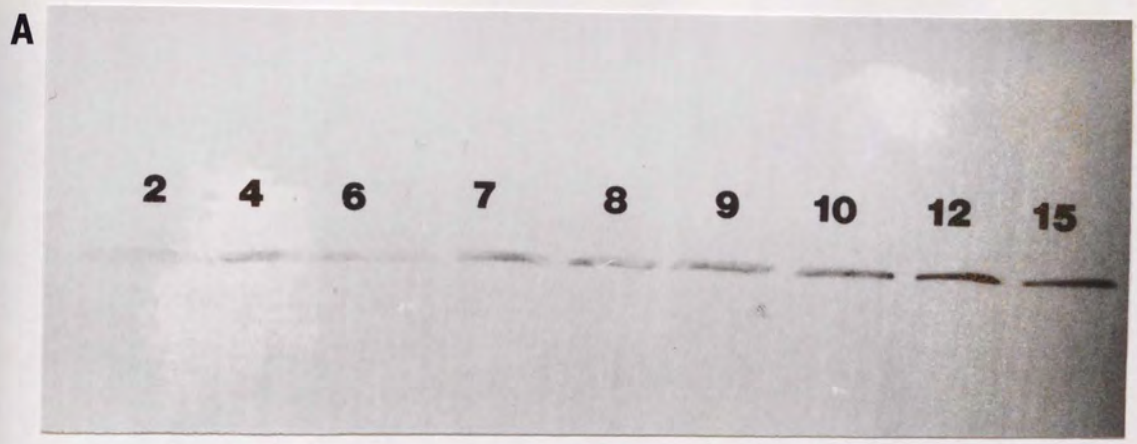


Figure 3.3 Calibration blot for AC88 antibody

Increasing numbers of HL60 cells separated by SDS-polyacrylamide gel electrophoresis then electroblotted. The nitrocellulose blot was probed with AC88 antibody (A). Cell numbers are $x10^5$.
Laser densitometry of calibration blot to determine the number of cells to be used (B).



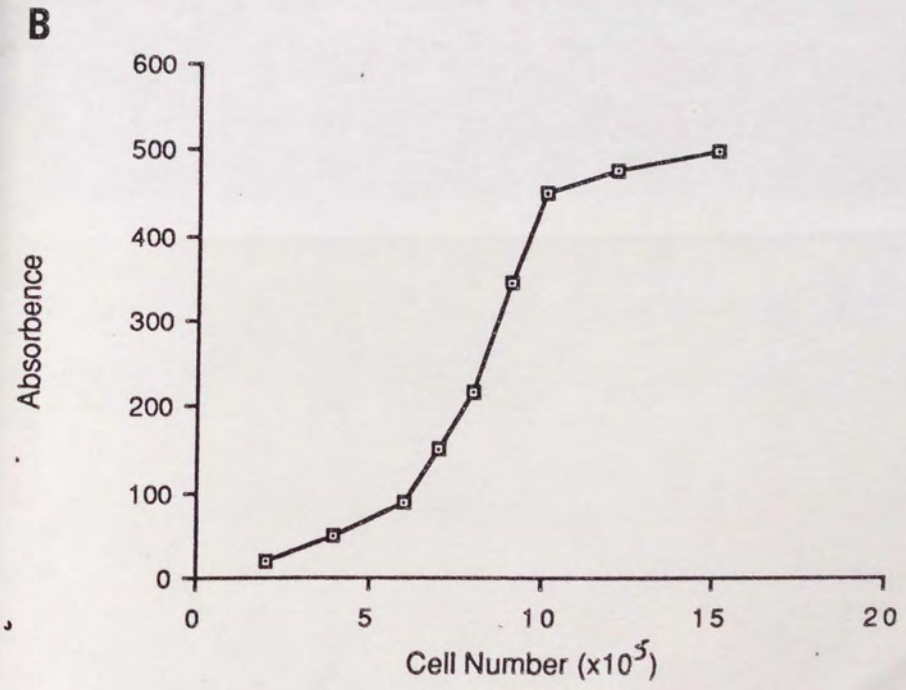
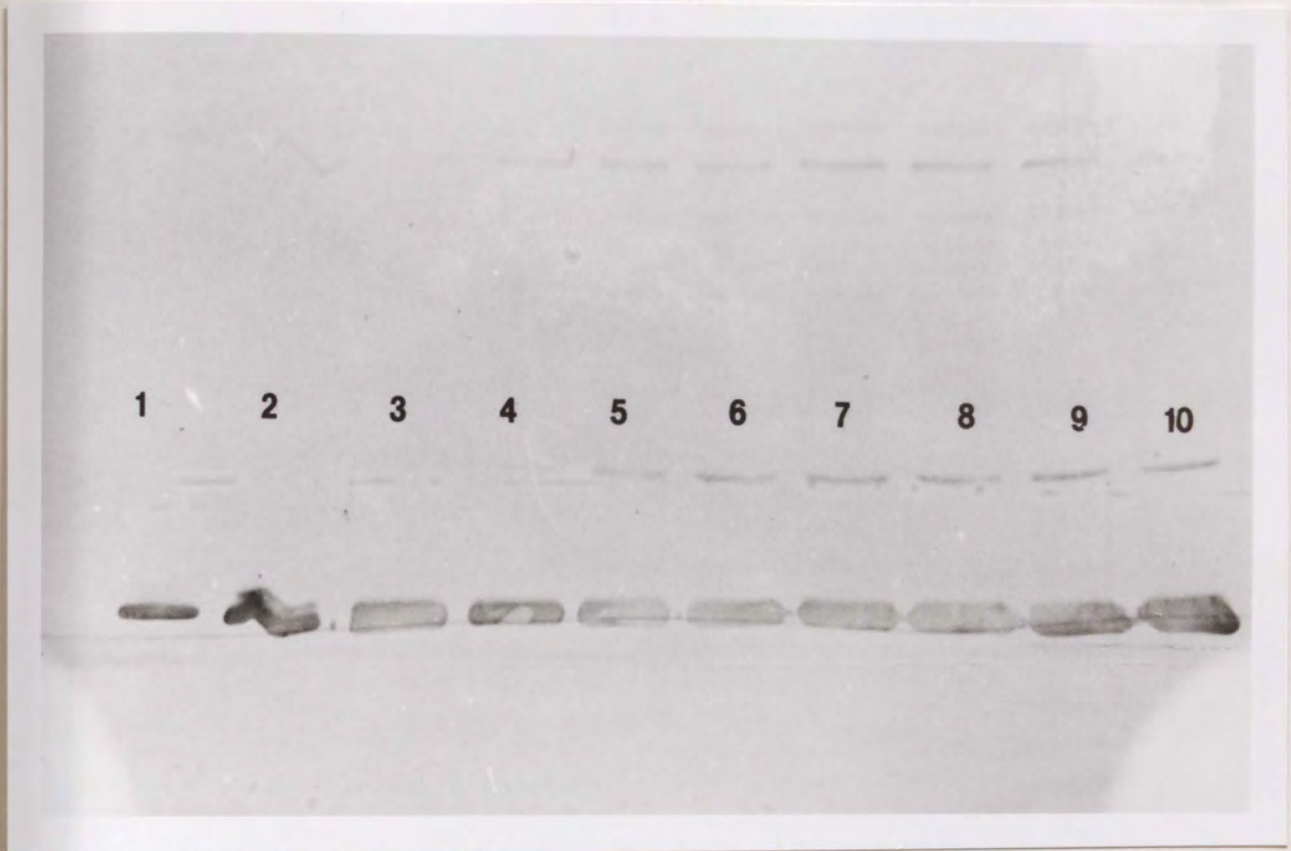


Figure 3.4 Calibration blot for actin antibody

Increasing numbers of HL60 cells separated by SDS-polyacrylamide gel electrophoresis then electroblotted. The nitrocellulose blot was probed with an anti-actin antibody. Cell numbers are $x10^5$.



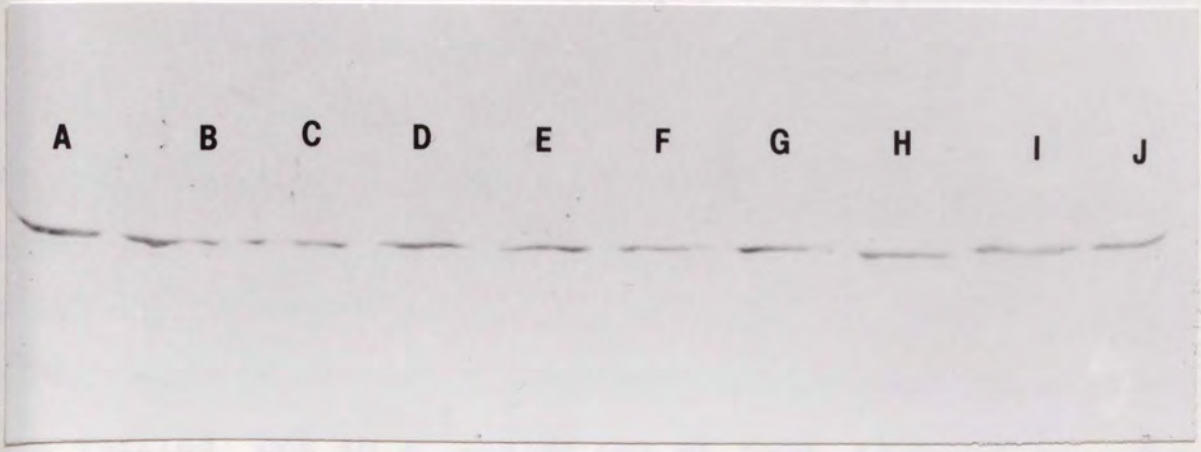
3.5.2 Immunological identification of cell proteins

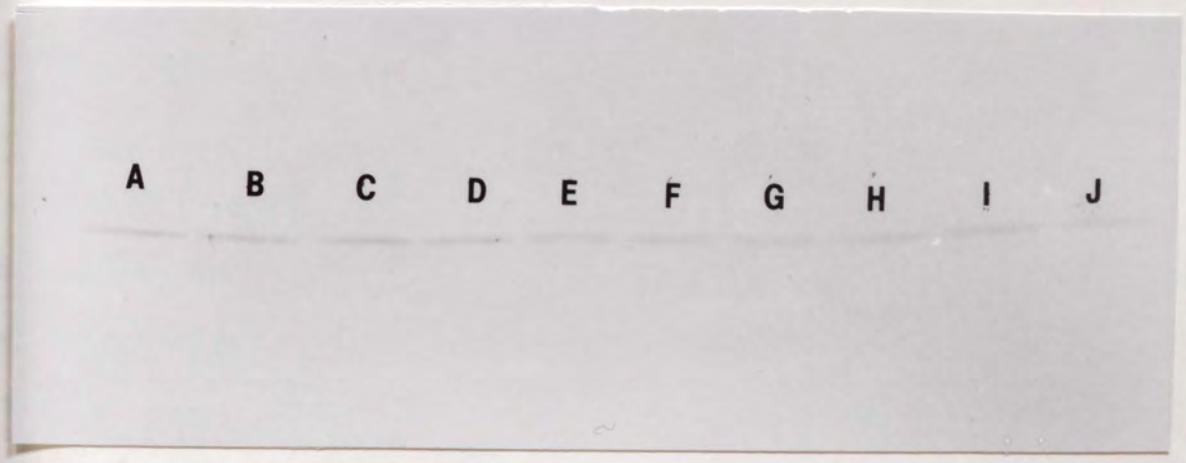
Initial experiments were performed to determine that any staining of protein bands observed was specific for the antibody in use. This was done by incubating the blocked nitrocellulose membrane with the biotinylated sheep anti-mouse IgG followed by the streptavidin-peroxidase (section 3.5.3). When this blot was incubated with developing solution no staining was observed. A blot that had been incubated with primary antibody was also incubated with streptavidin-peroxidase followed by developing solution; again no staining was observed. Control experiments such as these were performed using all of the cell lines described.

It was important to establish that antibodies bound with the same efficiency across all of the membrane since comparisons were to be made between lanes. 7×10^5 HL60 cells were electroblotted and the blocked membrane incubated with 72kD monoclonal antibody. The blot was then developed as described in section 3.5.3. From laser densitometry it was shown that the intensity of the stained protein bands was comparable across the width of the blot (figure 3.5).

Figure 3.5 Immunoblot of HL60 cells to demonstrate uniform transfer of protein during electroblotting and uniform staining with antibody across a blot.

Nitrocellulose blot of equal numbers of HL60 cells separated by SDS-polyacrylamide gel electrophoresis then electroblotted and probed with 72kD antibody.





3.5.2.1 Members of the hsp70 family

Blocked nitrocellulose membranes were incubated overnight in either a 1:500 dilution of Amersham 72kD monoclonal antibody which recognises hsp70, or a 1:1000 dilution of 7.10 monoclonal antibody (kindly provided by Dr. S. Lindquist) which recognises all four members of the hsp 70 family (Velazquez et al. 1983), or a 1:5 dilution of antibody 5A5 which recognises hsp70 and p72 (kindly provided by Dr. R. Morimoto. Antibodies were diluted in sterile PBS containing 0.1% BSA with a minimum of 5 ml of antibody solution per 16 cm square membrane; all antibody incubations were carried out in a sealed bag at room temperature. The membranes were washed three times in 0.1% Tween 20 in PBS following the overnight incubation.

3.5.2.2 Members of the hsp90 family

A monoclonal antibody raised against hsp 90 and grp94 was kindly provided by Dr. D. Toft. The lyophilised antibody was diluted in 1 ml of sterile PBS to give a concentration of 1 mg/ml and 50ul aliquots were stored at -70°C until required.

The blocked nitrocellulose membrane was incubated overnight with 50 ug of hsp90 in 5 ml of sterile PBS. Following this the membrane was washed 3 times in PBS/Tween 20.

3.5.2.3 Actin

A monoclonal antibody that recognised actin was used to determine the amounts of a "house-keeping" protein following treatment with toxic concentrations of drugs. It was also used to determine where one cytoskeletal protein was isolated following fractionation.

Protein samples that were subsequently probed for actin were separated on a 15% polyacrylamide gel before electroblotting. The blocked nitrocellulose membrane was incubated for an hour in a 1:750 dilution in PBS of actin monoclonal antibody (Amersham, UK) and then washed 3 times in PBS/Tween 20.

3.5.2.4 Histone H2B

A mouse monoclonal IgM coded 3D9-B11-1D9, which was specific for mammalian histone H2B, was kindly provided by Dr. B. Turner, University of Birmingham. This antibody was used to determine the location of histone proteins in the cell following treatment with heat and toxic concentrations of drugs.

The blocked nitrocellulose was incubated overnight in culture medium that had been collected from the antibody producing hybridoma cells. The blot was washed as previously described for other antibodies.

3.5.3 Antibody detection

The antibodies were detected using the biotin-streptavidin system supplied by Amersham International. The anti hsp 72, 7.10, hsp 90 and actin antibodies were all mouse ascities fluid and were detected by means of a 1:330 dilution of biotinyalated sheep anti-mouse IgG. The membrane was incubated for 2 hours at room temperature on a shaker. The H2B antibody was of the IgM class so the biotinylated anti-IgG could not be used as the secondary antibody; in its place a goat anti-mouse IgM linked to biotin was used at a dilution of 1:330. All antibody dilutions were made in sterile PBS with a minimum of 5 ml of solution per 16 cm square membrane and the incubations were carried out in a sealed plastic bag.

After incubating the membranes in the biotin solutions they were washed 3 times in PBS/Tween 20 before incubating in a 1:330 dilution of streptavidin-peroxidase for 30 minutes, again in a sealed bag. This stage and the subsequent stages were the same regardless of the first antibody used. The membranes were washed twice in PBS/Tween 20 and twice in PBS for a total of 40 minutes before visualising the bands. A developing solution was prepared as described by Nakene et al (1968) by mixing solution A, consisting of 1.5 g NaCl, 1.0 ml 1M Tris HCl pH 7.5 (section 2.2.14), and distilled water to 50 ml, to which 50 ul 30% hydrogen peroxide was added, with solution B, consisting of 30 mg 4-chloro-1-napthol dissolved

in 10ml methanol. The developing solution was added to the washed membrane under constant shaking. Purple-blue bands appeared after approximately 5 minutes and once they had appeared the membrane was washed 3 times in distilled water before drying between Whatman 3MM paper and storing at room temperature in the dark. The nitrocellulose blots were photographed using Kodak Technical Pan film.

If required, blots were then stained for the total protein transferred by means of the dye Ponceau S. The nitrocellulose blot was incubated for 2 minutes in a solution of Ponceau S (section 2.2.13) then destained in 0.1% acetic acid until the background was pale and bands of protein were clearly visible.

3.6 Determination of cytoplasmic free calcium in HL-60 cells

3.6.1 Loading of HL60 with quin 2

The concentration of cytoplasmic free calcium in HL60 cells was used as a measure of membrane integrity and was assessed by quin 2 fluorescence.

Following drug-treatment 1.5×10^7 cells were harvested, resuspended in 10 ml of fresh RPMI-1640 containing 10% foetal calf serum and 20um quin-2 acetoxylmethyl ester (quin 2), a highly selective and fluorescent Ca^{2+} indicator, and incubated at $37^{o}C$ for 60 minutes. This acetoxymethyl ester form of quin 2 readily permeates the cell membrane but once

it is in the cell it is hydrolysed in the cytoplasm and trapped within the cell. The cells were then resuspended in 2 ml of neutrophil buffer (section 2.2.15) and transferred to a 1 cm^2 quartz cuvette. The fluorescence was monitored using a Perkin-Elmer LS-5 spectrometer equipped with a thermally controlled cuvette holder and a magnetic stirrer; the excitation and emission wavelengths were 339 and 492 nM. To measure the intracellular free calcium the method described by Tsien et al. (1982) was used. The cells were incubated in the spectrofluorimeter for 10 minutes to obtain a steady-state fluorescence reading (F) before lysing with 20 ul of a 10% solution of Triton X-100 in distilled water. This led to a rise in fluorescence (F_{max}) as calcium from the medium was able to enter the cell and bind to free quin 2. Finally all the calcium was chelated by the addition of 200 ul of 250 nM EGTA causing a decrease in fluorescence (F_{min}). The intracellular calcium concentration was calculated as follows:-

$$[Ca2+]_i = Kd \frac{(F - F_{min})}{(F_{max} - F)}$$

where Kd is the effective dissociation constant of quin 2 from Ca^{2+} and had a value of 120.

Resting calcium concentrations of less than 200nM were considered to be indicative of healthy cells.

3.6.2 Incorporation of Calcium 45

The cytoplasmic free calcium in HL60 cells that had been treated with Adriamycin could not be measured using quin 2 due to the fluorescence of Adriamycin at 492nM. As a result of this the calcium levels were measured by uptake of radiolabelled calcium.

15×10^6 cells were harvested from control samples and from samples following incubations with 1uM Adriamycin. These samples were washed twice in calcium-free neutrophil buffer (section 2.2.15) by centrifugation at 1000 rpm for 5 minutes and then resuspended in 2 ml of the same buffer. 15 ul of calcium 45 (specific activity 40mCi/mg calcium) was added to the cells which were then incubated at 37°C; 400 ul aliquots were removed at 1, 3, 5, 10 and 15 minute intervals and filtered on Whatman GF/C glass fibre filters. The filter was washed 3 times with calcium free neutrophil buffer, once with methanol and then dried in an oven at approximately 20°C for 5 minutes. 10 ml of Optiphase (Fisons, Loughborough, UK) was added to each sample and the radioactivity counted using a Packard 2000 Tricarb scintillation counter.

3.7 Cloning of HL60 cells in semi-solid media

Due to poor cloning efficiencies (around 10%) achieved when HL60 cells were cloned in agar, low viscosity carboxymethylcellulose was used as the supportive media.

Conditioned medium was routinely collected from exponentially growing stocks of HL60 cells and was stored at $4^{o}C$; before use the media was supplemented with 2mM glutamine. A stock solution of carboxymethylcellulose (section 2.2.16) was diluted to a final concentration of 0.83% by the addition of 5% foetal calf serum and conditioned media and was prewarmed to $37^{o}C$ before use. Following drug treatment the cells were washed twice in PBS and 500 cells were added to 1ml carboxymethylcellulose and aliquoted out into 3.5cm multiwell dishes; cells were plated out in triplicate and were incubated at $37^{o}C$ in an atmosphere of 5% CO_2 in air for 10 days when colonies of greater than 50 cells were scored. Untreated cells were cloned in parallel and the results were expressed as follows

$$\frac{\text{number of colonies in treated wells}}{\text{number of colonies in control wells}} \times 100\%$$

The mean plating efficiency, that is the mean number of colonies that grew in the control wells as a percentage of the number of cells plated out was 57.6% (+/- 9.59%).

3.8 Preparation of cells for cell cycle analysis by flow cytometry

Cell cycle analysis was performed by flow cytometry (FCM) according to the method of Gray and Coffino (1979). FCM enables simultaneous analysis of multiple parameters and the rapid observation of individual cells in aqueous suspension at the rate of several thousand per second.

After drug treatment 10^6 cells were harvested per sample and washed twice by centrifugation in ice-cold PBS. The cells were then fixed in 1 ml 70% ethanol whilst under constant agitation to prevent the cells clumping, and stored at $4^{o}C$ in the dark for up to 2 weeks.

The fixed cells were centrifuged at 1500 rpm, the ethanol removed, and the cells washed once in PBS. 0.5 ml of propidium iodide staining solution (100ug/ml) was added along with 0.5 ml of a solution of RNA-ase in PBS (100ul/ml); the RNAase degraded the RNA so that only DNA intercalation with the flurochrome was recorded. The cells were transferred into Falcon 2054 tubes and flow cytometric analysis carried out using a Becton Dickinson 440 Fluorescence Activated Cell Sorter with an argon laser in the Dept. of Immunology, Birmingham University. 10^4 individual cells were analysed per sample at a flow rate of 10^3 cells per second. Cells were stained with the propidium iodide no more than 12 hours before analysis due to decay of the fluorescent signal.

The fluorescence (540nm excitation and 625 nm emission

for propidium iodide, related to DNA content per cell) was determined for each cell and the results plotted as a histogram of the number of cells against fluorescence using a Consort 40 computer; the fluorescence histogram enabled the cell cycle phase of the cells to be determined. The peak to the left corresponded to cells with a diploid DNA content, that is, cells in G_o or G_1 phase, the central plateau corresponded to cells synthesising DNA in S phase, and the peak to the right represented tetraploid cells in G_2 and M phases. The percentage of cells in each phase of the cell cycle was determined from the area under the curve assuming a normal population. The cells with low DNA fluorescence were considered non-viable and were excluded from cell cycle calculations but those cells were used as a measure of cell viability to confirm the results obtained with trypan blue.

3.9 Localisation of proteins in HL60 cells following heat shock or treatment with toxic concentrations of drugs

Crude nuclear and cytoplasmic fractions of HL60 cells were immunoblotted and the blots were probed with either 72kD, anti H2B or an actin antibody.

Following either a heat shock (43.5°C for 1 hour then 37°C for 2 hours) or incubation with drugs, $1x10^7$ cells were washed twice in PBS then lysed in 250ul of ice-cold hypotonic buffer (section 2.2.17) for 5 minutes. Following lysis the cells were homogenised on ice for 2 minutes; to confirm that

all the cells had been lysed, a 5 ul aliquot was removed and viewed under a microscope as described in section 3.1.5. To prepare a cytosolic and a nuclear fraction the homogenate was centrifuged at 2000g for 10 minutes at 4^{o}C after which the supernatant (cytosolic fraction) was removed. The pellet (nuclear fraction) was resuspended in 250 ul hypotonic buffer to ensure equal salt concentration in both fractions, and to both fractions was added 50 ul 3x sample buffer (section 2.2.4). A parallel sample of control cells was also fractionated. 50 ul of both fractions were loaded onto a 7.5% polyacrylamide gel for hsp70 analysis or onto a 15% gel for analysis of actin and H2B, and then electroblotted as described in section 3.5.1. The nitrocellulose membranes were probed with antibodies against hsp70 (section 3.5.2.1), actin (section 3.5.2.3) or H2B (section 3.5.2.4).

3.10 Determination of the half-life of hsp 70

3.10.1 Pulse-chase labelling of cell proteins

The half life of hsp 70 under different conditions was calculated by immunoprecipitating pulse chased samples with the 72 kD antibody, running the precipitate on a 10% polyacrylamide gel and determining the time course of the decay of the radioactive signal.

HL60 cells in an exponential phase of growth were left untreated or treated as required so that 1 x 10^{7} cells were

harvested at the end of the treatment. The cells were washed twice in methionine free RPMI-1640 medium by centrifugation at 1000 rpm for 5 minutes, incubated in methionine free RPMI-1640 for 30 minutes to deplete the methionine pools, and then resuspended in 1000ul of methionine free RPMI-1640. 150 uCi [$L^{35}S$] methionine was added to the cells and incubated at 37°C for 60 minutes shaking continuously. Following this the cells washed twice with RPMI-1640 and resuspended in 40 ml RPMI-1640 containing 10% foetal calf serum and incubated at 37°C. At time points 0, 30, 60 and 120 minutes 2 x 10^6 cells were removed, washed twice in sterile PBS and resuspended in 150ul Laemmli sample buffer without dye (section 2.2.18). The samples were boiled for 5 minutes and sonicated for 30 seconds before freezing at -70°C until required.

3.10.2 Immunoprecipitation of hsp 70

The cell lysate was diluted 1:10 with RIPA buffer (section 2.2.19) so that the final concentration of SDS was 0.1% before preabsorbing with 50 ul of 50% protein-A agarose in RIPA/SDS (section 2.2.19) for 30 minutes at room temperature, shaking continuously. The solution was clarified by centrifuging at 4000 rpm for 5 minutes and the protein-A beads discarded.

The Amersham anti-hsp 72 antibody was diluted 1:5 in sterile PBS and 3 ul of this diluted antibody added to the supernatant of each sample overnight at room temperature.

Following this incubation 5 ul of sheep anti-mouse secondary antibody was added for 2 hours followed by 50 ul of 50% protein-A agarose in RIPA for 60 minutes; both incubations were carried out at room temperature. The protein-A beads were then pelleted by centrifugation at 4000 rpm for 5 minutes and washed 5 times in 1ml of RIPA/SDS buffer. After the final wash the pelleted beads were resuspended in 35 ul of 2 x Laemmli sample with dyes (section 2.2.4) and boiled for 10 minutes. The beads were then pelleted and the supernatant removed and saved. 35 ul of distilled water was added to the beads and they were boiled for a further 10 minutes before centrifuging and adding the supernatant to the first supernatant which contained the dye.

The samples were run on a 10% polyacrylamide gel as described in section 3.4.2 and stained/destained. The stained gel was then dried and autoradiographed for 7 days as outlined in section 3.4.6. The intensity of the bands corresponding to hsp70 was determined by laser densitometry.

3.10.3 Recovery of radioactive counts from the polyacrylamide gel

Following autoradiography the dried gel was rehydrated and the radioactivity recovered by solubilising the gel.

Each hsp 70 band was excised from the gel and rehydrated by adding 0.5 ml distilled water and incubating for 30 minutes at 37°C. 1.5 ml of Protosol (NEN) was added to each

sample and the solution incubated at 37°c for a further 2 hours. 10 ml of Optiphase (Fisons, UK), was added to each sample and the solutions left for 2 days at room temperature before counting on a Tri Carb 2000 scintillation counter.

3.11 Analysis of DNA

The presence or absence of DNA cleavage in HL60 cells after treatment with toxic concentrations of either N-methylformamide, CB3717 or Adriamycin was detected by lysing the cells and separating the intact DNA from any that had been degraded, on an agarose gel.

After drug treatment 10^6 cells were harvested per sample, transferred to a cold sterile microfuge and washed once in cold tris buffered saline pH 7.4 (section 2.2.20). The cells were pelleted by centrifugation at 6000 rpm and all the supernatant removed; all these steps were carried out at 4°C. At this point the cells could be stored frozen at -70°C until required or used immediately.

The cells were lysed by resuspending in 20 ul lysis buffer (section 2.2.21) containing 0.01 mg proteinase K and incubating at 50°C for 24 hours after which the condensate was spun down from the lid. The lysate was extracted once in a 1:1 solution of phenol chloroform then 10 ul of 0.5 mg/ml RNAase A was added to digest the RNA and the cell lysate incubated at 50°C for a further hour. The samples were then heated to 70°C, the condensate spun down from the lid and 10

ul loading buffer (section 2.2.22) added before vortexing the tube and reheating to 70°C. All of the sample was loaded into the dry wells of a 2% agarose gel in tris-phosphate (section 2.2.23) containing 0.1ug ethidium bromide, using a siliconised pipette tip. The agarose in the samples was allowed to solidify for 5 minutes and any air bubbles removed. The gel was finally submerged in tris phosphate buffer and run at 40 mV for approximately 2 hours to allow the bromophenol blue dye front to migrate 3-4 cm. The DNA could be visualised using an ultra-violet trans-illuminator. If any cleavage had occurred low molecular weight oligonucleotide fluorescent bands could be seen on the gel; if no laddering had occurred and the DNA was intact, an intense band of fluorescence could be visualised at the top of the gel, and if cellular necrosis had occurred the degraded DNA formed a "smear" down the length of the gel.

3.12 Electron microscopy

3.12.1 Preparation of cells for scanning electron microscopy

To investigate the effects of NMF, Adriamycin and CB3717 on HL60 morphology, the cells were viewed by scanning electron microscopy (SEM).

Following drug-treatment 10^6 cells were harvested, pelleted by centrifugation at 1500 rpm and washed once in E.M. PBS (section 2.2.24). The cells were resuspended in 10

ml of ice-cold E.M. PBS containing 2.5% gluteraldehyde (section 2.2.25) and fixed for 1 hour at 4°C. After fixing the cells were washed twice in E.M. PBS to remove the gluteraldehyde fixing solution and twice in sterile distilled water to remove the phosphate crystals. The cell pellet was resuspended in 50 ul sterile distilled water and 25 ul of the suspension dropped onto each of two formvar coated grids which were then left to dry overnight at room temperature.

The grids were splutter-coated with platinum and scanned by Lesley Tompkins, Physiology Dept., Birmingham University.

3.12.2 Preparation of cells for transmission electron microscopy

Transmission electron microscopy (TEM) was performed to assess the effect of NMF, Adriamycin, and CB3717 on HL60 ultrastucture.

2×10^6 cells were harvested, pelleted by centrifugation at 1000 rpm, washed once in E.M. PBS (section 2.2.24), resuspended in PBS then fixed using a solution of 2.5% gluteraldehyde in E.M. PBS (section 2.2.25) for 1 hour at 4°C. The cells were washed in E.M. PBS and the cell pellet transferred to a 0.4 ml microfuge tube. Sections of cells of approximately 2mm in thickness were made by cutting through the microfuge tube. The discs were washed in a 1% solution of ozmium tetroxide in PBS for 1 hour following which the cells were removed from the microfuge disc using a

microspatula. The pellet was washed twice in E.M. PBS and then dehydrated by washing for a minimum of 20 minutes in solutions of 50%, 70%, 80%, 90% then 100% ethanol. The cell pellet was washed twice in propylene oxide for 30 minutes, then for 2 hours in a 1:1 solution of embedding resin (section 2.2.26) and propylene oxide before leaving overnight in a 3:1 solution of embedding resin and propylene oxide. Finally, the cell pellet was transferred to a plastic bottle cap filled with embedding resin and left overnight at 60°C.

The embedded cells were sectioned by Derek Mills, Electron Microscopy Dept., Birmingham University and viewed with his assistance.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Heat shock and differentiation

4.1.1 Induction of HL60 differentiation by a heat shock

Francis Richards in this laboratory had demonstrated that a heat shock of 43.5°C for 60 minutes followed by a further 72 hour incubation at 37°C could induce differentiation, as assessed by reduction of the dye NBT, in 20% of HL60 cells and at this temperature the cells synthesised heat shock proteins of molecular weight 70 and 90kD (Richards et al 1988). It had also been demonstrated by Richards (PhD. thesis 1987) that known inducers of stress proteins or hsps were able to induce, to varying degrees, HL60 differentiation.

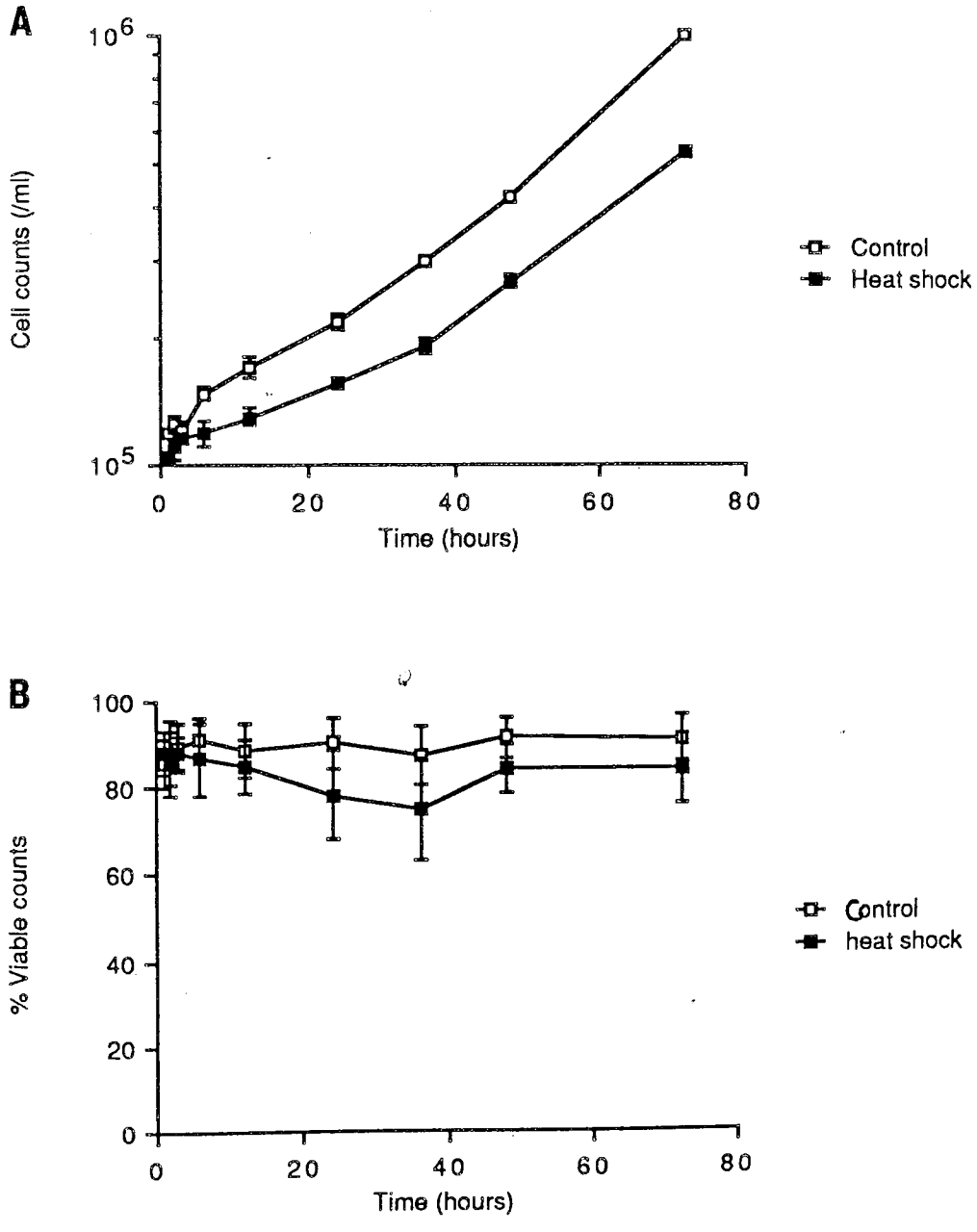
Heat shock proteins are produced when cultured cells or whole organisms are exposed to a variety of stresses such as a heat shock or heavy metals (section 1.6). As discussed in section 1.5, it is conceivable that it is the response of cells to a sub-toxic concentration of a drug that may induce differentiation following the observations made by Chow et al (1987). A role for stress proteins in differentiation is therefore possible since these proteins are thought to aid in the cells recovery from sub-lethal damage.

Initial experiments were carried out to determine the time course of induction of differentiation by a heat shock. HL60 cells provided by Dr. G. Brown, University of Birmingham

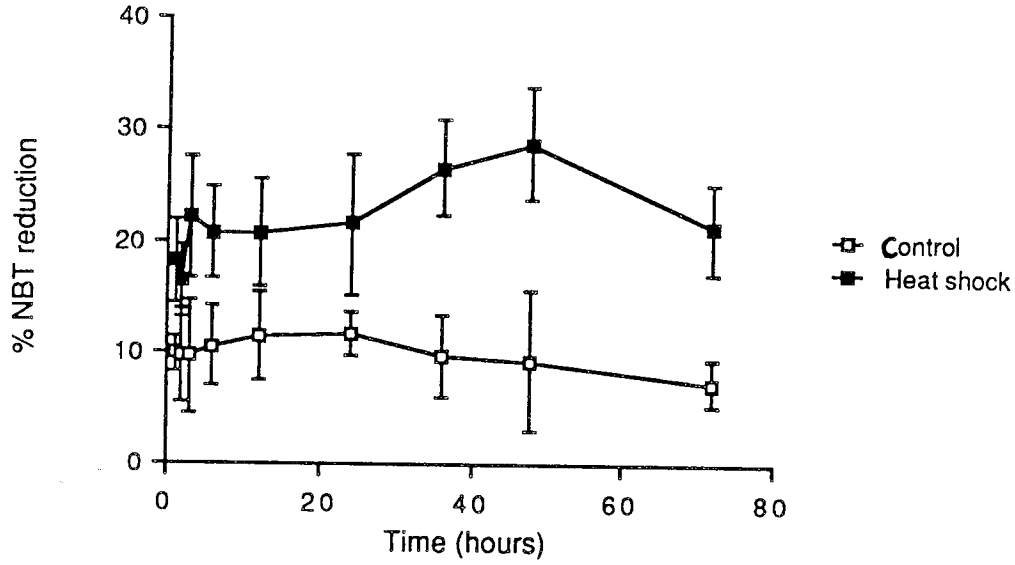
were subjected to a heat shock of 42.5°C for 60 minutes (a heat shock of 43.5°c for 60 minutes was found to be toxic to this subline of cells, data not shown). Cells that had been heat shocked were growth inhibited for the first 6 hours but then grew exponentially with a doubling time of approximately 18 hours which was comparable to that in control cells (figure 4.1). There was no significant loss in viability with heat shocked cells greater than 70% viable throughout the time course. After two hours 18.04% of cells were able to reduce NBT with maximum differentiation (28.6%) at 48 hours after a heat shock. Although the percentage of control cells able to reduce NBT was high, the percentage of positive heat shocked cells was significantly higher as determined by the Students T-test ($p<0.05$ at 2 hours and $p<0.005$ at 48 hours: $n=3$). Neither the control or heat shocked cells expressed non-specific esterase activity at pH 5.8 to 6.2. There was no significant fall in the incorporation of ^{3}H-thymidine following a heat shock but due to the 75% of cells that were uncommitted it is not possible using this method to confirm whether or not the 25% of cells that were expressing markers of a more mature phenotype were terminally differentiated. However since the cells are only exposed to heat for an hour and still continue to express markers of differentiation 72 hours later it can be assumed that they have terminally differentiated.

Figure 4.1 The induction of differentiation in HL60 cells heat shocked at 42.5°c

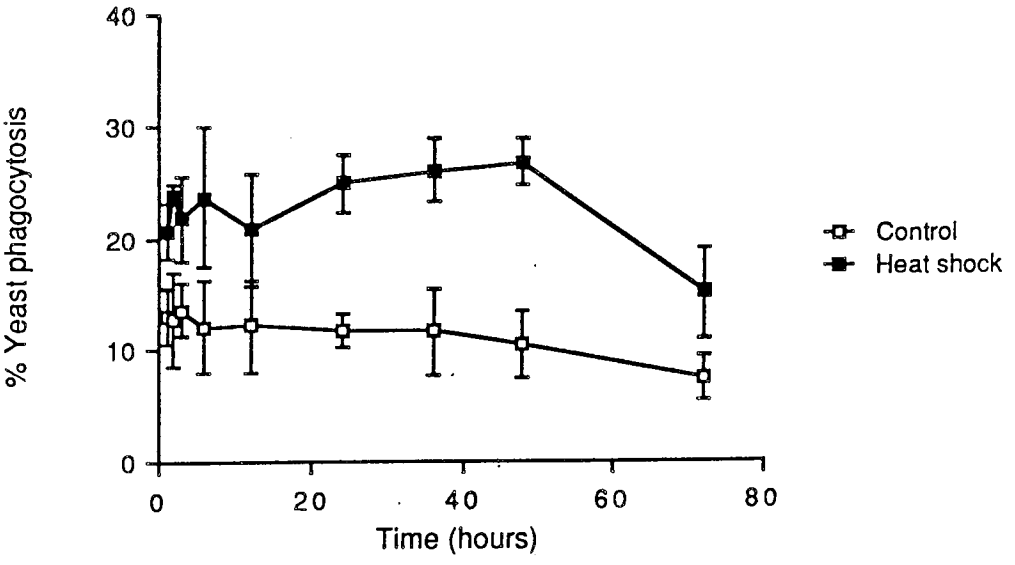
Cells were heated to 42.5°c for 1 hour then returned to 37°c and cell growth (A), cell viability (B), NBT reduction (C) and yeast phagocytosis (D) were determined at intervals up to 72 hours after the heat shock.



C


40
30
20
10
0
% NBT reduction
0
20
40
60
80
Time (hours)
Control
Heat shock

D


40
30
20
10
0
% Yeast phagocytosis
0
20
40
60
80
Time (hours)
Control
Heat shock

4.1.2 Discussion

This data agrees with that previously obtained by Francis Richards, using a different subline of HL60 cells, and shows that heat was a weak inducer of differentiation and that induction of differentiation occurred at a temperature which was marginally below that which was cytotoxic. What was surprising about the results was that the markers of differentiation were expressed after 2 hours and that the cells were committed to terminal differentiation after only a one hour exposure to the inducer.

The fact that the percentage of cells that differentiated after a heat shock was low may be due to the inhibitory effect of a heat shock on normal protein synthesis (Ashburner and Bonner 1979). For a cell to differentiate there must be a requirement for protein synthesis for the expression of a mature phenotype; in heat shocked cells inhibition of the synthesis of these proteins may well prevent many cells from differentiating. Heat shock is also known to cause unfolding of tertiary protein structure (Pfeil 1987) which would have an adverse effect on the ability of cells to produce the functional proteins necessary for differentiation. Low levels of differentiation may also be due to the non-specificity of a heat shock in terms of the stress proteins that it induces, and the intracellular localisation of the damaged protein. The major proteins synthesised immediately following a heat shock are hsp70,

hsp72, hsp90 and hsp110 with synthesis of grp78 a much later event. Hsp70 is known to translocate to the nucleus after a heat shock (Welch et al 1985), possibly as the result of damaged protein that had been observed within the nucleolus; however it may be that it is events in the endoplasmic reticulum or cell membrane that are more important in differentiation which would imply modulation of the glucose regulated proteins. Reports from a meeting on Differentiation Therapy (edited by Lotan et al 1990) have implicated events at the cell membrane and in the nucleus in the transduction of signals initiated by differentiation-inducing agents. Several investigators have reported changes in the expression of cell surface glycoproteins during the induction of differentiation of leukemia cells (reviewed by Sartorelli 1985).

The rapid commitment to differentiation by a heat shock is possibly due to the speed with which damage accumulates within the cell. A rapid accumulation of damage may explain why agents such as TPA are able to commit HL60 cells to differentiate more quickly than agents such as NMF.

To investigate further whether stress proteins were regulated during differentiation their synthesis and amounts were measured after HL60 cells were induced to differentiate with the antitumour agent NMF. Since heat was a poor inducer of differentiation it was not considered to be a good model to study protein changes during differentiation whereas NMF induces greater than 70% of HL60 cells to undergo

granulocytic differentiation (Langdon et al 1987).

4.2 HL60 drug-induced differentiation

4.2.1 Characterisation of differentiation and cytotoxicity induced by N-methylformamide in HL60 cells

NMF is a planar-polar solvent which induces the terminal differentiation of a number of cell lines in vitro, possesses activity against murine tumours and has been reported to have good, broad-spectrum activity against certain human tumour xenografts grown in mice (Gesher et al 1982). Its precise site of action within the cell is unknown but effects at the cell membrane have been implicated in the promotion of differentiation by the polar solvents. HL60 cells can be induced to undergo terminal differentiation to form granulocytic like cells with NMF (Collins et al. 1978 and Langdon et al. 1987). The characteristics of the differentiation process and NMF cytotoxicity were studied to investigate any relationship between stress proteins, differentiation and drug toxicity.

Incubation of cells for 96 hours with increasing concentrations of NMF inhibited cell proliferation, with total inhibition at 200mM (figure 4.2). Viability started to decrease at concentrations greater than 180mM, with viability falling below 50% at a concentration of 225mM. The percentage of differentiated cells increased from less than

5% with concentrations of NMF greater than 50mM with the optimum concentration for differentiation being 170mM when approximately 70% of the cells expressed markers of a more mature phenotype; at concentrations of NMF greater than 170mM the percentage of differentiated cells decreased rapidly. These results were in agreement with those obtained by Langdon and Hickman 1987 and showed that optimum differentiation was obtained when HL60 cells were treated with a concentration of NMF that was marginally below that which was cytotoxic.

Having determined the optimum concentration of NMF required for the differentiation of HL60 cells, the time course of the development of the differentiated phenotype was studied (figure 4.3). Following a 24 hour incubation with 170mM NMF the percentage of differentiated cells increased to 15% and this increase continued in a time dependant manner; there was no significant differentiation observed in cells treated for less than 12 hours with 170mM NMF. The percentage of cells able to reduce NBT exceeded those able to phagocytose the yeast particles throughout the time course with 75% cells NBT positive after 96 hours compared with 69% cells able to phagocytose. This is due to the relatively late expression of the C3b complement receptor in the maturation of myeloid cells (Werb, 1984). Over a 96 hour time course the cells underwent one doubling which took place during the first 48 hours and there was no significant loss in viability.

Figure 4.2 The effect of NMF on HL60 cell growth and differentiation

Cells were treated with various concentrations of NMF and cell growth (A) and viability (B) were determined after 96 hours. Differentiation was assessed (C) by reduction of NBT (▲) and yeast phagocytosis (△).

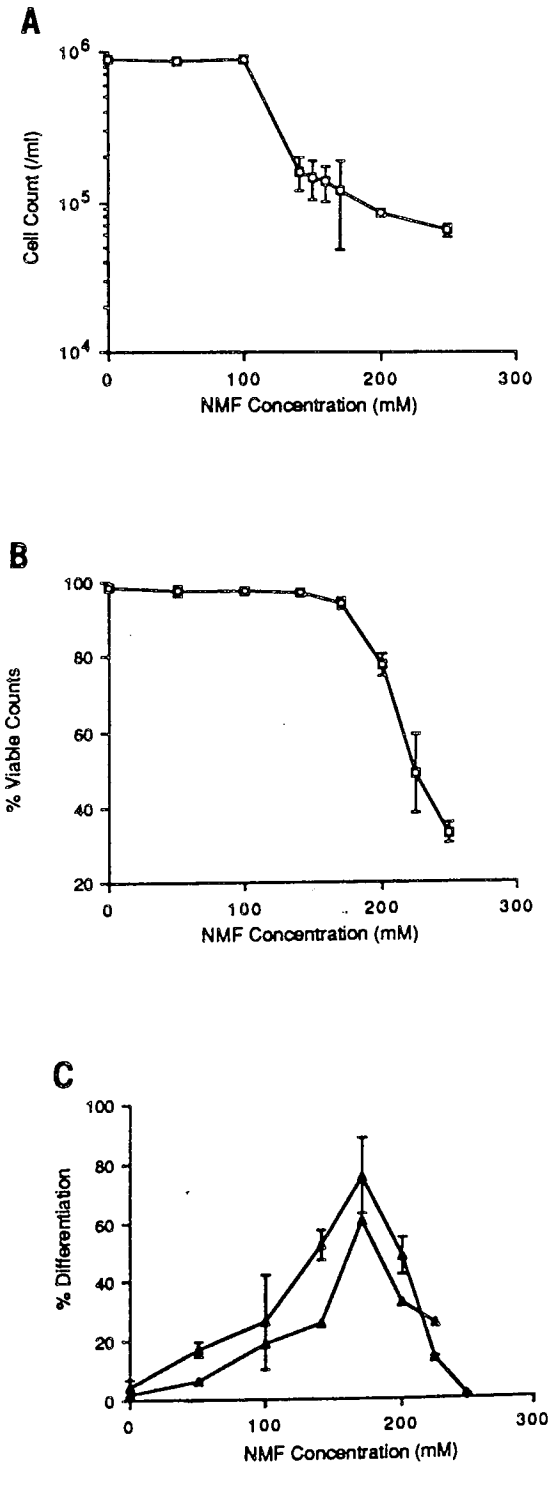
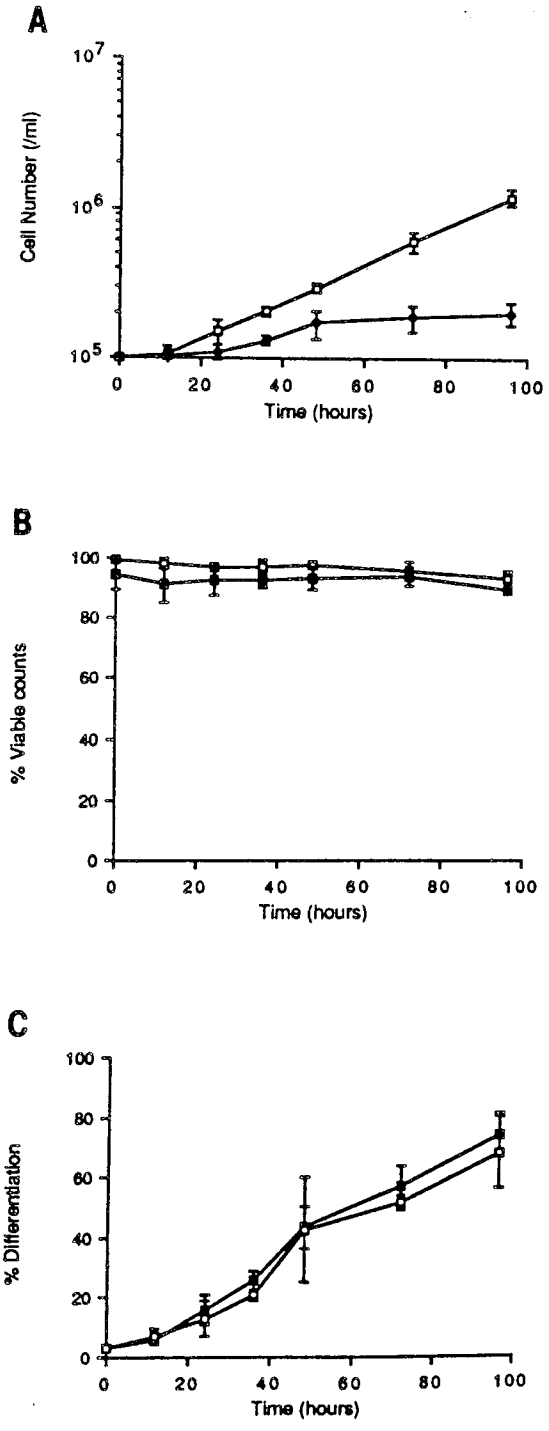


Figure 4.3 Time course of the induction of differentiation of HL60 cells treated with NMF

Cells were incubated in the presence (■) or absence (□) of 170mM NMF and cell growth (A) and viability (B) were assessed daily. Differentiation was assessed (C) by reduction of NBT (■) and yeast phagocytosis (□).



4.2.2. Analysis of heat shock proteins in HL60 cells induced to differentiate with NMF

Experiments by Richards had shown that incubation of HL60 cells with 170mM NMF decreased the synthesis of hsp70 after 54 hours. It was decided to repeat this experiment since a different sub-line of HL60 cells was now being used, and also to continue the period of incubation with 170mM NMF to 96 hours since it was at 96 hours that maximum differentiation was observed (figure 4.3).

A heat shock of 42.5^{o}C had been used on the subline of HL60 cells provided by Dr. G. Brown but this temperature did not induce large increases in the synthesis of hsps in the cells from the Paterson Institute; hence a heat shock of 43.5^{o}C was used to induce hsps for assessment of synthesis and amounts.

All autoradiographs and Western blots are representative results from 3 or more experiments except for those using the antibody AC88 where in some cases it was only possible to carry out two experiments due to antibody availability (in such cases no change in hsp90/grp94 amounts had been observed in either of the two experiments). Molecular weights were calculated from the Rf values of the molecular weight standards.

HL60 cells were treated with 170mM NMF for 12, 24, 36 48, 72 and 96 hours then harvested and labelled with ^{35}S methionine. The proteins were separated according to their

molecular weight using one dimensional SDS-polyacrylamide gel electrophoresis and then fluorographed. The autoradiograph of the fluorographed gel (figure 4.4) shows that the control cells (lane A), that is cells that had not been treated with NMF, synthesised constitutive amounts of both hsp70 and hsp90. They also synthesised grp78 and grp94, the two major glucose regulated proteins. After a heat shock the molecular weights of the major proteins synthesised were 70 and 90kD (lane B). These proteins correspond to the hsps 70, and 90kD as previously described in other mammalian cells (reviewed by Lindquist et al. 1989). The cells that had been subjected to a heat shock of 43.5°C for one hour did not totally repress the synthesis of all other cellular proteins as is the case in other cells (Lindquist et al 1987), but synthesised decreased amounts. Following incubation with 170mM NMF there was no change in the synthesis of hsp70 or hsp90 but the synthesis of a protein of molecular weight 78kD decreased in a transient manner after 24 hours and then increased to constitutive levels after 36 hours. There was also a decrease in synthesis after 12 hours of a protein of approximately 90kD, which was probably grp94.

Protein synthesis does not, however, always reflect the total cellular amounts of a particular protein. The cellular amounts of a protein are not governed only by the rate of synthesis but also by the rate of degradation. The rate of turnover of a particular protein depends on the protein structure, the metabolic state of the cell, any

covalent modifications undergone by the protein and whether or not the protein is bound to some cellular structure (Rivett 1986). It was therefore important to study the cellular amounts of hsp70 and hsp90 as well as their synthesis; this was done by Western blotting.

When the amounts of hsp70 were measured by probing the nitrocellulose paper with the 72kD antibody purchased from Amersham, constitutive amounts of hsp70 could be detected (figure 4.5); an increase in the cellular amounts of hsp70 following a heat shock was detected reflecting the increase in synthesis. However the amounts of hsp70 detected increased above constitutive amounts between 24 and 48 hours incubation with 170mM NMF and then decreased at 72 and 96 hours.

Figure 4.6, a Western blot using the antibody AC88 which recognised hsp90 and grp94, showed that the decrease in grp94 synthesis after 12 hours was accompanied by a decrease in the amounts of hsp90/grp94 after 48 hours. The time that the decrease in amounts of protein occurred did vary. Only one band was visualised with AC88 indicating poor resolution during transfer which was probably due to the amount of cells required to be loaded in order to achieve a visable band. In general there was no increase above control (lane A) in amounts of immunodetectable protein following a heat shock (lane B).

To investigate whether there were any early changes in the synthesis of hsp70 and hsp90 during the precommitment

period, cells were treated with 170mM NMF for up to 6 hours and then their proteins labelled with ^{35}S methionine. Cells were also harvested for assessment of the total amount of hsp70.

The fluorograph (figure 4.7) showed no change in the synthesis of hsp70, hsp90 or either of the glucose-regulated proteins in any of the NMF treated samples; there was also no change in amounts of hsp70 (figure 4.8).

HL60 cells were also treated with 100mM NMF for up to 96 hours. This concentration had no effect on cell growth or viability and only 20% of cells were induced to differentiate at the end of the time course (figure 4.3). At the end of the time course cells were harvested for labelling with ^{35}S methionine and for Western blotting.

The fluorograph (figure 4.9) showed no change in the synthesis of any of members of the 70kD family or the 90kD family. Probing a Western blot with the 72kD antibody however revealed an increase in amounts of hsp70 after 36 hours (figure 4.10).

Hsp70 synthesis was not regulated when HL60 cells are induced to differentiate with 170mM NMF but the cellular amounts of the protein were; it would initially seem that it is the synthesis of the glucose-regulated proteins that were more important during differentiation although no changes occurred during the first 6 hours of treatment. A non-toxic concentration of NMF, 100mM, did not affect hsp or grp synthesis but did however bring about an increase in the

cellular amounts of hsp70 after a 36 hour incubation.

The original hypothesis, set out in the aim of this thesis was that it is the modulation of different members of the stress protein family that bring about drug-induced differentiation or toxicity. This hypothesis was proposed in the light of published work (section 1.6). Compounds such as tunicamycin, shown by Watowich et al. (1988) to modulate the glucose regulated proteins, have been reported by Nakayasu et al. (1980) to induce HL60 myeloid differentiation (as assessed by the ability for phagocytosis). Experiments were therefore undertaken to study whether known modulators of grps brought about differentiation, and whether modulators of hsp70 were inactive in inducing differentiation. From work described by Watowich (1988) and previous studies by Welch et al. (1986) it was well established that the amino acid analogue azetadine carboxylic acid induced hsp70 so this compound was used for the hsp70 modulator.

Figure 4.4 Protein synthesis in HL60 cells induced to differentiate with NMF

Fluorograph of equal numbers of ^{35}S methionine labelled cells separated by SDS-PAGE. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 170mM NMF for 12 hours (C); 24 hours (D); 36 hours (E); 48 hours (F); 72 hours (G) or 96 hours (H).

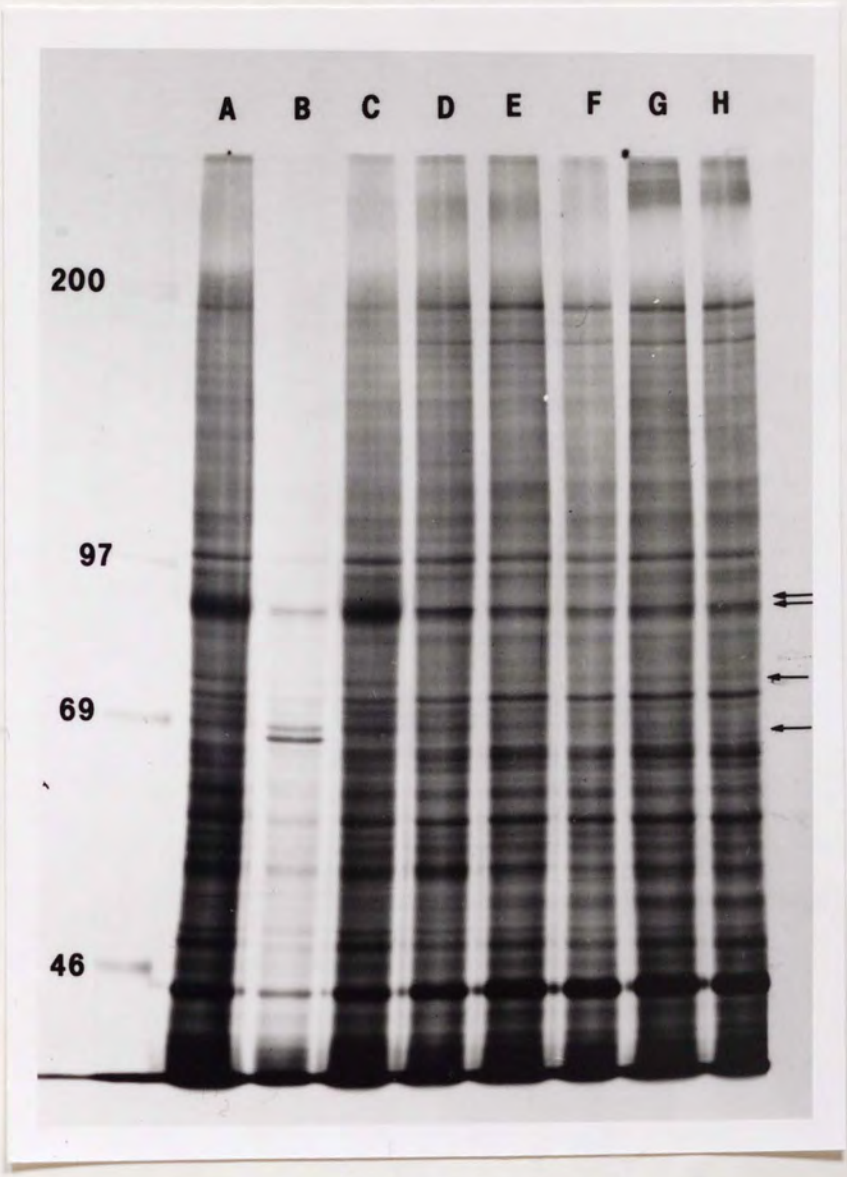


Figure 4.5 Amounts of hsp70 in HL60 cells induced to differentiate with NMF

Immunoblots of NMF treated HL60 probed with 72kD. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B) ; or treated with 170mM NMF for 12 hours (C); 24 hours (D); 36 hours (E); 48 hours (F); 72 hours (G); 96 hours (H).

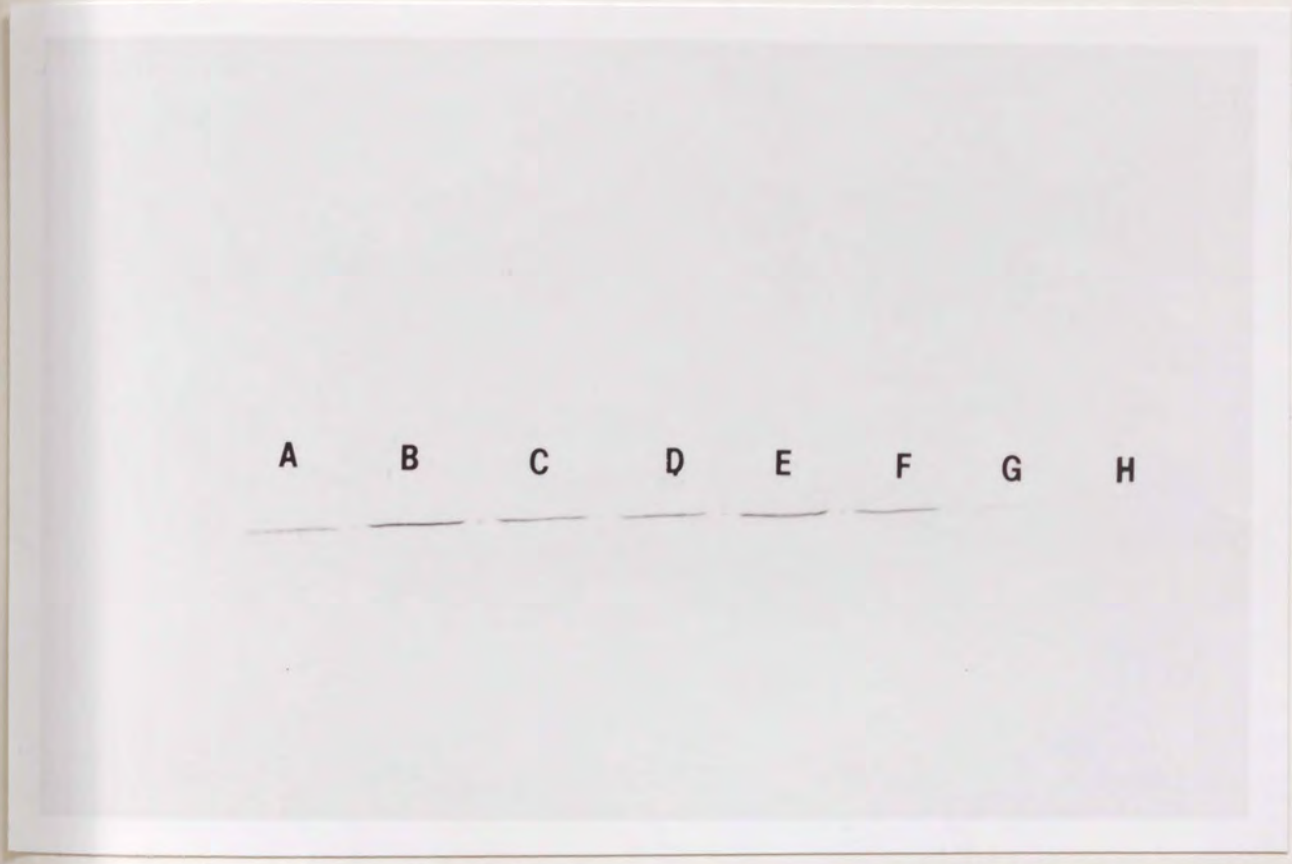


Figure 4.6 Amounts of hsp90/grp94 in HL60 cells induced to differentiate with NMF

Immunoblots of NMF-treated HL60 cells probed with AC88 antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 170mM NMF for 12 hours (C); 24 hours (D); 36 hours (E); 48 hours (F); 72 hours (G); 96 hours (H).

Figure 4.7 The effect of 170mM NMF for 1-6 hours on protein synthesis in HL60 cells

Fluorograph of equal numbers of ^{35}S-methionine labelled cells separated by SDS-PAGE. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 170mM NMF for 1 hour (C); 2 hours (D); 3 hours (E); 4 hours (F); 5 hours (G); 6 hours (H).

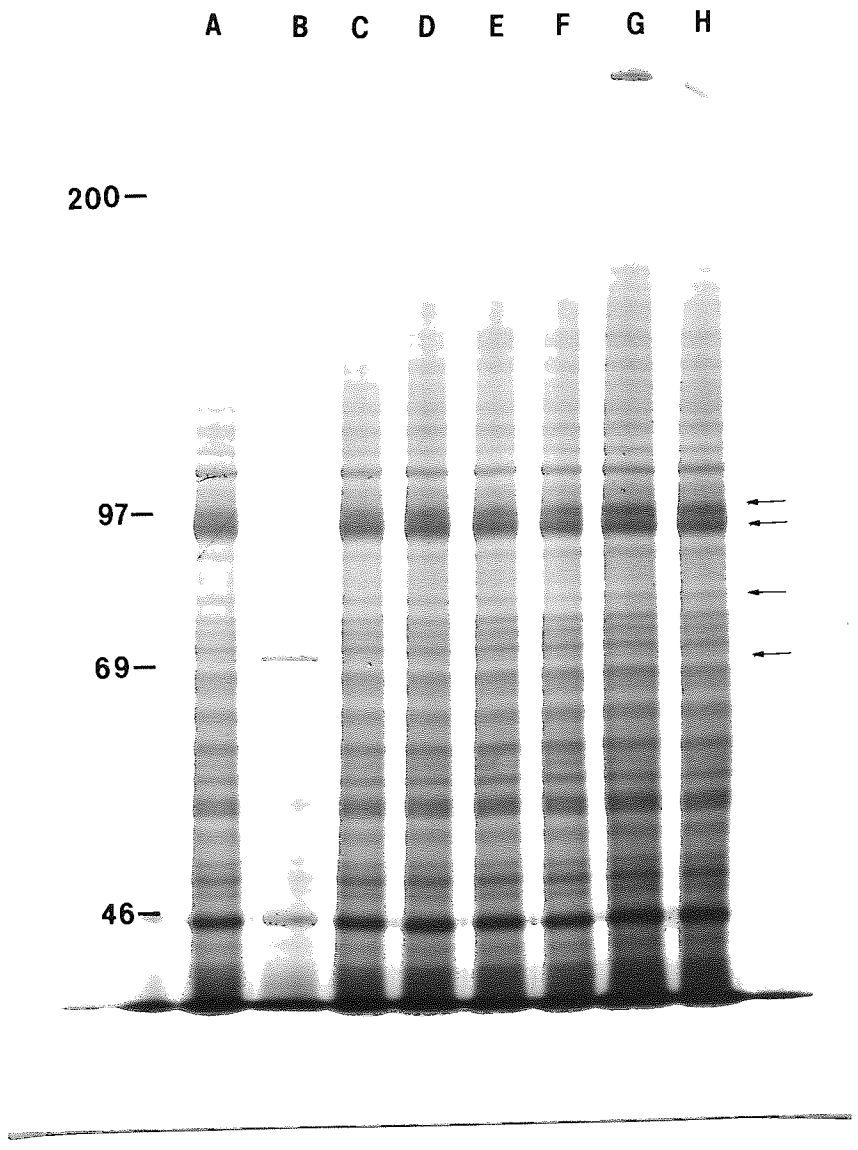


Figure 4.8 The effect of 170mM NMF for 1-6 hours on amounts of hsp70 protein in HL60 cells

Immunoblots of NMF-treated HL60 cells probed with 72kD antibody. Cells were heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (A); untreated (B) ; or treated with 170mM NMF for 1 hour (C); 2 hours (D); 3 hours (E); 4 hours (F); 5 hours (G); 6 hours (H).

Figure 4.9 The effect of 100mM NMF on protein synthesis in HL60 cells

Fluorograph of equal numbers of ^{35}S-methionine labelled cells separated by SDS-PAGE. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 100mM NMF for 12 hours (C); 24 hours (D); 36 hours (E); 48 hours (F); 72 hours (G); 96 hours (H).

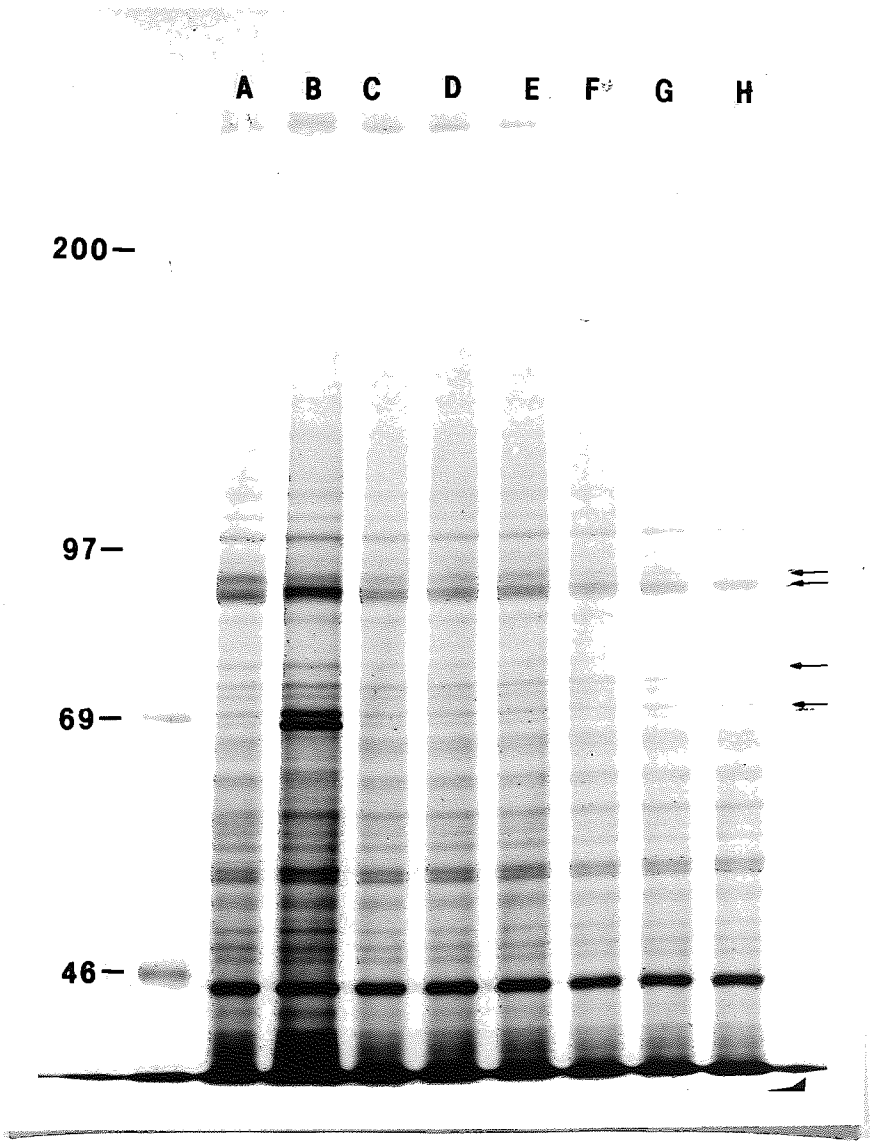


Figure 4.10 The effect of 100mM NMF on amounts of hsp70 in HL60 cells

Immunoblots of NMF treated HL60 probed with 72kD. Cells were untreated (A); or treated with 100mM NMF for 12 hours (B); 24 hours (C); 36 hours (D); 48 hours (E); 72 hours (F); 96 hours (G); or heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (H).

4.2.3. Characterisation of differentiation and cytotoxicity induced by tunicamycin and azetadine carboxylic acid in HL60 cells

As already mentioned, it has been established by Nakayasu (1980) that tunicamycin induces HL60 cells to phagocytose polystyrene latex particles and to develop a more mature phenotype. Tunicamycin is an antibiotic which specifically inhibits the synthesis of N-acetylglucosaminyl pyrophosphoryl polyisoprenol leading to inhibition of protein glycosylation. To determine the concentration of tunicamycin required to bring about maximum differentiation of HL60 cells the relationship between concentration and differentiation was determined. The optimum concentration of tunicamycin for the induction of differentiation, as assessed by reduction of NBT, was 0.5µg/ml (figure 4.11), and at this concentration viability remained at greater than 70% with 64% of the cells becoming NBT positive after 96 hours. The cells did not express non-specific esterase activity at any concentration of drug. At concentrations of antibiotic greater than 0.5µg/ml proliferation was inhibited and cell viability decreased.

Azetadine carboxylic acid, an analogue of the essential amino acid proline, is incorporated into polypeptides giving rise to non-functional proteins. Continuous incubation of HL60 cells for 96 hours with azetadine carboxylic acid did not induce either granulocytic differentiation, as assessed

by NBT reduction, or any evidence of monocytic differentiation, as assessed by non-specific esterase activity. Viability and cell growth were reduced in a progressive manner as concentrations of azetadine were increased (figure 4.12).

Figure 4.11 The effect of tunicamycin on HL60 growth and differentiation

HL60 cells were treated with various concentrations of tunicamycin, and cell growth (A), and cell viability (B) were determined after 96 hours. Differentiation was assessed (C) by reduction of NBT (□) and yeast phagocytosis (■).

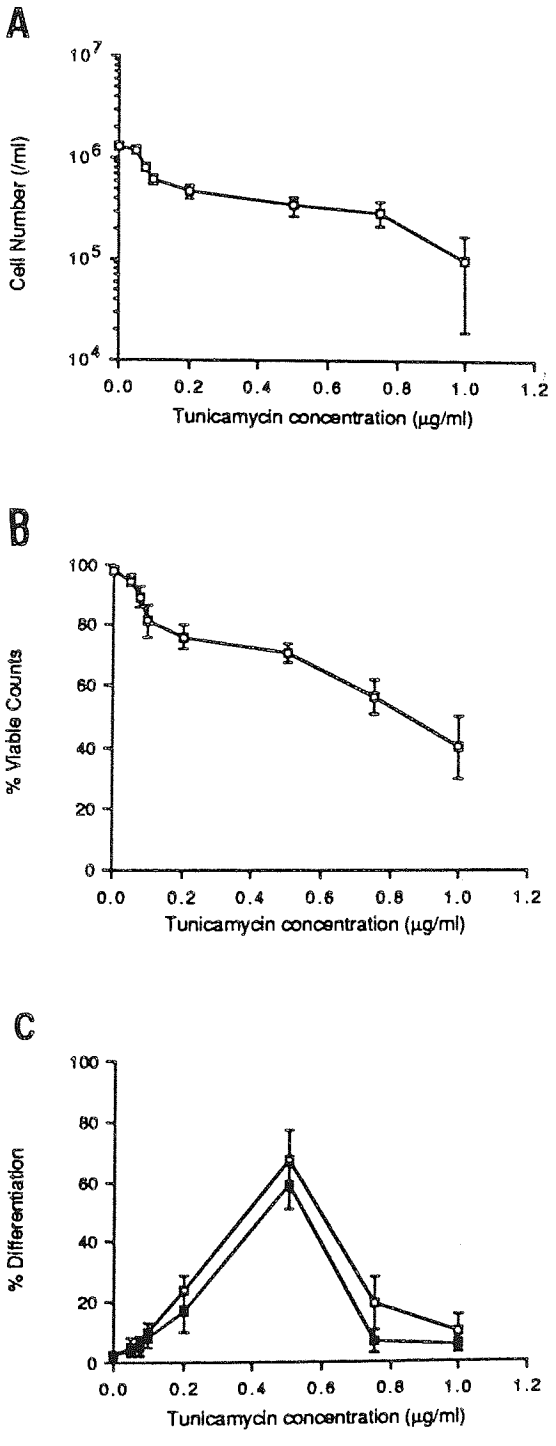
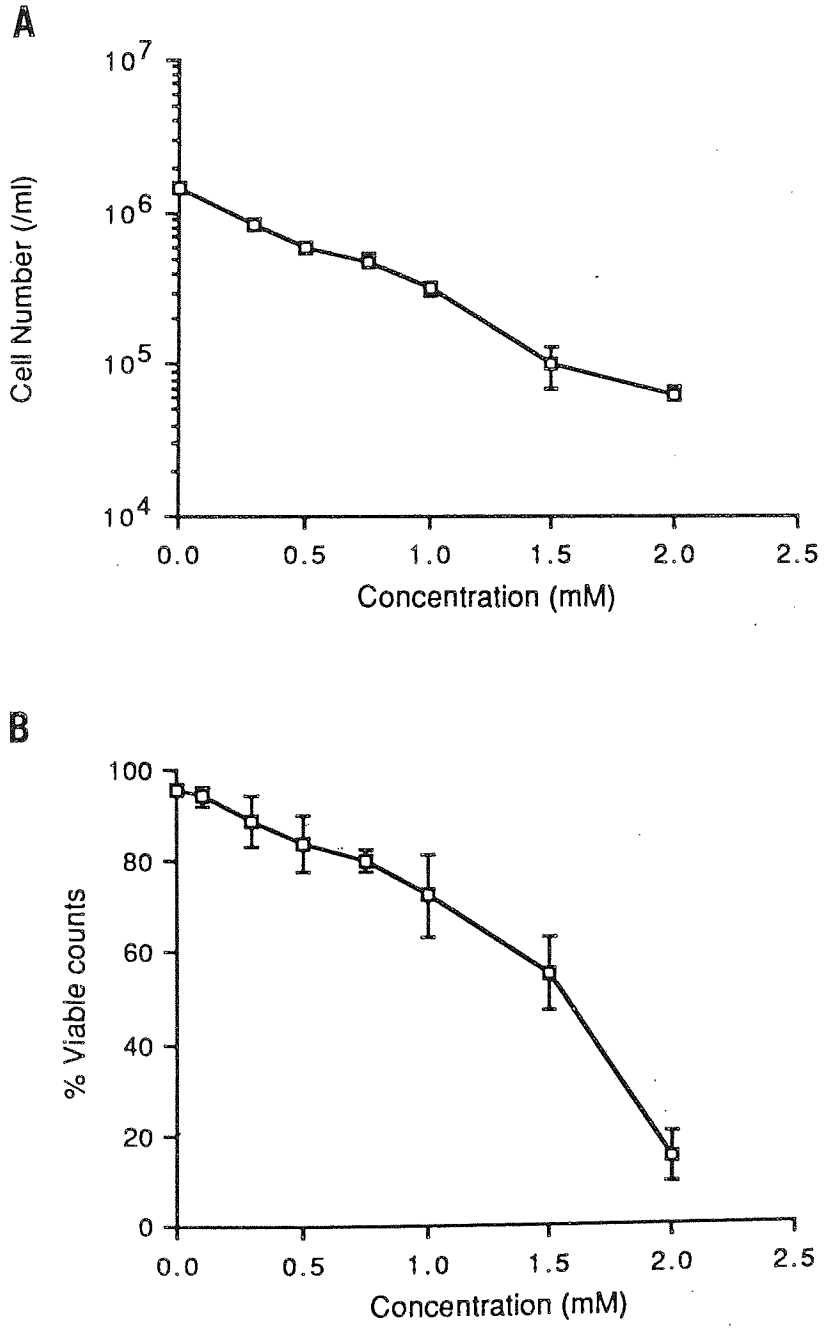


Figure 4.12 The effect of azetadine carboxylic acid on HL60 cell growth

HL60 cells were treated with various concentrations of azetadine carboxylic acid, and cell growth (A), and cell viability (B) were determined after 96 hours.



4.2.4 Comparison of heat shock proteins in HL60 cells treated with azetadine carboxylic acid, NMF and tunicamycin

A comparison of the proteins synthesised after incubation of HL60 cells with tunicamycin and azetadine was made by labelling cells with ^{35}S-methionine following a 24 hour incubation with 0.5µg/ml tunicamycin or 1mM azetadine carboxylic acid; samples were also harvested in parallel for Western blotting. The concentration of azetadine carboxylic acid was chosen because it induced similar loss in viability to that brought about by 0.5µg/ml tunicamycin. A sample of cells was also labelled with 170mM NMF for 24 hours.

From the fluorograph, figure 4.13, the differences in protein synthesis caused by the three drugs are demonstrated. Tunicamycin did not bring about any change in the synthesis of grp78 but interestingly a decrease in the synthesis of grp94 was observed; azetadine carboxylic acid did not induce the synthesis of either of these proteins or hsp70 above constitutive levels. As previously described in section 4.2.2, NMF brought about a decrease in grp78 and grp94 synthesis after 24 hours. Western blot analysis using the 72kD antibody, figure 4.14, showed an increase in the amounts of hsp70 in the cells treated with azetadine carboxylic acid compared to the amounts in tunicamycin treated cells.

In summary it appears therefore that agents that modulate glucose regulated proteins can induce HL60

differentiation.

Figure 4.13 Comparison of protein synthesis in HL60 cells treated with azetadine carboxylic acid, NMF and tunicamycin

Fluorograph of ^{35}S-methionine labelled cells separated by SDS-PAGE. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated for 24 hours with 1mM azetadine carboxylic acid (C); 170mM NMF (D); 0.5µg/ml tunicamycin (E).

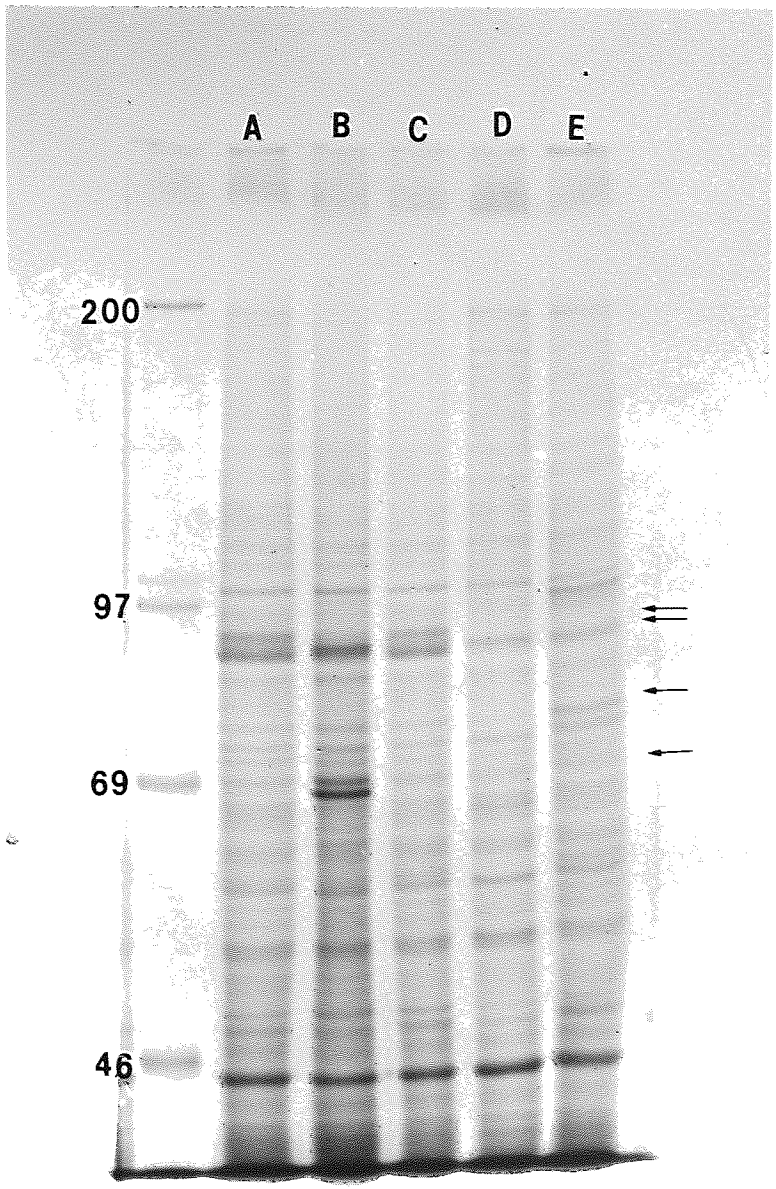
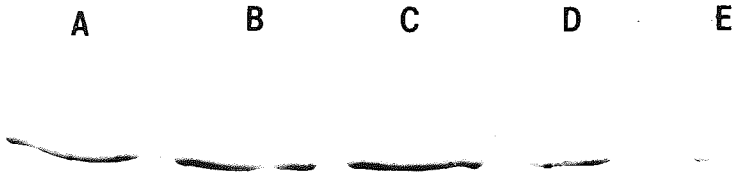


Figure 4.14 Comparison of amounts of hsp70 protein in HL60 cells treated with azetadine carboxylic acid, NMF and tunicamycin

Immunoblots of treated HL60 probed with 72kD. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated for 24 hours with 1mM azetadine carboxylic acid (C); 170mM NMF (D); 0.5ug/ml tunicamycin (E).



4.2.5 Discussion

HL60 cells synthesise constitutive amounts of the stress proteins hsp70, p72, grp78, hsp90 and grp94 and these proteins were able to be resolved using one-dimensional SDS-PAGE. Hsp70 and members of the 90kD family could also be detected quantitatively using Western blotting techniques. Following a heat shock the cells increased synthesis of hsp70 but not of hsp90; synthesis and amounts of hsp90 remained at comparable levels in control and heat shocked cells which is contrary to observations in other cell types (Lindquist et al 1988).

The differentiation of HL60 cells into granulocytic -like cells is induced by sub-toxic concentrations of drugs, and involves early changes in the synthesis of the glucose-regulated proteins which in the case of grp78, after induction with NMF, is transient. This decrease in synthesis correlates with the commitment to differentiate; no changes in either grp78 or grp94 were observed during the first 6 hours of exposure to 170mM NMF, which has a commitment time of 24 hours. There was no apparent decrease in the synthesis of hsp70 during the time course of differentiation with 170mM NMF as had been observed by Richards (1987) in a different subline of HL60 cells and by Hensold et al (1988) in MEL cells induced to differentiate with DMSO. The reasons for this are as yet unclear, but 2-dimensional electrophoresis may provide a clearer result due to better separation of

proteins using this technique. However using Western blotting techniques to assess the total amount of hsp70 there was a decrease in amount of hsp70 protein after 48 hours, in agreement with Richards. This decrease in hsp70 amounts was preceded by an increase in amounts of hsp70 protein, in the absence of any increase in synthesis; this increase in amounts may well reflect the subtoxic concentration of NMF required to induce differentiation since hsps are induced in response to a stress (section 1.6). It is unlikely that this increase is a consequence of changes in cell cycle since synthesis of hsp70 is elevated in S phase (Milarski et al 1986); HL60 cells are blocked in the G_1 phase of the cell cycle after 48 hours exposure to 170mM NMF (Richards 1987). The lack of correlation between synthesis and amounts of hsp70 highlights the importance of measuring the total amounts of a protein rather than just transcription and translation products and may well relate to a change in half-life of hsp70 such that it is degraded at a slower rate.

What was also interesting was that 100mM NMF caused an accumulation in amounts of hsp70 after 72 hours in the absence of any increase in synthesis; 100mM NMF was unable to induce differentiation. Previous work by Richards (1987) which investigated the induction of differentiation in HL60 cells, suggested that differentiation was a two stage process that involved the inhibition of proliferation and the presence of damaged protein which provided the signal to differentiate. 170mM NMF, a sub-toxic concentration, was

able to provide both of the signals whereas 100mM NMF could induce differentiation only when the proliferative capacity of the cell was inhibited. The results presented here demonstrate that 100mM NMF was able to cause the accumulation of some damaged protein, as indicated by the increase in amounts of hsp70, and this may well explain the results obtained by Richards.

The decrease in the synthesis of grp94 during HL60 differentiation agrees with Yufu et al (1990). Unfortunately the Western blot was unable to resolve hsp90 from grp94 so the decrease observed at 48 hours was the result of the amounts of both hsp90 and grp94. Grp94 is thought to play a role in intracellular calcium sequestration (section 1.6.2) and signalling. A decrease in grp94 may well therefore affect calcium signalling during differentiation. Okazaki et al (1986) showed that calcium-free media and verapamil enhanced HL60 differentiation after DMSO, retinoic acid and 1,25-dihydroxy vitamin D_3, and they suggested that this enhancing effect was related to the inhibition of calcium mobilisation in the cells, perhaps inhibiting the ability of the cells to proliferate. However Yen and co-workers (1986) argued against a role for calcium in the control of HL60 cell differentiation.

Hsps have been shown to bind to a number of oncogene products, possibly contributing to the maintenance of the transformed state (as discussed in section 1.6.5). From the results of the Western blot it would appear that the amounts

of hsp70 and hsp90/grp94 decrease to almost immuno-undetectable levels in differentiated HL60 cells; if for example, c-myc was bound to hsp70 in HL60 cells, which resulted in an extension of the half-life of c-myc, a decrease in hsp70 protein would lead to a decrease in the amount of myc protein, so enabling the cell to undergo differentiation. Associations might also be taking place between other oncogene products, such as src and hsp90 (Brugge et al 1981); increases in src activity during HL60 differentiation have been noted. This increase in activity was not due to an increase in expression of the gene, but was due either to an increase in activity of the protein itself or an increase in the amount of protein (Barnekow and Gessler 1986). Associations between hsp90 and src have been observed at the cell membrane and as discussed in section 4.1.2 events at the cell membrane may well be important in the transduction of signals initiated by differentiation-inducing agents.

As already mentioned hsp70, grp78, hsp90 and grp94 could be detected in exponentially growing HL60 cells; this is in contrast to some other cell types (Lindquist et al 1988) where levels of the stress proteins are low. High levels of these proteins in HL60 cells may be a reflection of their transformed state and the presence of abnormal proteins, for example high levels of oncogene proteins or due to the proliferative potential of the cells.

Azetadine carboxylic acid which is less selective in

the induction of the 70kD family of proteins (Watowich and Morimoto 1988), was inactive as an inducer of differentiation over a range of concentrations which included both sub-toxic and toxic concentrations. This was surprising as it was predicted that azetadine would induce low levels of differentiation, such as in the case of a heat shock, since in some cell types it was known to modulate both hsp70 and grp78 (Welch et al 1986). The amounts of hsp70 in HL60 cells following treatment with azetadine carboxylic acid were comparable to those obtained following a heat shock and were greater than the amounts in tunicamycin treated cells. The inability of azetadine to induce differentiation may be due to the localisation of hsp70 following treatment. Welch et al (1986), using immunocytochemistry, demonstrated that hsp70 was localised to the nucleolus in HeLa cells after treatment with azetadine; a similar pattern of hsp70 distribution was observed in heat shocked cells. Since modulation of grps suggest events that are associated with the endoplasmic reticulum are important in differentiation, azetadine carboxylic acid, which induces nuclear damage, may be unable to cause the necessary changes required for changes in grps and subsequent differentiation. Although other groups have reported modulation of grps in response to azetadine (Welch et al 1986 and Watowich et al 1988), high concentrations (5mM) were used. This may mean that the workers were looking at the events associated with toxicity; no data was given on cell viability by either group.

Changes in the endoplasmic reticulum may well be important in the commitment to HL60 differentiation, as demonstrated by early, specific modulation of the glucose-regulated proteins. To determine whether these changes in heat shock proteins were specific for granulocytic induced differentiation of HL60 cells the synthesis and amounts of hsps during differentiation of a second human leukemia cell line were studied.

4.3 K562 induced differentiation

4.3.1 Characterisation of the differentiation and cytotoxicity induced by cytosine arabinoside (ara-C) in K562 cells

K562 cells are considered to be representative of early haemopoietic stem cells, are more primitive than the HL60 cell, and will undergo differentiation to erythroid-like cells and synthesis haemoglobin when incubated with the anticancer agent cytosine arabinoside (Rowley et al. 1981). This compound is a cytidine analogue and inhibits DNA synthesis. It is generally thought that the active form of the drug, ara-CTP, acts by inhibiting mammalian DNA polymerases in a manner which is competitive with the natural substrate cytidine 5' triphosphate (Graham et al 1970). It has already been reported by Singh and Yu (1984) that hsp70 is synthesised in K562 cells induced to differentiate with haemin, but it should be noted that haemin does not induce terminal differentiation and once the inducer is removed the cells will revert to an undifferentiated phenotype. The changes that Singh and Yu observed may not therefore be correlated with terminal differentiation.

K562 cells from Charing Cross Hospital, London, were treated with increasing concentrations of ara-C to determine the concentration required to bring about maximum differentiation. These cells underwent approximately 3

divisions in 3 days indicating a population doubling time of 24 hours. When treated with ara-C for 72 hours the percentage of benzidine-positive cells increased in a linear manner from less than 5% in the untreated cells to 57% in cells treated with 3µg/ml (figure 4.15). At concentrations greater than 3µg/ml the degree of differentiation decreased. The viability of the cells was greater than 80% until the concentration of ara-C exceeded 7.5µg/ml whereon it fell to less than 50%; growth was inhibited at concentrations greater than 3µg/ml. Since the optimum concentration of ara-C required to bring about erythroid differentiation was 3µg/ml, this concentration was used to determine the time course of the development of the differentiated phenotype.

K562 cells were incubated with 3µg/ml ara-C for up to 96 hours and cell number, cell viability and the percentage differentiation assessed (figure 4.16). At this concentration cell growth was inhibited but cell viability remained greater than 80%. The percentage differentiation increased at an almost linear rate after 24 hours exposure to the drug.

To confirm that ara-C did in fact bring about terminal differentiation and to determine whether a commitment to differentiation occurred more rapidly than the production of haemoglobin, experiments were performed to determine the length of incubation with 3µg/ml ara-C required to commit the cells to differentiate even in the absence of the inducer

(figure 4.17). The extent of differentiation was assessed 72 hours after the start of the experiment, and it was shown that no significant commitment occurred during the first 12 hours of incubation with ara-C. From this point onwards there was a linear increase in the number of cells committed to differentiate with no loss in viability. Cells that had been incubated for 24 hours with drug were not able to undergo cell division. Since treated cells did not revert to none haemoglobin producing cells, it could be concluded that exposure to ara-C caused terminal differentiation.

Figure 4.15 The induction of erythroid differentiation of K562 cells after incubation with ara-C

K562 cells were treated with different concentrations of ara-C, and cell growth (A), cell viability (B) and differentiation (C) were determined after 72 hours.

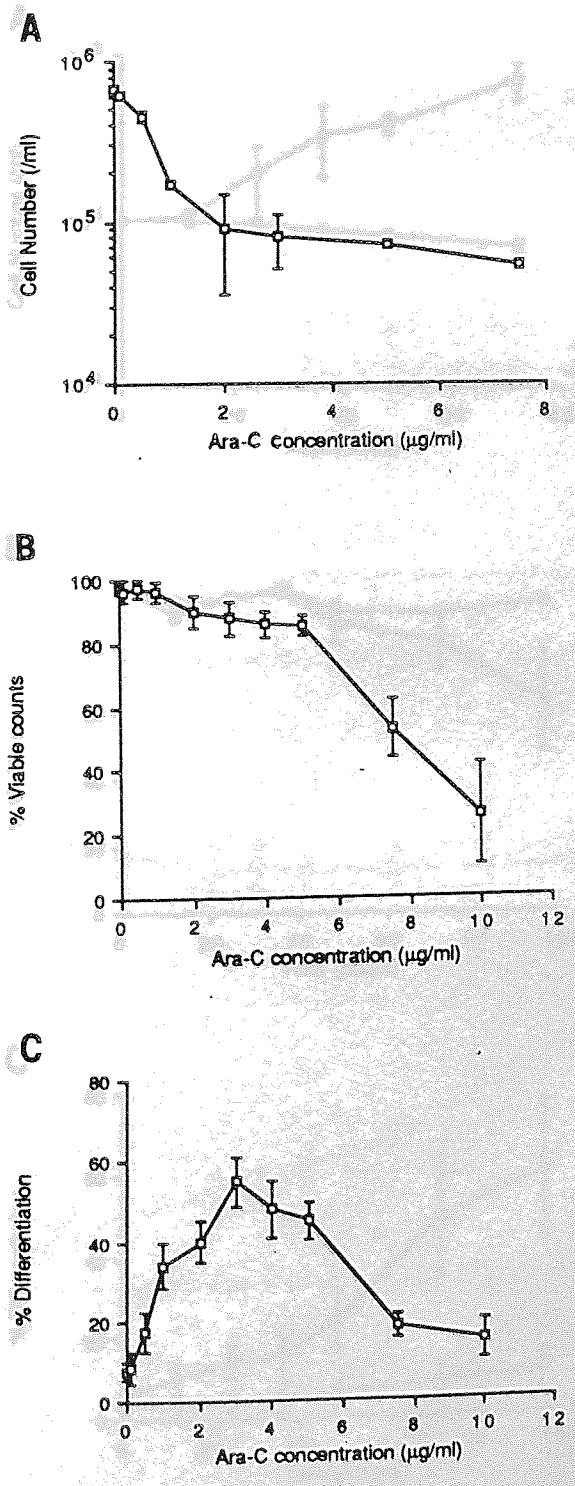


Figure 4.16 Time course of the induction of differentiation of K562 cells treated with ara-C

K562 cells were treated in the presence (■) or absence (□) of 3ug/ml ara-C and cell growth (A), cell viability (B) and differentiation (C), were monitored on a daily basis.

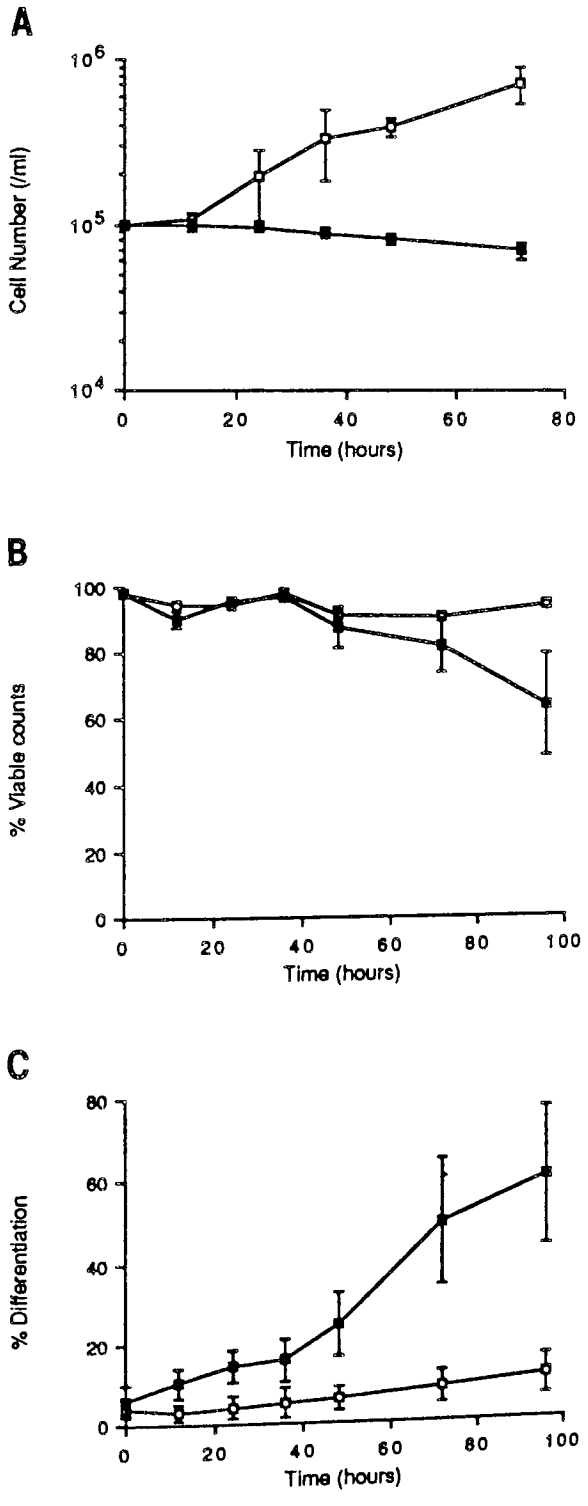
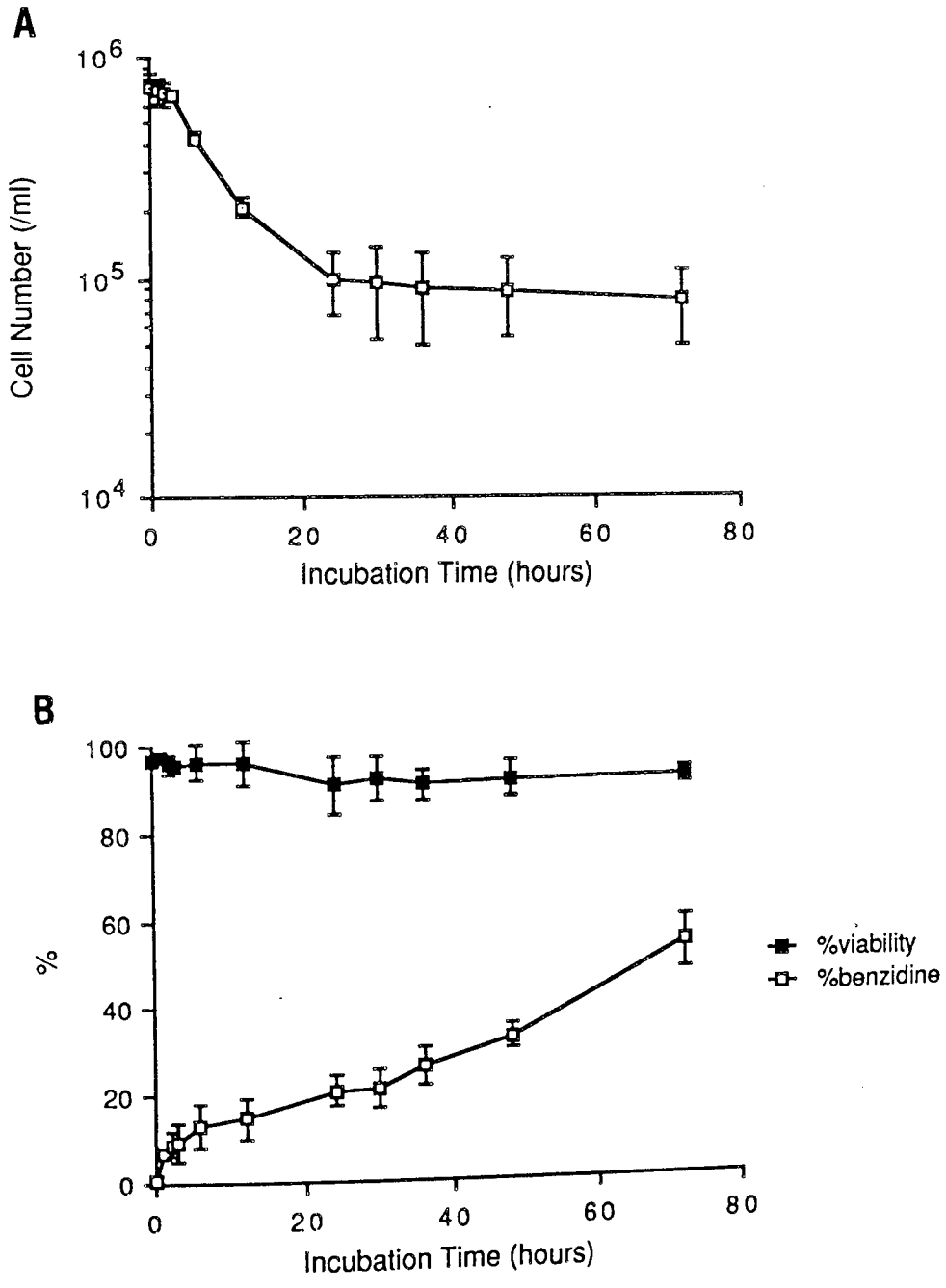


Figure 4.17 Commitment of K562 cells induced to differentiate with ara-C

K562 cells were treated with 3ug/ml ara-C for various times following which the cells were washed and incubated in the absence of any inducer. Cell growth (A), cell viability (B) and differentiation (B) were assessed after 72 hours.



4.3.2 Analysis of hsps in K562 cells induced to differentiate with ara-C

To determine whether any changes in hsps occurred during the time course of K562 terminal differentiation, cells were labelled with ^{35}S-methionine for analysis of protein synthesis, and harvested for Western blot analysis, at time periods up to 96 hours after the addition of ara-C. Cells were also heat shocked for 60 minutes at 43.5^{o}C then incubated at 37^{o}C for 2 hours. The fluorograph (figure 4.18) shows, as expected, heat shock induced elevated synthesis of heat shock proteins of molecular weight 70 and 90kD. There was no decrease in the synthesis of the other cellular proteins but it may be that the recovery period of K562 cells, following a heat shock, is less than two hours so that normal protein synthesis had been re-established at the time of labelling. It has been also demonstrated by Mivechi (1989), that K562 cells are more resistant to heat than HL60 cells. After 72 and 96 hours incubation with 3µg/ml ara-C there was an elevated synthesis of hsp70, although in one experiment it was noted that at 96 hours the synthesis of hsp70 had decreased. The cells synthesised constitutive amounts of a protein of approximately 72kD and the synthesis of this protein was greater than that of hsp70; this protein was probably the clatherin-uncoating ATPase. Unlike HL60 cells K562 do not synthesise detectable amounts of grp94.

The blot using the anti-hsp70 antibody (figure 4.19)

demonstrated the increased amounts of hsp70 above constitutive levels following a heat shock, as predicted. The samples treated with ara-C for up to 96 hours showed very little variation in their amounts of hsp70, as assessed by laser densitometry, with the amounts of hsp70 being elevated above constitutive amounts after 12 hours incubation and this amount remaining constant throughout the time course. These early increases in amounts of hsp70 was reminiscent of the accumulation of hsp70, without increased synthesis, observed during NMF-induced differentiation of HL60 cells (section 4.2.2). Figure 4.20 shows that amounts of hsp90/grp94 did not change as the cells differentiated; as with HL60 cells only one band was resolved at a molecular weight of 90kD. When blots of K562 cells were probed with AC88 several bands of lower molecular weight than 90kD were visualised. Control experiments had indicated that these bands were not the result of non-specific staining by the detection system so it can be assumed that it is the AC88 antibody that is the cause of the additional staining. Other authors have reported similar non-specific binding of lower molecular weight proteins in a variety of cell types using this antibody; their relationship to hsp90 is uncertain (Norten *et al* 1989).

Figure 4.18 Protein synthesis in K562 cells induced to differentiate with ara-C

Fluorograph of equal numbers of ^{35}S methionine labelled cells separated by SDS-PAGE. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 3ug/ml ara-C for 12 hours (C); 24 hours (D); 36 hours (E); 48 hours (F); 72 hours (G); 96 hours (H).

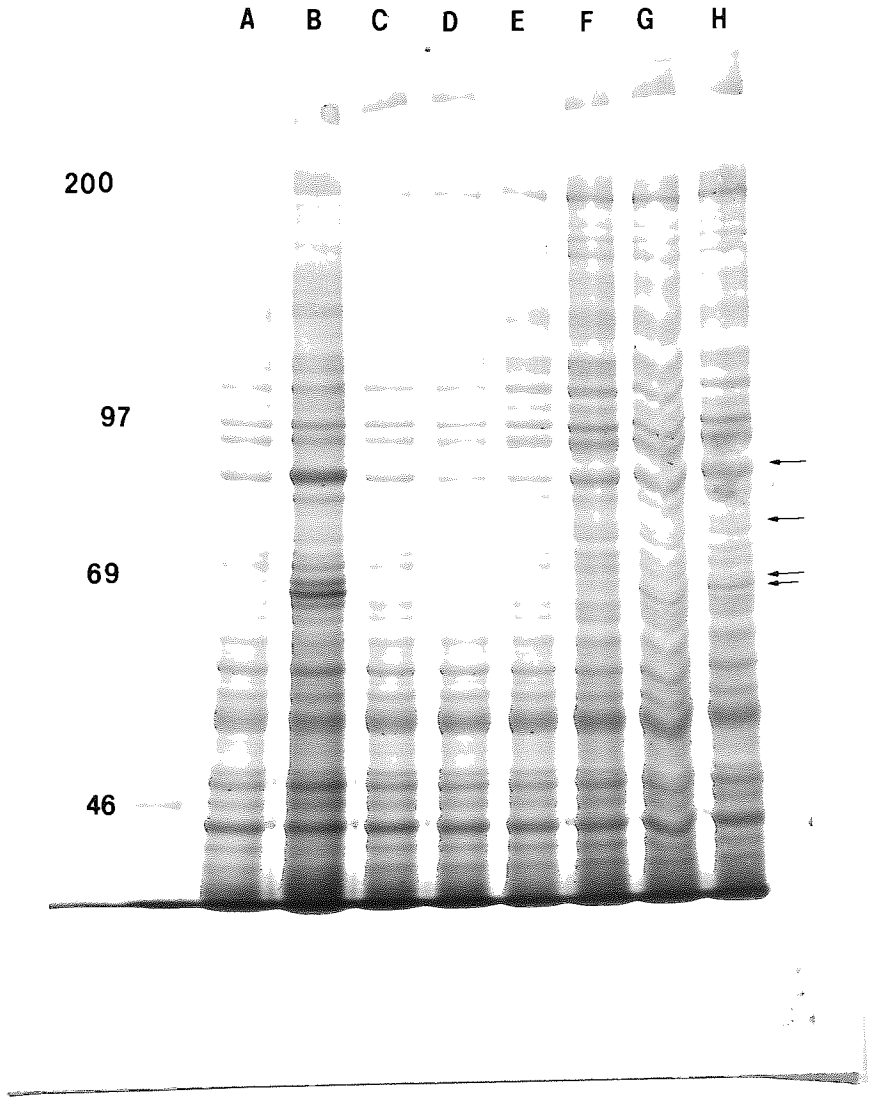


Figure 4.19 Amount of hsp70 protein in K562 cells induced to differentiate with ara-C

Immunoblot of K562 cells probed with 72kD. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 3ug/ml ara-C for 12 hours (C); 24 hours (D); 36 hours (E); 48 hours (F); 72 hours (G); 96 hours (H).

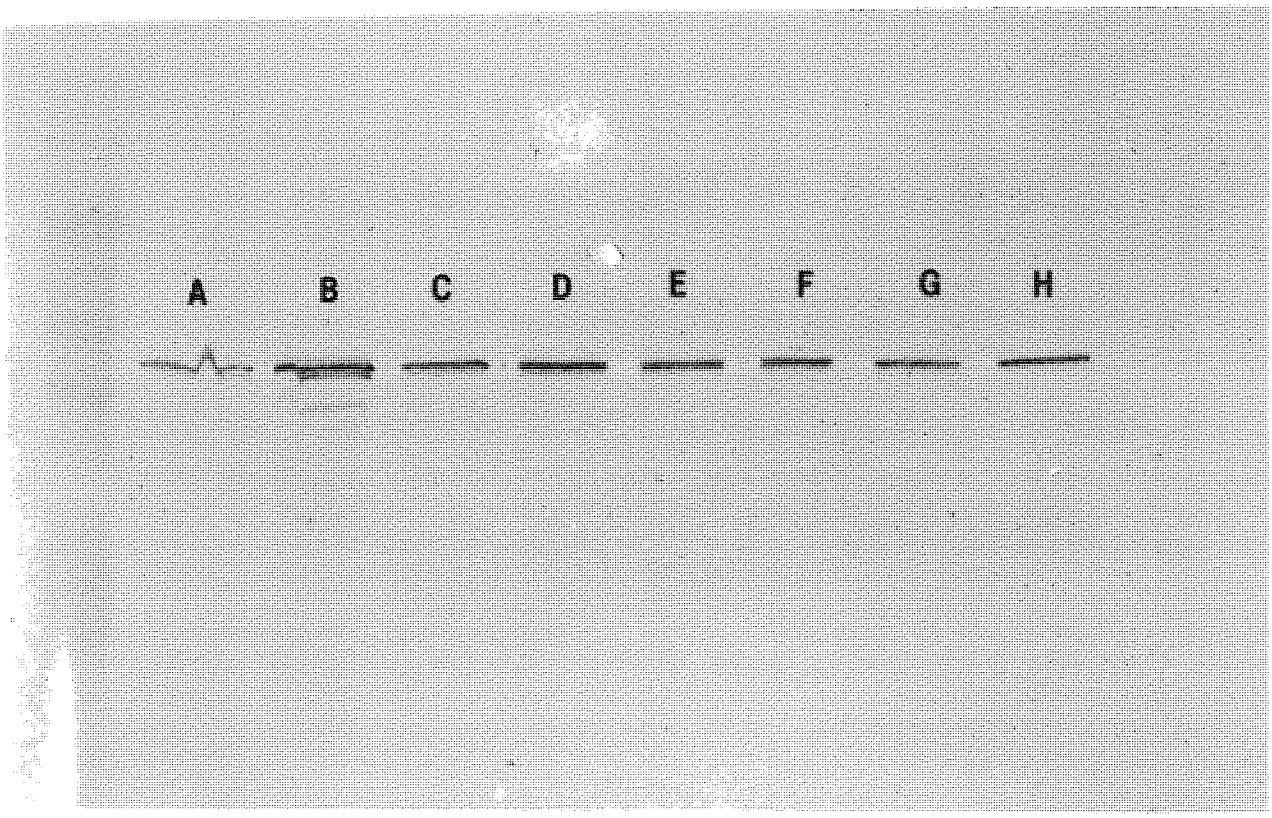
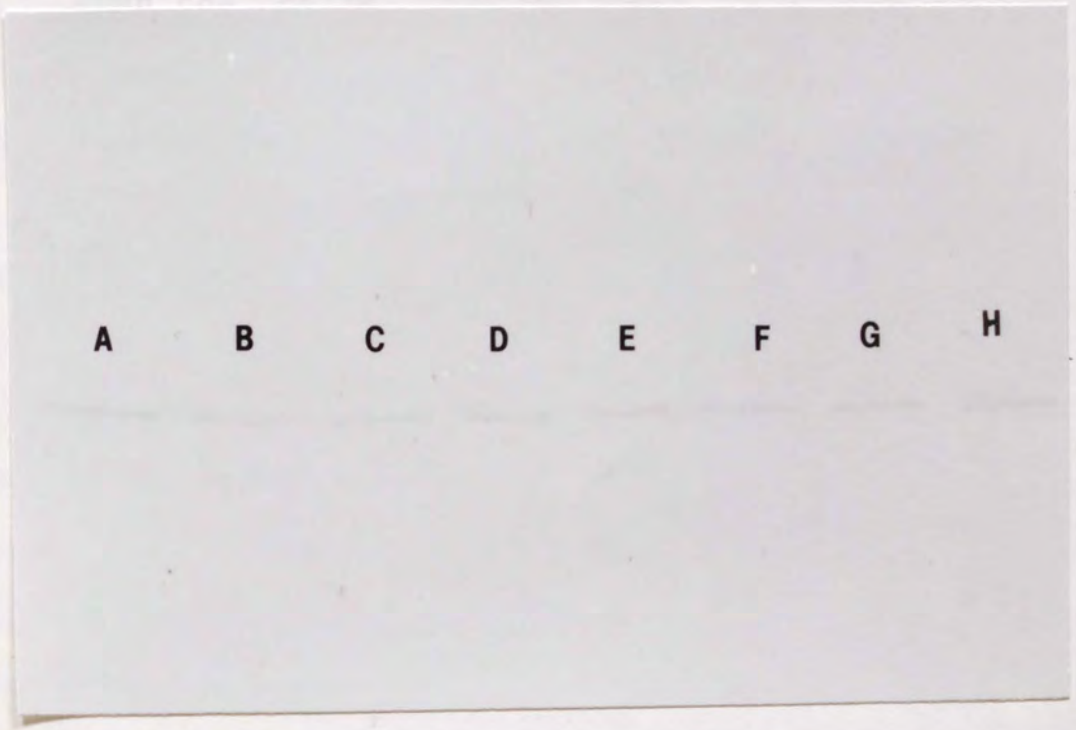


Figure 4.20 Amount of hsp90/grp94 protein in K562 cells induced to differentiate with ara-C

Immunoblot of K562 cells probed with AC88 antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 3µg/ml ara-C for 12 hours (C); 24 hours (D); 36 hours (E); 48 hours (F); 72 hours (G); 96 hours (H).



4.3.3 Discussion

Ara-C was able to induce terminal differentiation of K562 cells to erythroid-like cells, at a marginally sub-toxic concentration. There was an increase in hsp70 synthesis which accompanied an increase in amounts of hsp70 at time points when the cells were expressing markers of a more mature phenotype and after the cells had been committed to differentiate, suggesting that changes in hsp70 synthesis did not play a causative role in the induction of differentiation. There was no change in hsp90 or grp94 synthesis or amounts.

These results are in agreement with those obtained by Singh *et al* (1984) and Theodorakis *et al* (1989), and suggest that hsp70 synthesis is elevated when primitive erythroid-like cells are induced to differentiate. The use of ara-C extends these previous results by demonstrating that the increase in hsp70 synthesis also occurs during terminal differentiation.

From these results it does not appear as if the changes in the synthesis of the stress proteins grp94 and grp78 observed for HL60 granulocytic differentiation occur during K562 induced erythroid differentiation although the early accumulation of amounts of hsp70 protein does seem consistent for both cell lines. The work by Theodorakis *et al* (1989) has however shown a transcriptional activation of grp78 during haemin induced differentiation of K562 cells. The

fact that hsp70 amounts did not decrease in the K562 cells at later time points as was observed with HL60 differentiation may be due in part to the pathway of differentiation induced by ara-C or the cytotoxic effects of the drug. Also, as discussed in section 1.6.8, it has been reported that haemin induced differentiation of K562 cells is mediated via the stress-induced form of HSTF which in turn binds to the heat shock promoter at position -100; during basal expression and IL-2 inducibility the HSTF binds to the promoter at -68 (Morimoto et al 1990). Since ara-C inhibits DNA synthesis it may be inducing damage in the nucleus which may also account for the induction of hsp70. K562 differentiation would therefore appear to be more like a heat shock, in terms of the effects on stress proteins, than drug-induced differentiation of HL60 cells.

4.4 Analysis by fluorography and by Western blotting of heat shock proteins expressed in leukaemia cells after treatment with increasing concentrations of drugs.

4.4.1 Analysis of heat shock proteins in HL60 cells after treatment with increasing concentrations of NMF

Cells were treated with NMF for 24 hours, the minimum time required to commit HL60 cells to differentiate with 170mM NMF (Richards, PhD thesis 1987) to assess protein synthesis and the total amounts of hsp70 and hsp90; at this time cells were assessed as being >90% viable by trypan blue exclusion. The cell proteins which had been synthesised after treatment with increasing concentrations of NMF for 24 hours, were labelled with ^{35}S-methionine and equal numbers of cells were loaded per lane onto an SDS-polyacrylamide gel. Although treatment with the higher concentrations of NMF brought about cytotoxicity at later time points (96 hours) it was thought to be valid to load equal cell numbers rather than equal amounts of protein or equal numbers of radioactive counts in case treatment had caused some change in total protein. The cells did not show any increased synthesis of either hsp70 or hsp90 after treatment with concentrations of NMF between 50 and 250mM for 24 hours (figure 4.21).

When the amounts of hsp70 were measured by Western blotting an increase in the amount of hsp70 was observed when the concentration of NMF exceeded 140mM (figure 4.22, lanes

G-I) with the increase in concentration above this concentration being concomitant with an increase of hsp70. The low increase in amounts at 170mM was consistent with the results obtained during the time course experiments. There was no change following incubation with NMF, in the amounts of hsp90 or grp94 when a Western blot was probed with AC88 (figure 4.23).

Amounts of hsp70 were elevated to levels comparable to those obtained after a heat shock, in HL60 cells that had been treated with toxic concentrations of NMF despite the fact that there was no elevation in hsp70 protein synthesis. To investigate whether this accumulation of hsp70 without synthesis is specific for NMF induced toxicity, HL60 cells were treated with another agent that brings about differentiation but is thought to have a different site of action to NMF.

Figure 4.21 The effect of increasing concentrations of NMF on protein synthesis in HL60 cells

Fluorograph of equal numbers of ^{35}S methionine labelled cells separated by SDS-PAGE. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated for 24 hours with 50mM NMF (C); 100mM NMF (D); 140mM NMF (E); 170mM NMF (F); 200mM NMF (G); 225mM NMF (H); 250mM NMF (I); 300mM NMF (J).

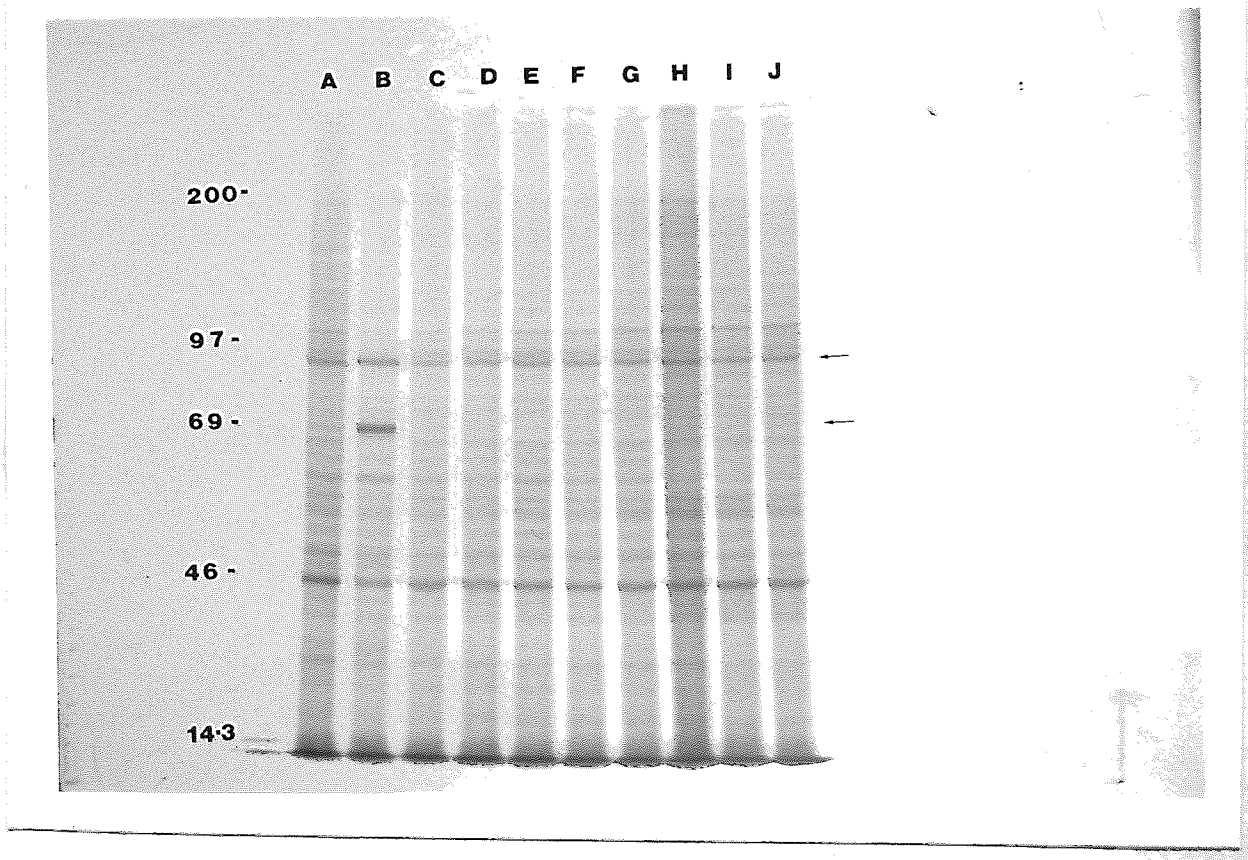


Figure 4.22 The effect of increasing concentrations of NMF on amounts of hsp70 in HL60 cells

Immunoblot of NMF treated HL60 cells probed with 72kD antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated for 24 hours with 50mM NMF (C); 100mM NMF (D); 140mM NMF (E); 170mM NMF (F); 200mM NMF (G); 225mM NMF (H); 250mM NMF (I).

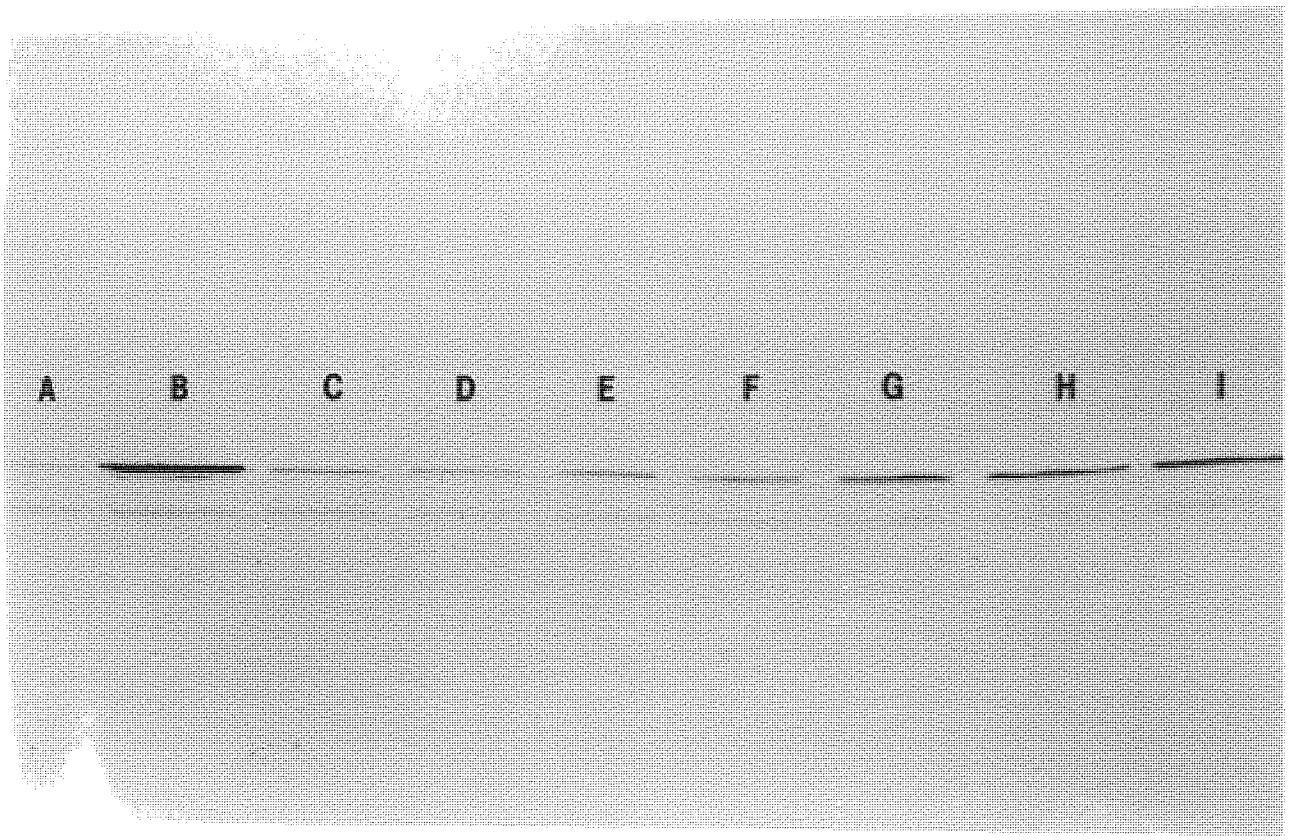


Figure 4.23 The effect of increasing concentrations of NMF on amounts of hsp90/grp94 in HL60 cells

Immunoblot of NMF treated HL60 cells probed with AC88 antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated for 24 hours with 50mM NMF (C); 100mM NMF (D); 140mM NMF (E); 170mM NMF (F); 200mM NMF (G); 225mM NMF (H); 250mM NMF (I); 300mM NMF (J).

4.4.2 The effect of TPA on HL60 cells

4.4.2.1 Characterisation of differentiation and cytotoxicity by TPA

TPA causes the rapid loss in proliferative capacity of HL60 cells and induces them to undergo terminal differentiation to monocyte or macrophage-like cells (Rovera et al. 1979). Indeed, most acute myeloid leukemia cells as well as chronic myelogenous leukemia blast crisis cells exhibit macrophage like differentiation when exposed to TPA (Fibach et al. 1981).

Incubation of HL60 cells with concentrations of TPA up to 50nM resulted in an immediate arrest of cell division and the adherence of cells to the plastic flask. The control cell population underwent 4 doublings over 96 hours, whereas the cells treated with TPA did not increase in cell number with the higher concentrations (>5nM) causing a decrease in cell number (figure 4.24). The viability of the control population remained in excess of 95%, and there was a decreased cell viability concomitant with increasing concentrations of TPA. At a concentration of TPA of 50nM less than 20% of the population remained viable.

The induction of differentiation by increasing concentrations of TPA after 72 hours indicates that maximum non-specific esterase activity and phagocytic activity was achieved with 5nM TPA and at this concentration there was

no adverse effect on cell viability with greater than 70% of the cells able to exclude trypan blue. At concentrations of TPA greater than 5nM the percentage of differentiated cells decreased rapidly. The NBT reduction assay was not utilised to assess TPA-induced HL60 differentiation because it had been reported by Newburger et al, (1981) that the TPA treated cells were not capable of reducing NBT or that there was a decreased reaction.

The optimum concentration of 5nM TPA, which caused greater than 80% of the cells to differentiate was then used to determine the amount of time TPA had to be present before the cells became committed to differentiate. Reports of the commitment time for TPA monocytic induced differentiation has varied from 20 minutes (Rovera et al, 1979) to 48 hours (Fibach et al, 1982).

Differentiation was assessed 72 hours after the addition of 5nM TPA with the cells being washed 6 times to remove the inducers before being resuspended in fresh medium. Such stringent washing conditions were used as work by Hughes (PhD thesis) had shown that at least 5 washes was required to remove the majority of the TPA from the cells. An increase in monocytic differentiation occurred after only 5 hours incubation and reached a maximum after 24 hours exposure. The cell viability remained in excess of 70% and there was a decrease in the proliferative potential of the cells after a 2 hour exposure (figure 4.25).

The cells became committed to differentiate very rapidly

and in the absence of cell replication which was in contrast to the inducers of granulocytic differentiation.

Figure 4.24 The effect of TPA on HL60 cell growth and differentiation

HL60 cells were treated with different concentrations of TPA and cell growth (A), and cell viability (B) were assessed after 72 hours. Differentiation was determined (C) by yeast phagocytosis (□) and production of non-specific esterases (■).

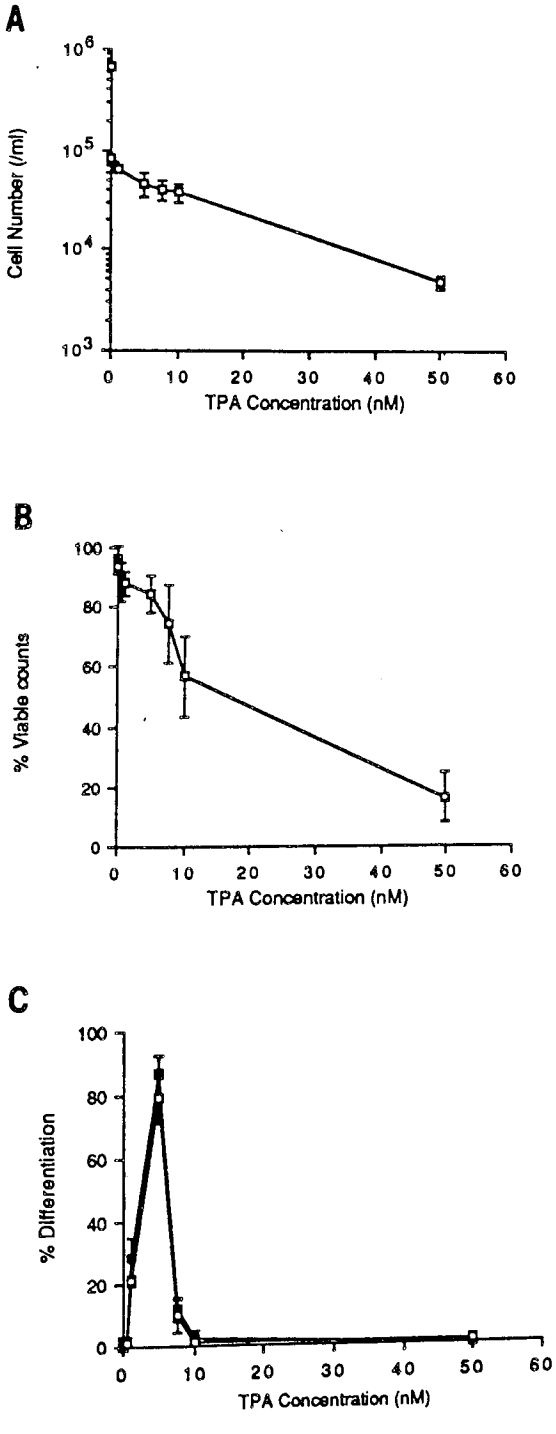
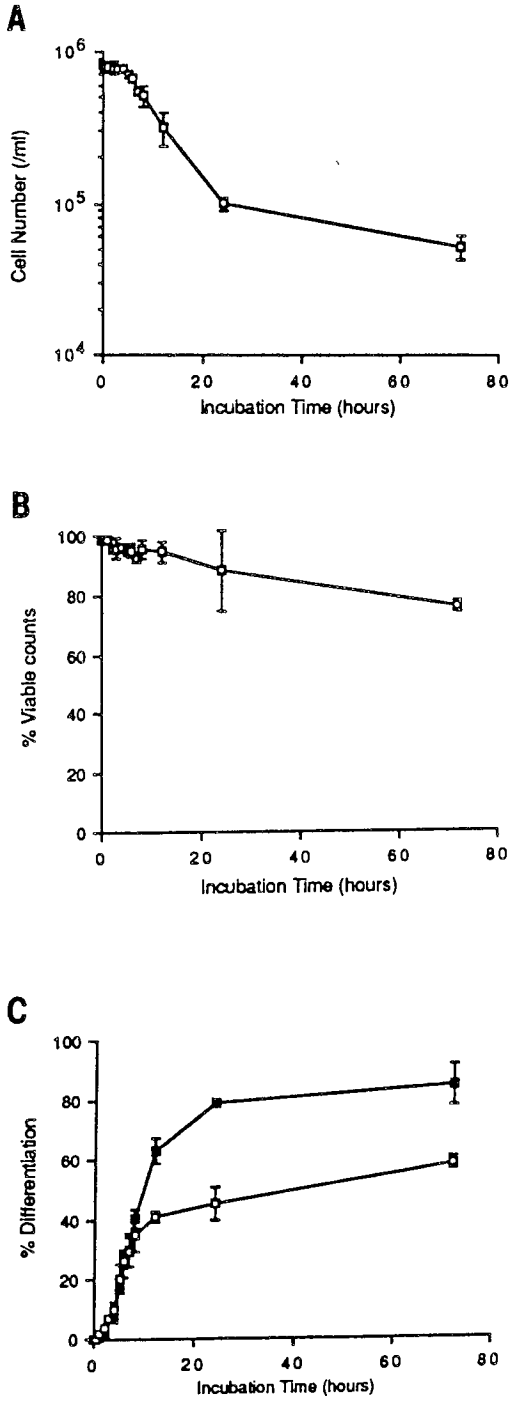


Figure 4.25 Commitment of HL60 cells induced to differentiate with TPA

HL60 cells were treated with 5nM TPA for various times following which the cells were washed and incubated in the absence of any inducer. Cell growth (A) and cell viability (B) were assessed after 72 hours. Differentiation was assessed (C) by yeast phagocytosis (□) and the production of non-specific esterases (■).



4.4.2.2. Analysis of heat shock proteins in HL60 cells after treatment with increasing concentrations of TPA

HL60 cells were treated with increasing concentrations of TPA, up to 50nM, for 5 hours before cells were harvested for analysis of protein synthesis and amounts of hsp70 and hsp90/grp94.

Concentrations of TPA up to 50nM, a toxic concentration, were added to HL60 cells for 5 hours, following which cells were labelled with ^{35}S methionine for 2 hours. As with NMF treated cells equal numbers of TPA treated cells were loaded onto SDS-PAGE. Figure 4.26, a representative fluorograph shows that there was no detectable increase in synthesis in any of the stress proteins, even at the high concentrations of TPA; typically there was an increase in synthesis of hsp70 following a heat shock. At a concentration of 5nM, that which was optimum for differentiation, there was no decrease in synthesis of either of the glucose regulated proteins as had been observed for granulocytic differentiation.

Figure 4.27, the immunoblot using the 72kD antibody showed an increase above constitutive in amounts of hsp70 at the higher concentrations of TPA (7.5, 10 and 50nM); previous results had shown that when incubated continuously for 72 hours these concentrations had brought about a loss in viability. The concentrations below 5nM, which had no significant effect on viability or differentiation, did not bring about any increase in amounts of hsp70. The

concentration of TPA that brought about maximum differentiation caused a consistent increase in the amount of hsp70 when cells were exposed for the commitment time. Probing a parallel nitrocellulose blot with AC88 indicated that there was no change in amounts of hsp90/grp94 (figure 4.28).

Figure 4.26 The effect of increasing concentrations of TPA on protein synthesis in HL60 cells

Fluorograph of equal numbers of ^{35}S methionine labelled cells separated by SDS-PAGE. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated for 5 hours with 0.1nM TPA (C); 0.5nM TPA (D); 1nM TPA (E); 5nM TPA (F); 7.5nM TPA (G); 10nM TPA (H); 50nM TPA (I).

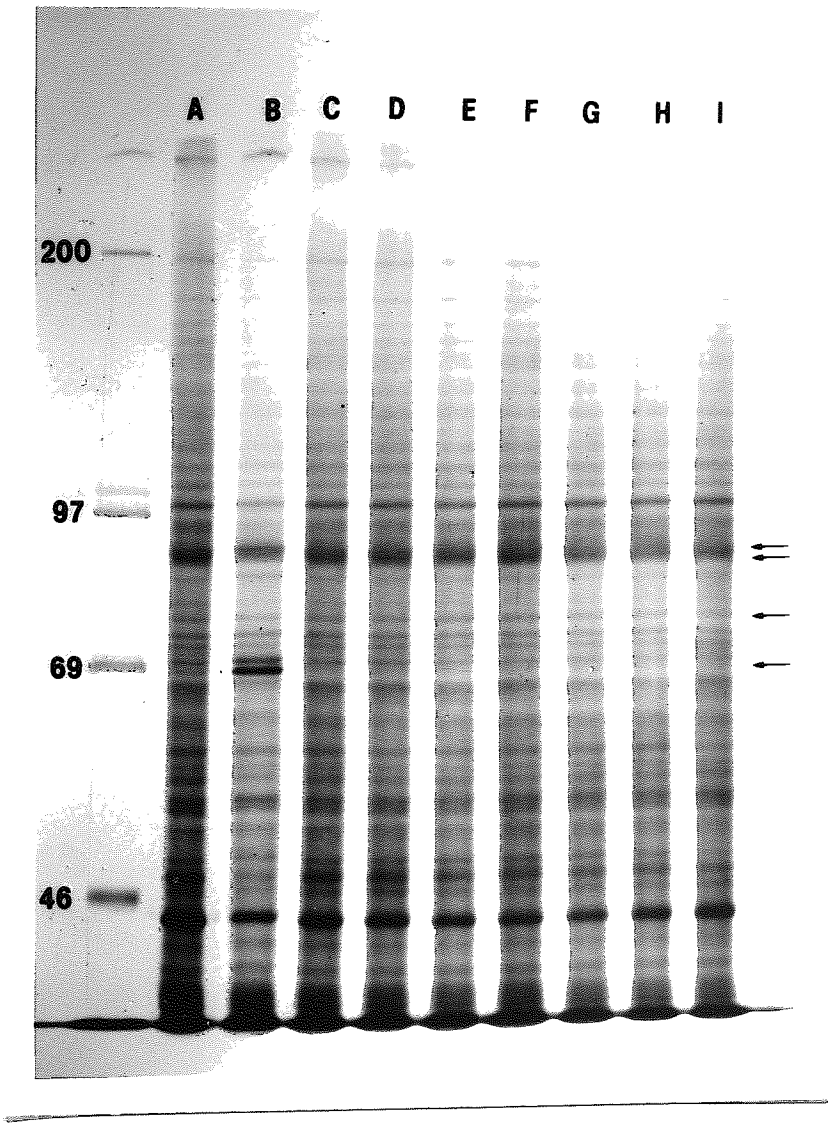
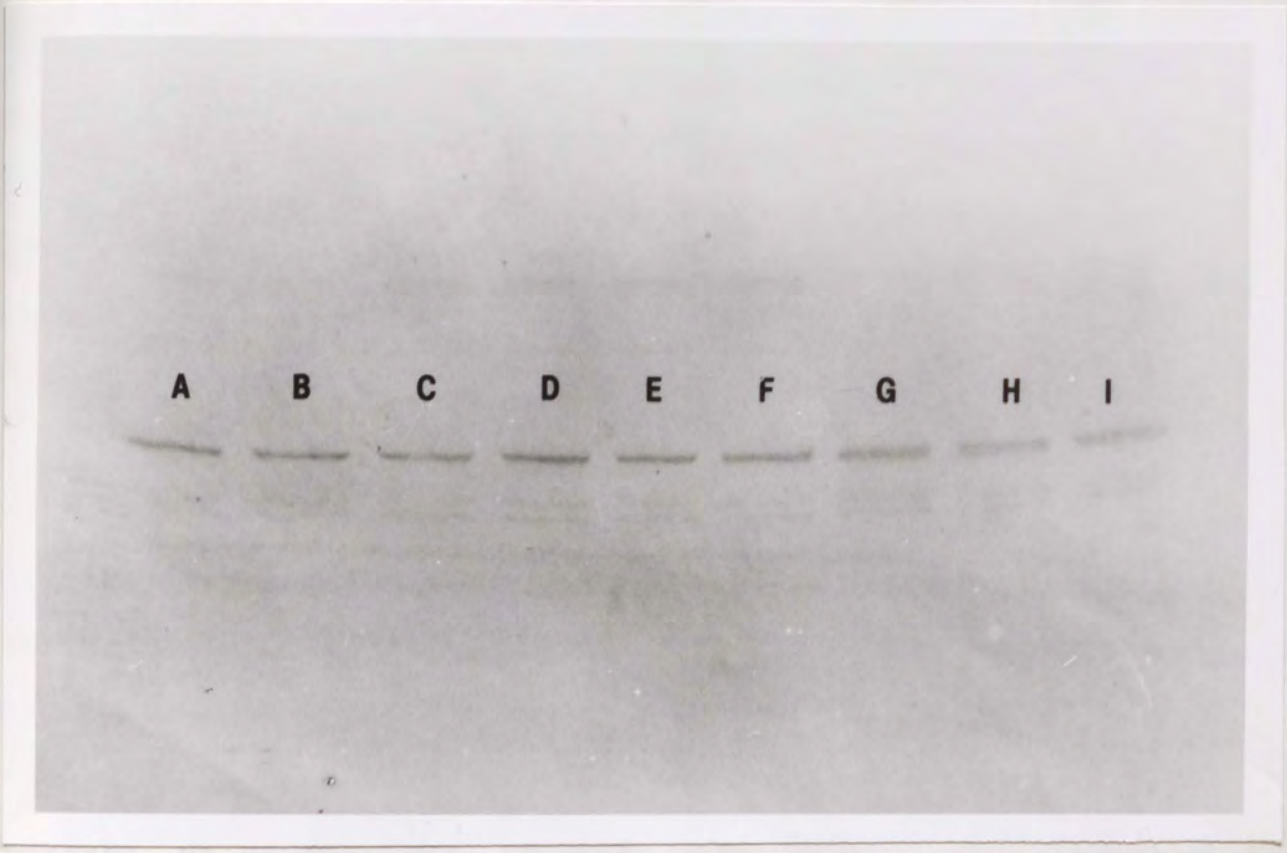


Figure 4.27 The effect of increasing concentrations of TPA on amounts of hsp70 in HL60 cells

Immunoblot of TPA treated HL60 cells probed with 72kD antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated for 5 hours with 0.1nM TPA (C); 0.5nM TPA (D); 1nM TPA (E); 5nM TPA (F); 7.5nM TPA (G); 10nM TPA (H); 50nM TPA (I).

Figure 4.28 The effect of increasing concentrations of TPA on amounts of hsp90/grp94 in HL60 cells

Immunoblot of TPA treated HL60 cells probed with AC88 antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated for 5 hours with 0.1nM TPA (C); 0.5nM TPA (D); 1nM TPA (E); 5nM TPA (F); 7.5nM TPA (G); 10nM TPA (H); 50nM TPA (I).



4.4.3 Analysis of heat shock proteins in K562 cells after treatment with increasing concentrations of ara-C

K562 cells were incubated for 24 hours with increasing concentrations of ara-C and protein synthesis and total amounts of hsp70 monitored. As was demonstrated in the time course experiment (section 4.3.2) K562 cells maintain almost normal levels of protein synthesis following a heat shock of 43.5°C and a recovery period of 2 hours at 37°C. Increasing concentrations of ara-C, up to 10µg/ml, did not have any effect on hsp synthesis (figure 4.29). Western blots using 72kD gave variable results; out of 4 blots, 2 indicated that there was an increase in amounts of hsp70 when concentrations of ara-C were increased above 5µg/ml, on one occasion there was no increase until 7.5µg/ml was used whereas one blot indicated that there was no significant change in amounts with an increase in concentration. Figure 4.30 demonstrates the results from two of the experiments

Blotting with AC88 indicated that there was no change in amounts of hsp90/grp94 (figure 4.31) with an increase in ara-C; as with the time course experiment there was some degree of non-specific binding when using this antibody.

Figure 4.29 The effect of increasing concentrations of ara-C on protein synthesis in K562 cells

Fluorograph of equal numbers of ^{35}S methionine labelled cells separated by SDS-PAGE. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated for 24 hours with 0.1µg/ml ara-C (C); 0.5µg/ml ara-C (D); 1µg/ml ara-C (E); 3µg/ml ara-C (F); 4µg/ml ara-C (G); 5µg/ml ara-C (H); 7.5µg/ml ara-C (I); 10µg/ml ara-C (J)

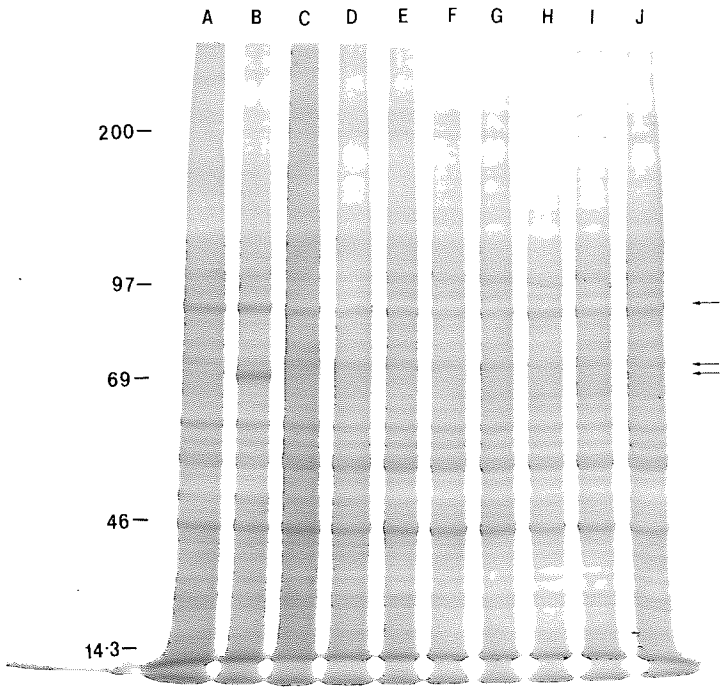
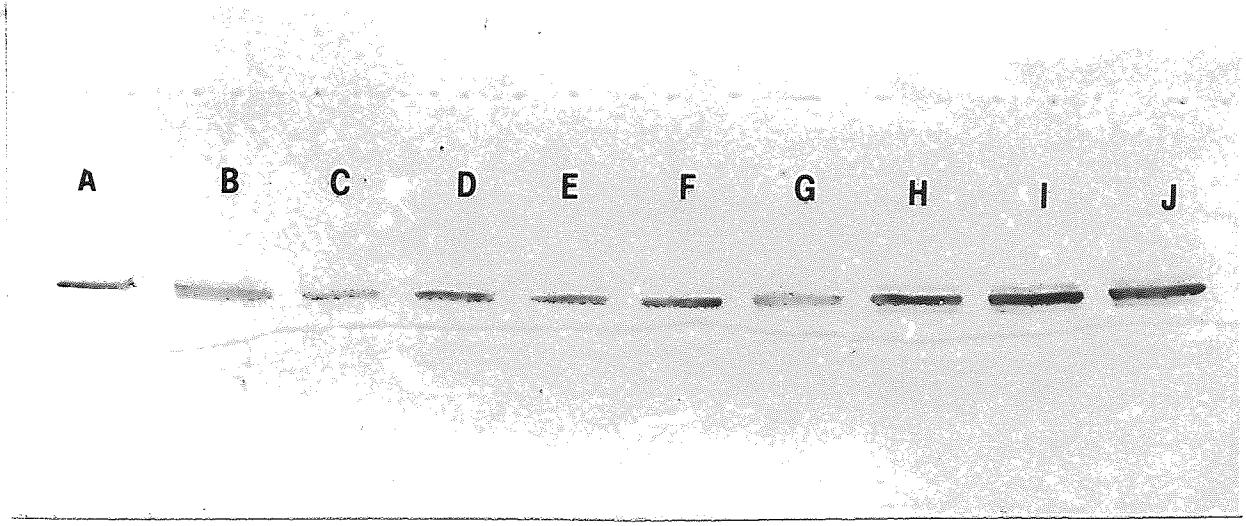


Figure 4.30 The effect of increasing concentrations of ara-C on amounts of hsp70 in K562 cells

Immunoblot of K562 cells probed with 72kD antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated for 24 hours with 0.1µg/ml ara-C (C); 0.5µg/ml ara-C (D); 1µg/ml ara-C (E); 3µg/ml ara-C (F); 4µg/ml ara-C (G); 5µg/ml ara-C (H); 7.5µg/ml ara-C (I); 10µg/ml ara-C (J). The results from two experiments are shown.



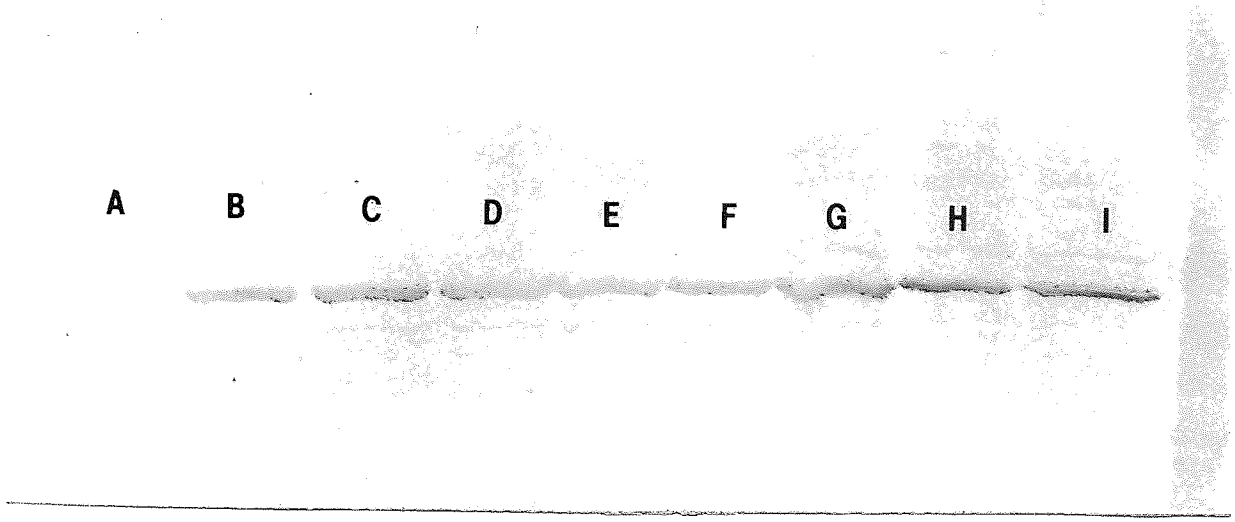
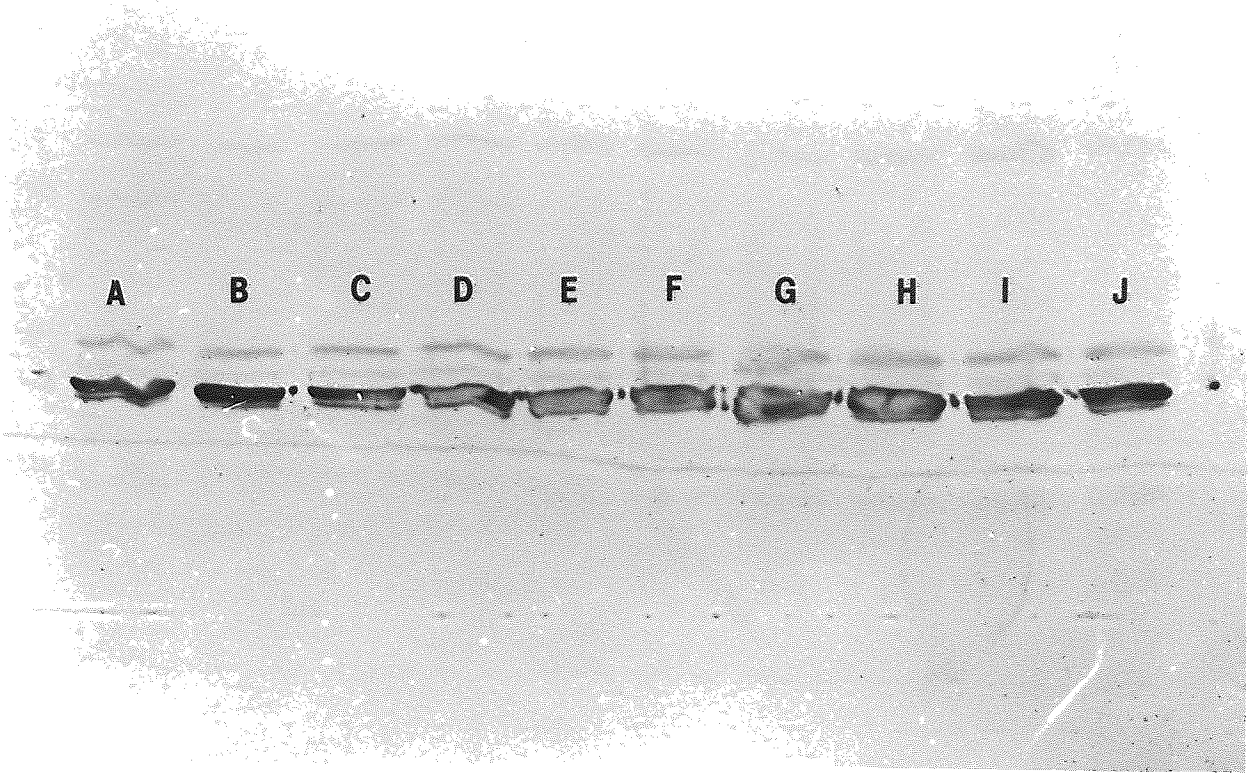


Figure 4.31 The effect of increasing concentrations of ara-C on amounts of hsp90/grp94 in K562 cells

Immunoblot of K562 cells probed with AC88 antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated for 24 hours with 0.1µg/ml ara-C (C); 0.5µg/ml ara-C (D); 1µg/ml ara-C (E); 3µg/ml ara-C (F); 4µg/ml ara-C (G); 5µg/ml ara-C (H); 7.5µg/ml ara-C (I); 10µg/ml ara-C (J)



4.4.4 Discussion

Increases in the amount of hsp70, in the absence of any increase in synthesis, were observed when HL60 and K562 cells were treated with high concentrations of NMF, TPA or ara-C, for the commitment time for each drug. There was no change in synthesis of grp78 and amounts and synthesis of hsp90, or grp94 at any of the concentrations, and the cells were >90% viable, as assessed by exclusion of trypan blue, when cells were harvested for protein labelling and Western blotting. From the initial experiments where the effect of different concentrations of NMF (section 4.2), and TPA (section 4.4.2), on HL60 cell growth, viability, and differentiation and the effect of ara-C on K562 cell growth, viability, and differentiation (section 4.3.1), were investigated, the increase in amounts of hsp70 occurred at concentrations of drug that induced differentiation or cytotoxicity. What was of particular interest was that the increases in amounts of hsp70 appeared to be an early indicator of a later loss in viability.

Increases in synthesis of stress proteins under conditions of cytotoxicity has been reported by several authors (section 1.6.7) and immunocytochemistry using a monoclonal antibody that recognises hsp70 has been used to detect neural injury (Gonzalez *et al* 1989). Since hsps are synthesised in response to a stress the results obtained with these two cell lines were not surprising as each drug did

induce a loss in viability at the concentrations which also brought about an increase in amounts of hsp70. Richards, in 1987, demonstrated that hsp70 and hsp90 were synthesised when HL60 cells were treated with very high concentrations of NMF (1M). The fact that no synthesis of hsp70 was observed in these experiments may be due to the fact that not such high concentrations of drug were used. Also other workers have used cell lines that did not express constitutive levels of hsp70 that were detectable either by immunoblotting or metabolic labelling, unlike HL60 cells. In cells where hsp70 levels are low, hsp70 would be required to be synthesised in response to a stress, to enable the cell to eliminate any damaged protein. Further discussions on the increase in amounts of hsp70 in the absence of any increase in hsp70 synthesis are presented in section 4.5.4. The fact that amounts of hsp70 in HL60 cells, following treatment with toxic concentrations of drug, were comparable to those in heat shocked cells, yet heat shock did not result in cytotoxicity, will be discussed in section 4.6.

The role of hsp70 in cells treated with toxic concentrations of drug is likely to be in binding to the damaged proteins that are produced as a direct or indirect consequence of the drugs. NMF and other solvents are thought to have profound effects on the "structure" of cellular water by interference with hydrogen bonding between water molecules (Kennedy et al 1987); presumably NMF could interact with hydrogen bonds in proteins in a similar manner, causing

changes in protein folding. TPA interacts with protein kinase C (Castagna et al 1982) and may induce conformational changes in the enzyme.

Evidence to support the stable binding of hsp70 to abnormal proteins has been provided by Beckmann et al (1990) who showed that after treatment with azetadine carboxylic acid significant amounts of ^{35}S labelled protein were co-precipitated with hsp70 and that these proteins still co-precipitated following a two hour incubation in the absence of azetadine and label; the amount of co-precipitating material in unstressed cells was markedly reduced following the two hour chase. The recent studies by Chiang et al (1989) suggest a role for hsp70 in the targeting of proteins for lysosomal degradation; damaged proteins such as would be produced in cells following treatment with toxic drugs, may be bound by hsp70 which then enables them to be degraded by lysosomal proteolysis.

In the light of these results it was proposed that when HL60 cells are treated with toxic concentrations of a drug, there is an increase in amounts of the heat shock protein hsp70, which is not necessarily accompanied by an increase in synthesis, and that this may be an early marker of a later loss in cell viability. Experiments were therefore designed to test this hypothesis (section 4.5). Since the results obtained with the K562 cells were variable it was decided to continue further studies with HL60 cells.

4.5 Investigations on the increase in amounts of hsp70 in HL60 cells treated with toxic concentrations of a drug

4.5.1 HL60 cells treated with NMF

Exponentially growing cultures of cells were treated for up to 8 hours with 225mM NMF. At the end of the 8 hour period cells were labelled with ^{35}S methionine for analysis of protein synthesis and harvested for Western blotting. A sample of cells from each time point and the control were washed twice in PBS, pre-warmed to 37°C, and resuspended in fresh medium at a density of 2 x 10^5 per ml. The flasks were gassed with 5% CO_2 and incubated at 37°C for 24 hours. Viability was measured by the exclusion of the dye trypan blue when the cells were initially harvested following the time course and after the 24 hour incubation in drug-free media. Intracellular calcium levels were also monitored as a measure of cell viability and membrane integrity. The ability of cells to proliferate after removal of NMF was determined by their ability to form colonies in semi-solid media.

The cells did not show any loss in membrane integrity, as indicated by their ability to extrude trypan blue, and by low (less than 200nM) resting calcium levels (figure 4.32), at the end of the 8 hour time course. Methionine labelling (figure 4.33) of cell proteins indicated that there was no change in the synthesis of any of the stress proteins but

figure 4.34, a Western blot of samples harvested in parallel, demonstrated that there was an increase in amounts of hsp70 after 4 hours. Cells that had been incubated with NMF for 8 hours had amounts of immunodetectable hsp70 comparable to those achieved after a heat shock (lanes B and J). Assessment of the cells that had been incubated for 24 hours in drug-free medium showed a time related loss in viability (figure 4.35) and that this loss in viability, up to 40% in those cells that had been exposed to NMF for 8 hours, was concomitant with the increase in amounts of hsp70 observed after Western blotting. Repetitions of the experiment demonstrated that although the time point at which hsp70 was elevated varied, the increase was accompanied by a later loss in viability of approximately 60%. What is interesting is that the wash procedure itself reduced cell viability; a 96 hour continuous incubation with 225mM NMF was required to induce a similar loss in viability to that brought about by the 8 hour incubation followed by washing. Similar results have been reported by Bill et al. 1988. Cells that had been treated with NMF for 5 hours showed a 50% loss in proliferative capacity, and cells that had been treated for 7 or more hours were unable to form colonies (figure 4.36).

To confirm that the results obtained with the 72kD antibody were reproducible, parallel blots were probed with antibodies that had been raised in a number of laboratories against different hsp70 epitopes. A blot stained with 72kD was used for a direct comparison. Figure 4.37 shows

representative blots using a) antibody 72kD

b) antibody 7.10

c) antibody 5A5-1

The results indicate that the same trend, that of an increase in amounts of hsp70 concomitant with a later loss in viability, was observed using each of the three antibodies. Hsp70 was the major band detected by 7.10 and 5A5-1 in each case.

Samples were also harvested for blotting after the 24 hour incubation in drug-free media. Probing a blot with the 72kD antibody showed that the remaining cells which all excluded trypan blue, did not have amounts of hsp70 that were elevated above control (figure 4.38), whereas cells that had been heat shocked then incubated for 24 hours at 37^{o}c still expressed elevated amounts of hsp70.

A blot was also probed with the antibody AC88 and figure 4.39 shows that there was no change in amounts of hsp90/grp94 following incubation with 225mM NMF.

To confirm that the results observed for hsp70 were not the consequence of a general stabilisation of all cell proteins, a Western blot was probed with an antibody raised against mouse actin. Out of 4 independent experiments an accumulation of actin was observed on only one occasion and this occurred after 6 hours exposure to 225mM NMF; figure 4.40 shows the results from two experiments including the one experiment where there was an increase in actin amounts (lanes H-J).

Since it is known that hsp70 is regulated during the cell cycle with a 10- to 15- fold rise during S phase (Milarski et al, 1986) it was considered important to determine whether or not the changes in amounts of hsp70 were the result of changes in cell cycle events.

HL60 cells were incubated with 225mM NMF for 3, 4 and 8 hours and then analysed by FCM (figure 4.41A). After 3 and 4 hours incubation there was no change in cell cycle but after 8 hours there was a small increase to 22.4% in cells in the G_2M phase of the cell cycle ($p<0.05$). A sample of cells from each time point was also analysed following a 24 hour incubation in drug-free media (figure 4.41B). In those cells that had been treated with NMF for 3 hours there was no change in cell cycle but for those samples that had been exposed for longer periods of time there was a significant increase in the proportion of cells in the G_1 phase of the cell cycle ($p<0.005$) with a concomitant decrease in the population of S phase cells. Representative DNA histograms of the HL60 cell samples are shown in figures 4.41 C and D. In the first set of time points it can be seen that there is only a small percentage of non-viable cells at lower DNA fluorescence which agrees with the results obtained with trypan blue indicating that greater than 80% of the cell population was viable. Figure 4.42 shows the cell size after NMF treatment and demonstrates that there was no change in the average cell size up to 8 hours treatment with 225mM NMF compared with control. Although size analysis was carried

out on fixed cells which may in its self bring about a reduction in size it was still thought valid to compare the control and NMF samples.

In the light of these results it was decided to investigate whether this increase in amounts of hsp70 accompanied by a later loss in viability could be repeated using other agents with different site of action.

Figure 4.32 Intracellular calcium concentration in HL60 cells treated with 225mM NMF

Intracellular calcium concentrations were determined by quin 2 loading after 3, 4 and 8 hours incubation with 225mM NMF (A). Intracellular calcium concentrations were also determined following a 24 hour incubation in drug-free media (B).

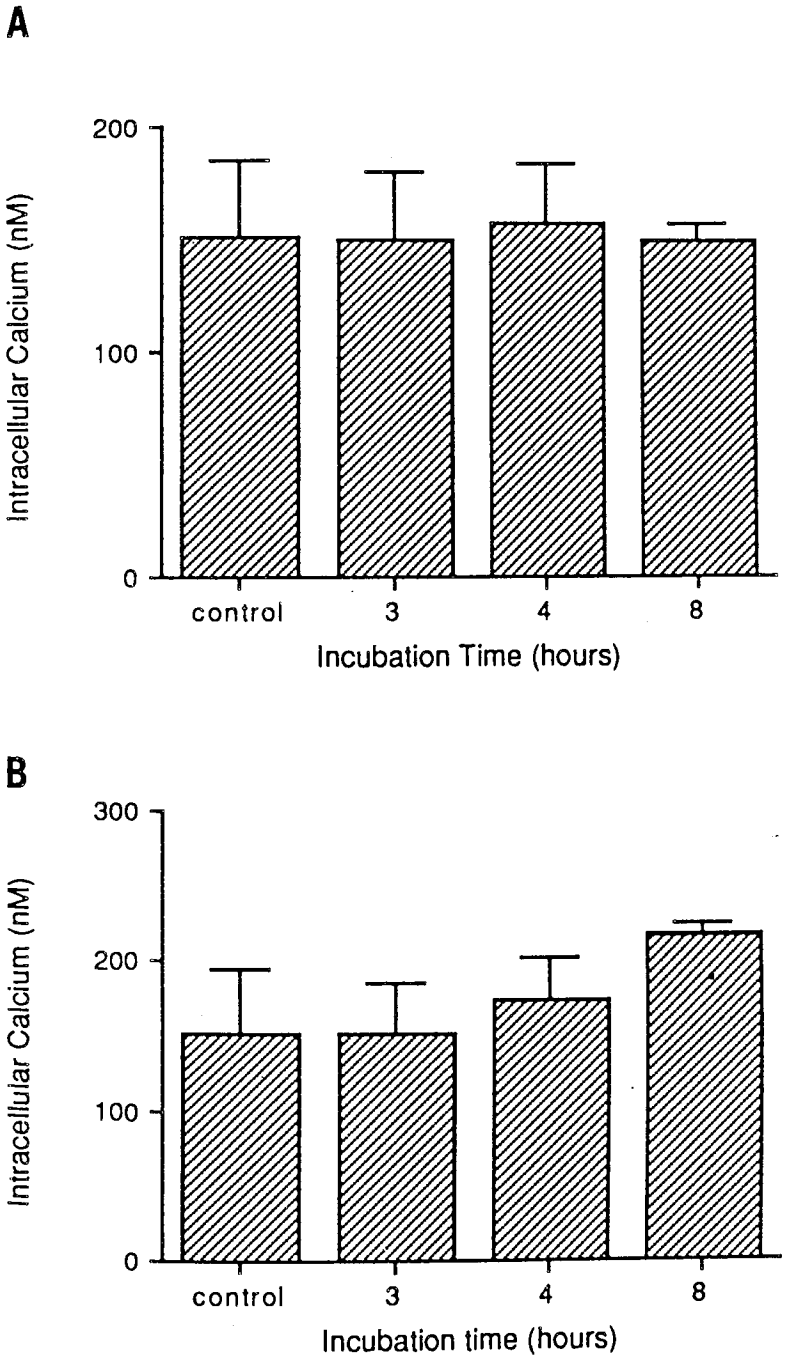


Figure 4.33 The effect of length of exposure to 225mM NMF on protein synthesis in HL60 cells

Fluorograph of equal numbers of ^{35}S methionine labelled cells separated by SDS-PAGE. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 225mM NMF for 1 hour (C); 2 hours (D); 3 hours (E); 4 hours (F); 5 hours (G); 6 hours (H); 7 hours (I); 8 hours (J).

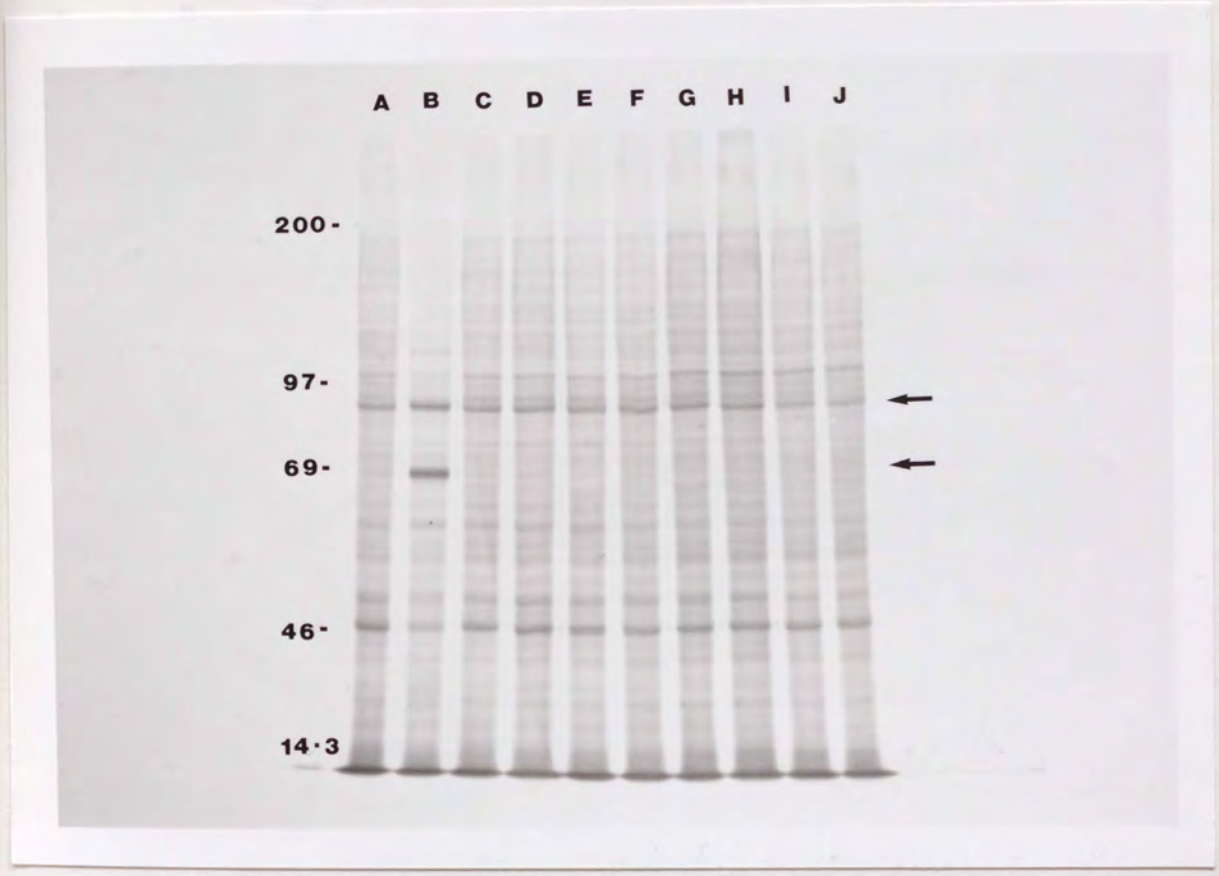


Figure 4.34 The effect of length of exposure to 225mM NMF on amounts of hsp70 in HL60 cells

Immunoblot of HL60 cells probed with 72kD antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 225mM NMF for 1 hour (C); 2 hours (D); 3 hours (E); 4 hours (F); 5 hours (G); 6 hours (H); 7 hours (I); 8 hours (J). The results from several experiments are shown; in experiments b and c there is no two hour time point.

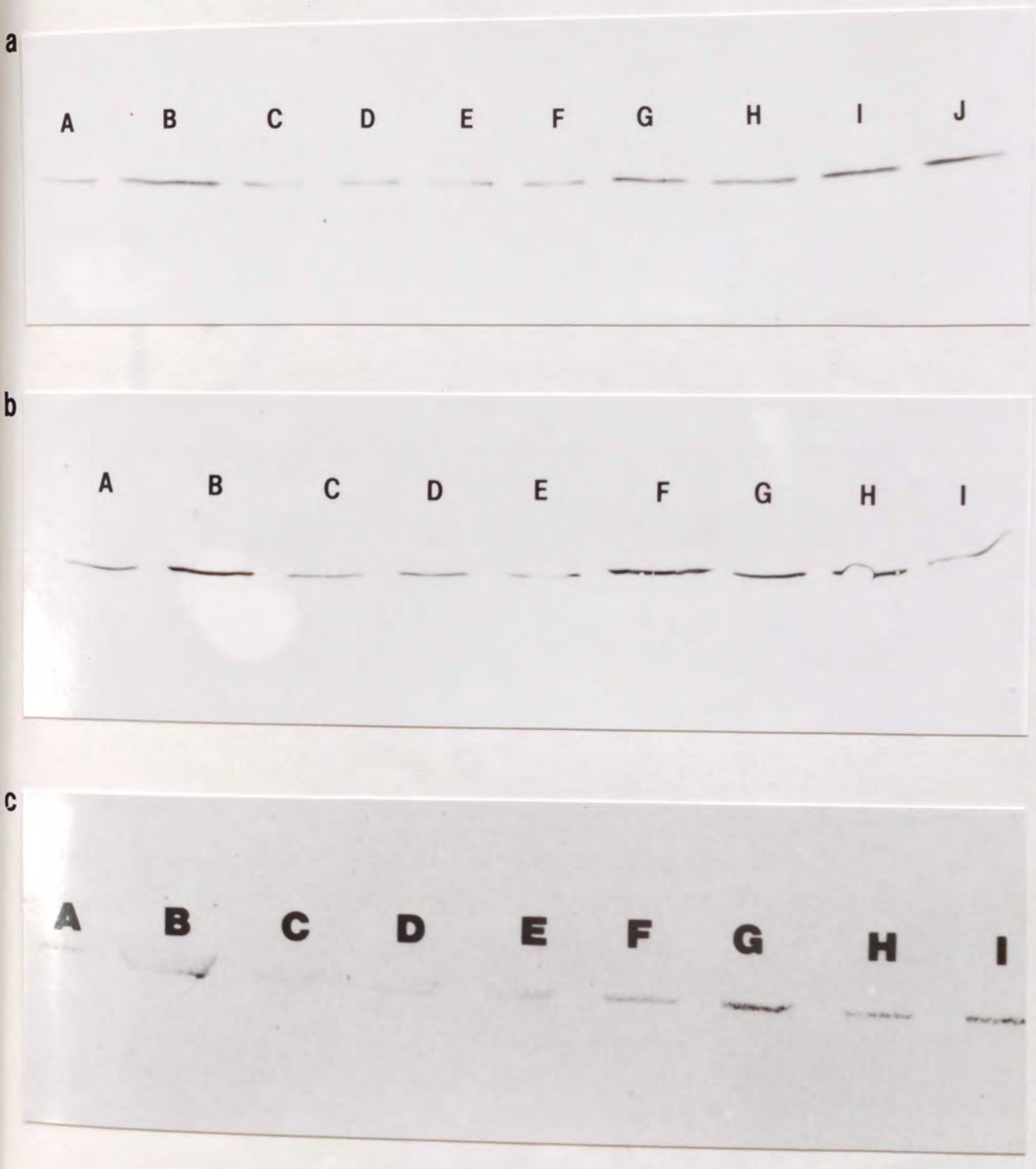


Figure 4.35 The effect on HL60 cell viability following a 24 hour incubation in drug-free medium after treatment with 225mM NMF

HL60 cells were treated with 225mM NMF for 1 to 8 hours following which the drug was removed. Viability was assessed by trypan blue exclusion after a further 24 hour incubation in the absence of NMF.

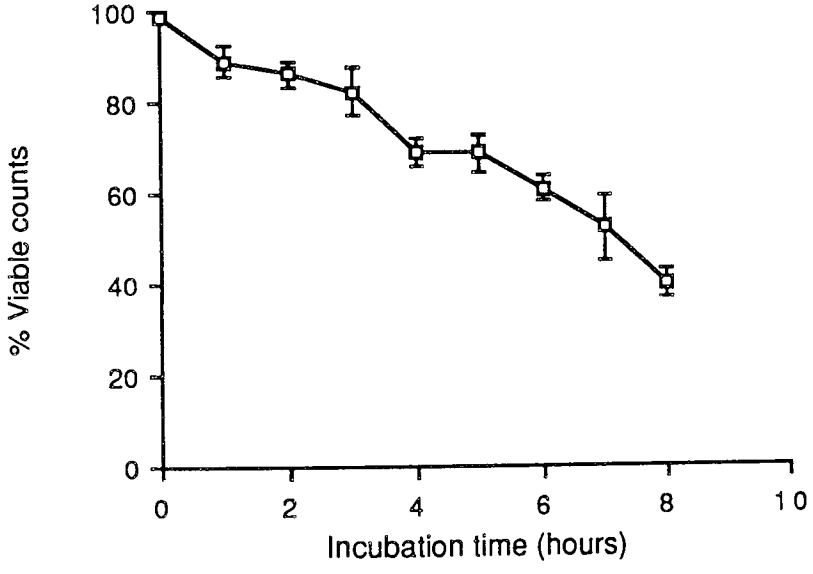


Figure 4.36 The effect of length of exposure to 225mM NMF on the cloning efficiency of HL60 cells

HL60 cells were cultured in suspension in the presence of 225mM NMF. At the times indicated the cells were washed and cloned in drug-free semi-solid medium. Colonies were counted on day 10.

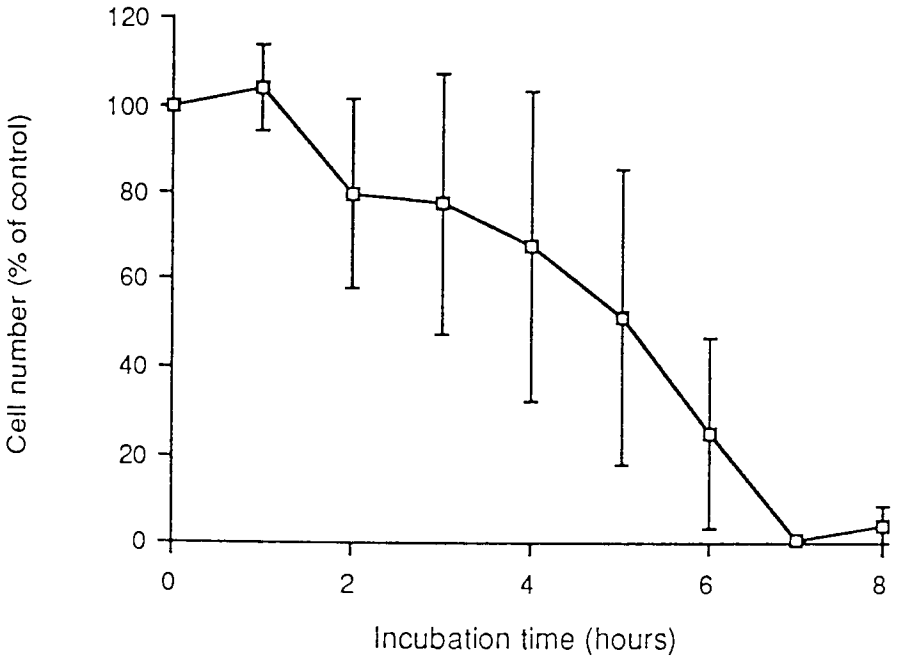


Figure 4.37 The effect of length of exposure to 225mM NMF on amounts of hsp70 in HL60 cells : comparison of three antibodies

Immunoblot of HL60 cells probed with 1. 72kD antibody, 2. 5A5 antibody, 3. 7.10 antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 225mM NMF for 1 hour (C); 2 hours (D); 3 hours (E); 4 hours (F); 5 hours (G); 6 hours (H); 7 hours (I); 8 hours (J).

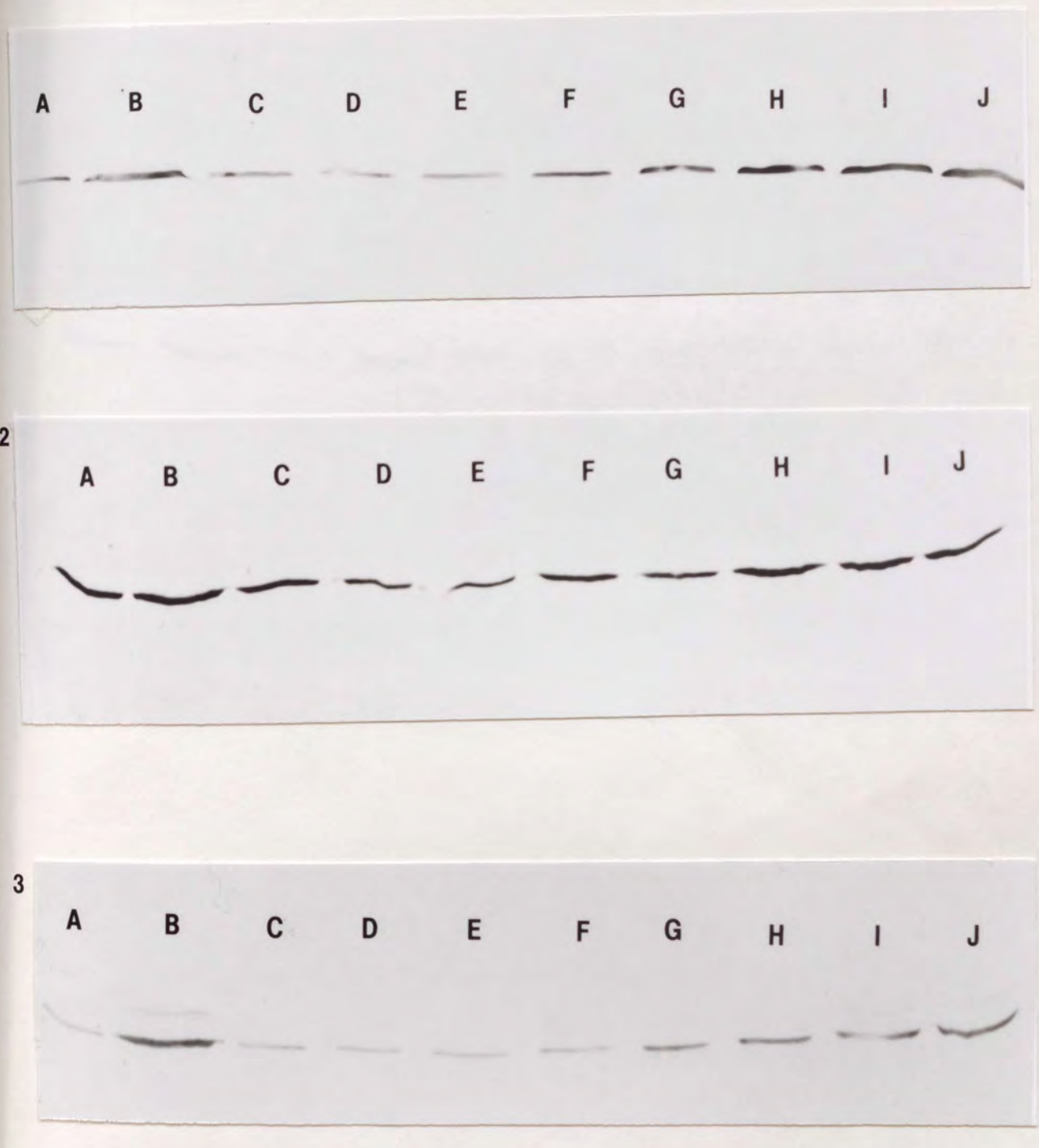


Figure 4.38 The effect of exposure to 225mM NMF on amounts of hsp90/grp94 in HL60 cells

Immunoblot of HL60 cells probed with AC88 antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 225mM NMF for 1 hour (C); 2 hours (D); 3 hours (E); 4 hours (F); 5 hours (G); 6 hours (H); 7 hours (I); 8 hours (J).

Figure 4.39 The amount of hsp70 in HL60 cells that are able to exclude trypan blue following a 24 hour incubation in drug-free medium after treatment with 225mM NMF

Immunoblot of trypan blue negative HL60 cells that had been treated with NMF for 1-8 hours then incubated for a further 24 hours in the absence of NMF. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 24 hours) (B); or treated with 225mM NMF for 1 hour (C); 2 hours (D); 3 hours (E); 4 hours (F); 5 hours (G); 6 hours (H); 7 hours (I); 8 hours (J).

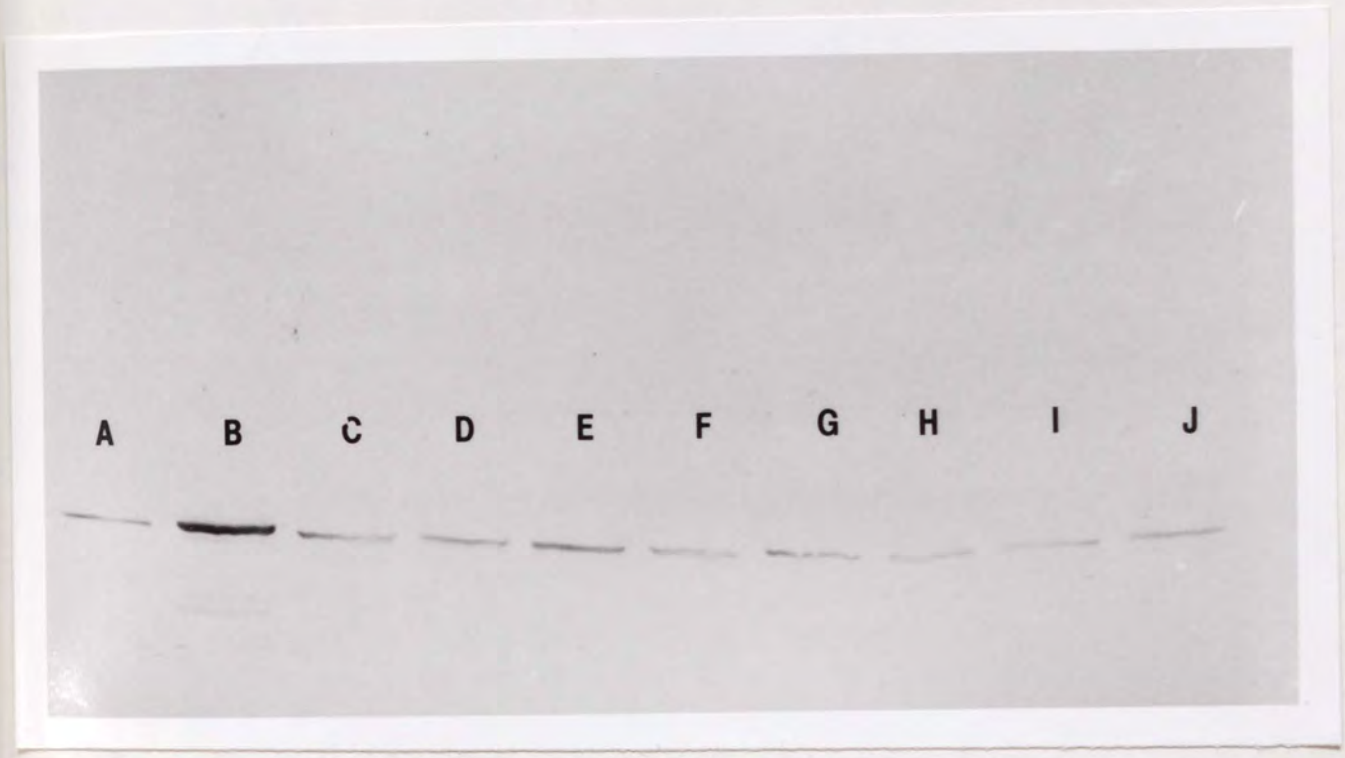


Figure 4.40 The effect of length of exposure to 225mM NMF on amounts of actin in HL60 cells

Immunoblot of HL60 cells probed with actin antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 225mM NMF for 1 hour (C); 2 hours (D); 3 hours (E); 4 hours (F); 5 hours (G); 6 hours (H); 7 hours (I); 8 hours (J). The results from two experiments are shown.

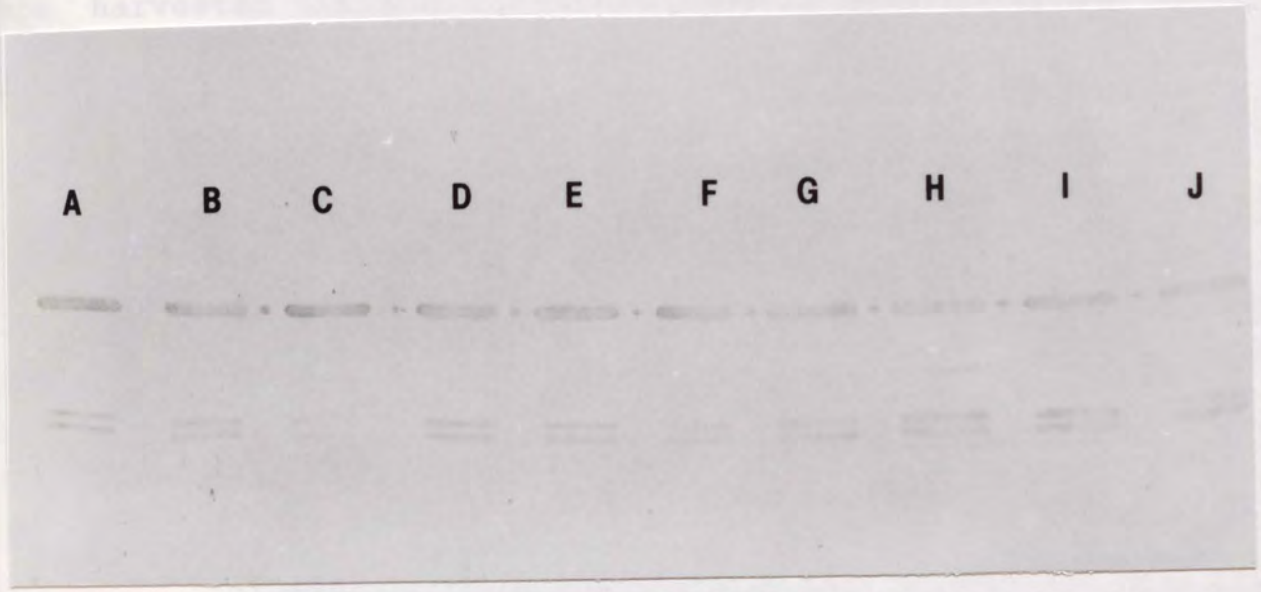
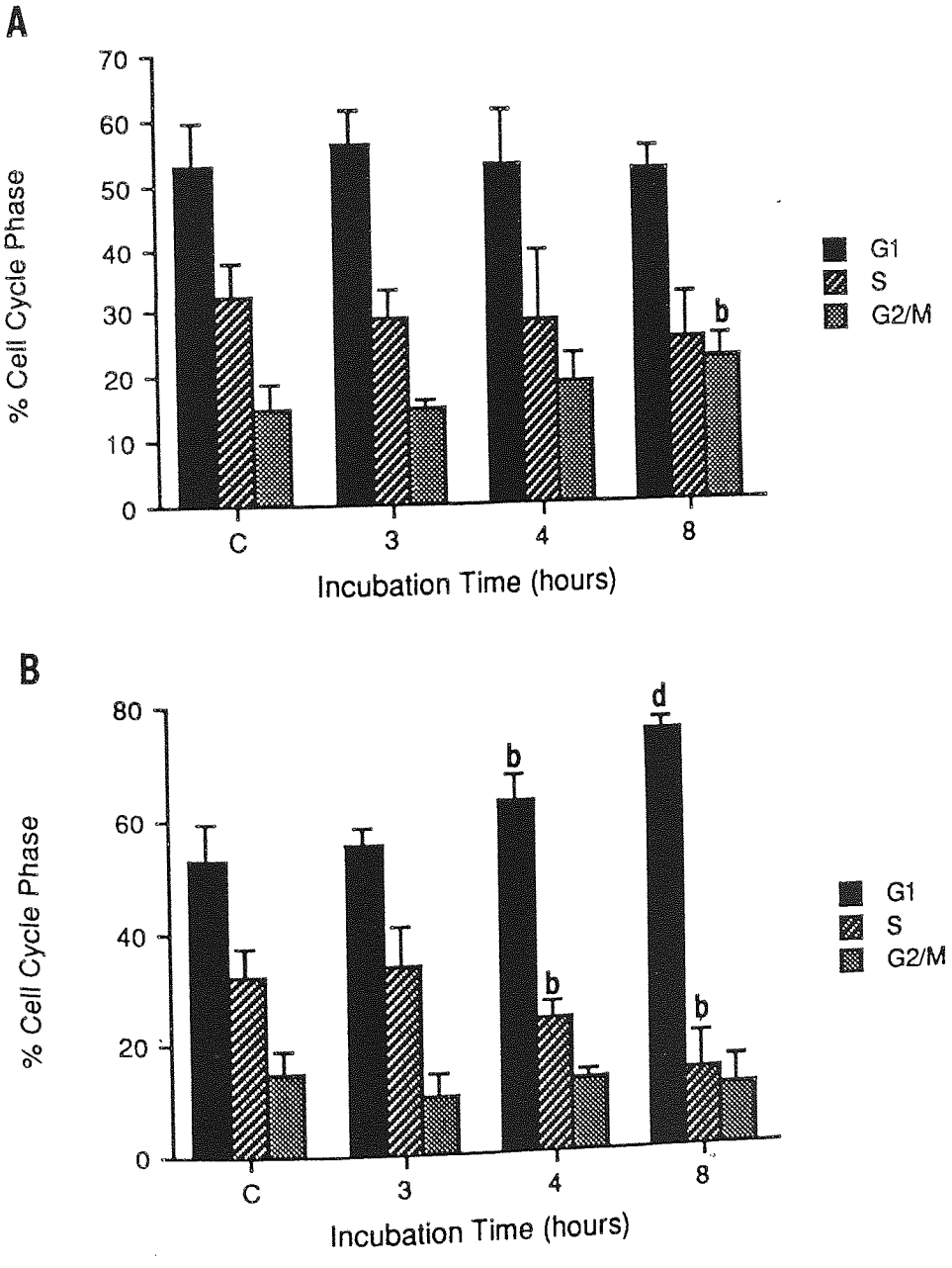
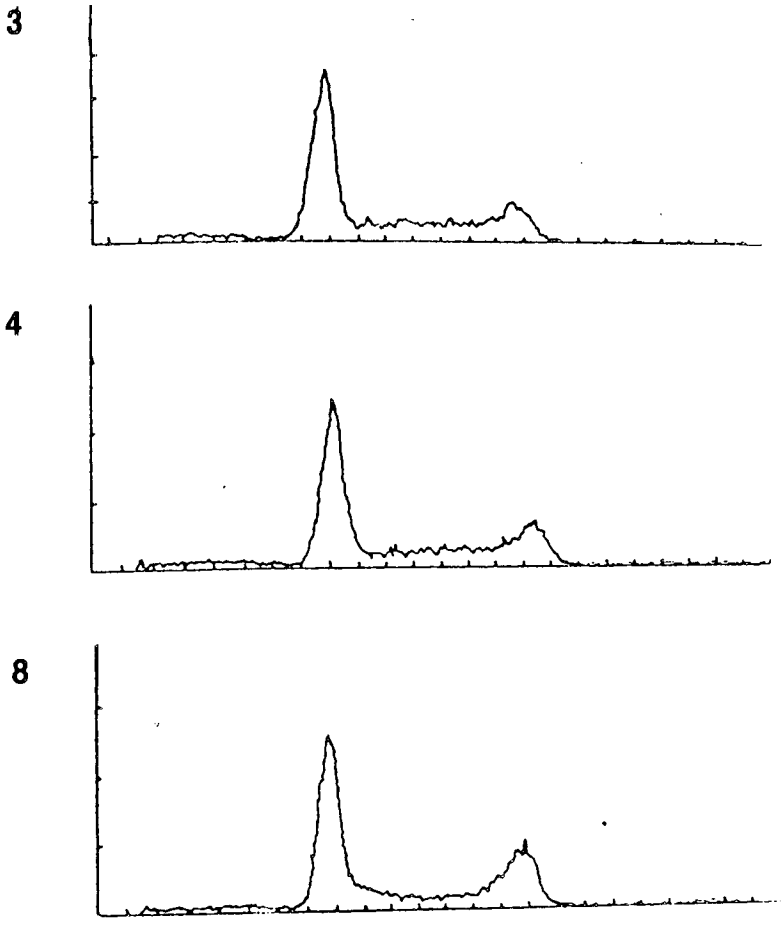


Figure 4.41 The effect of 225mM NMF on HL60 cell cycle

The percentage of HL60 cells in each phase of the cell cycle was determined after treatment with 225mM NMF for 3 hours; 4 hours; and 8 hours (A). Cell cycle analysis was also determined following a 24 hour incubation in the absence of NMF (B). The percentage of cells in each phase of the cell cycle after treatment were compared with control cells and levels of significance calculated using a Students T-test. Unless stated there was no significant difference between control and treated cells. a: $p<0.1$; b: $p<0.05$; c: $p<0.01$; d: $p<0.005$; e: $p<0.001$
Representative histograms of DNA content are shown; cells were harvested immediately following treatment (C); cells were harvested following a 24 hour incubation in the absence of NMF (D).



C



D

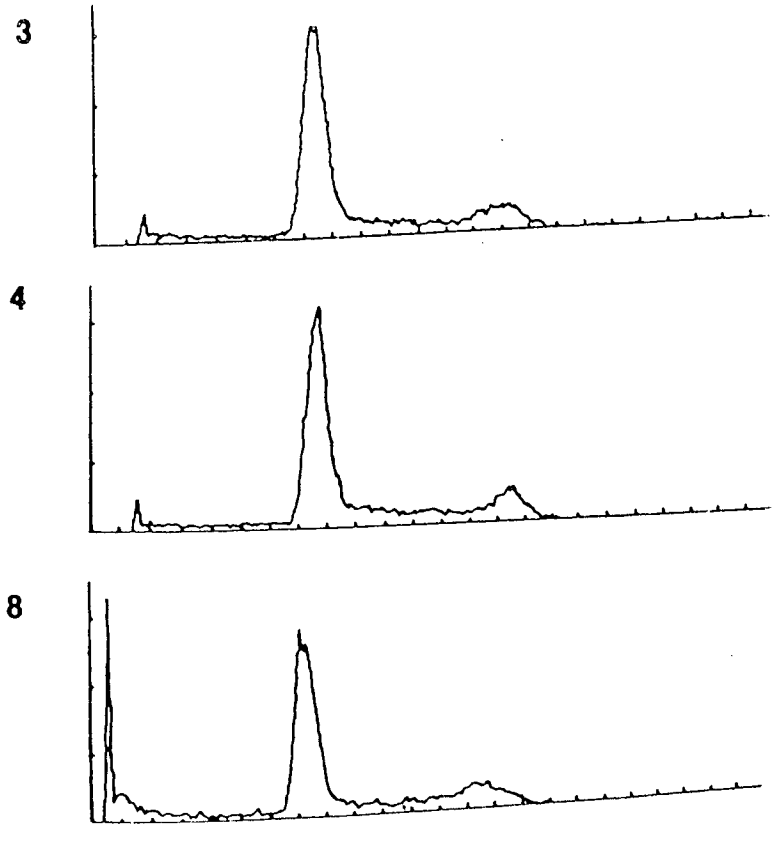
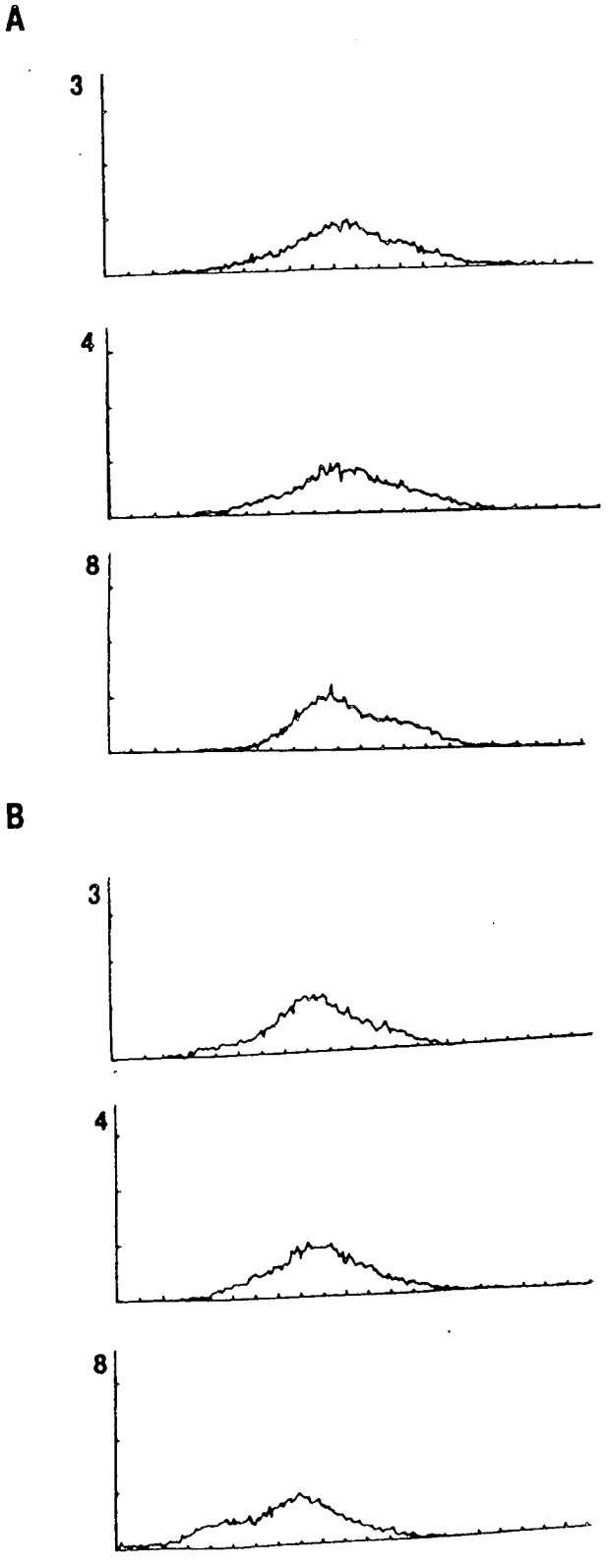


Figure 4.42 The effect of 225mM NMF on HL60 cell size

Histograms of HL60 cell size after treatment with 225mM NMF for 3 hours, 4 hours, and 8 hours (A). Cells were also harvested following a 24 hour incubation in drug-free medium (B).



4.5.2 HL60 cells treated with Adriamycin

Adriamycin, an anthracycline antibiotic, is used to treat haematological malignancies and a wide range of solid tumors (Pratt and Ruddon 1979). It interacts with nucleic acids and cell membranes although the molecular basis for its antitumour activity is unknown (Tritton et al 1982).

The relationship between the concentration of Adriamycin and HL60 cell growth and viability is shown in figure 4.43. Cells were incubated continuously with concentrations of drug up to 2uM and cell growth and viable counts were assessed on a daily basis over a 96 hour time course. Adriamycin, at concentrations of less than 100nM, did not significantly effect cell growth or viability whereas higher concentrations caused a progressive decrease in growth rate and viability (up to 2uM). Incubation of cells with 1uM Adriamycin produced a loss in viability of 80% and a total inhibition of proliferation (figure 4.44). No differentiation, either monocytic or granulocytic, was observed at any concentration.

Exponentially growing cells were incubated for time periods between 2 and 24 hours with 1uM Adriamycin and at the end of this period cells were labelled with ^{35}S methionine, and harvested for Western blotting. Viability was assessed as described for NMF treated samples, both at the end of the time course and following a 24 hour incubation in drug-free medium. Proliferative potential was determined

by colony formation in methylcellulose. A relatively high concentration of Adriamycin was used as this brought about a similar loss in viability over 96 hours as did 225mM NMF, and also it was of interest to see whether a high concentration of Adriamycin would increase synthesis of hsp70 as did 1M NMF (Richards et al. 1988).

The fluorographs, figure 4.45, show variable results; in general there was no increase above controls in hsp70 synthesis when cells were exposed to 1μM Adriamycin, except on one occasion (figure 4.45b), where there was an induction after 24 hours incubation. There was a decrease in overall protein synthesis after 8 to 16 hours. Western blotting (figure 4.46) with 72kD showed an increase in amounts of hsp70 after 8 hours incubation (lane F) and these increased amounts were present in the 16 and 24 hour samples; amounts of hsp70 in these cells were comparable to that in the heat shocked cells. There was a consistent decrease in hsp70 amounts below that in the control in the samples incubated for 2, 4 and 6 hours. At the time when samples were harvested for methionine labelling and Western blotting, cells were greater than 75% viable (there was an approximate 25% loss in viability in those samples that had been treated for 24 hours), and there was no difference in uptake of Ca45 between control and treated cells (data not shown). Following the 24 hour incubation there was a loss in viability in those samples that had been exposed to Adriamycin for 8 or more hours (figure 4.47). These cells

were unable to form colonies in the clonogenic assay (figure 4.48).

Western blotting for hsp90/grp94 (figure 4.49) and actin (figure 4.50), showed that there was no change in amounts of either of these proteins during the time course.

FCM analysis following 6 and 8 hours incubation with 1μM Adriamycin (figure 4.51A) showed a small decrease in cells from the G_1 phase of the cell cycle with a concomitant increase in the population of S phase cells, although these changes were not highly significant ($p=0.1$). Following the 24 hour incubation in drug-free media, analysis by FCM (figure 4.51B) of the cells that had been treated for 8 hours with Adriamycin, showed a build up of cells in the G_2M phase (38.1%) with a decrease in S phase cells. There were no significant cell cycle changes in the 6 hour samples. Representative histograms are shown in figure 4.51C.

Comparison of cell size after treatment with Adriamycin showed no change in the average population (figure 4.52A) when compared to control cells. After the 24 hour incubation there was a decrease in size in those samples that had been treated for 8 hours (figure 4.52B).

Figure 4.43 The effect of Adriamycin on HL60 cell growth

Cells were treated with various concentrations of Adriamycin and cell growth (A) and cell viability (B) were assessed after 96 hours.

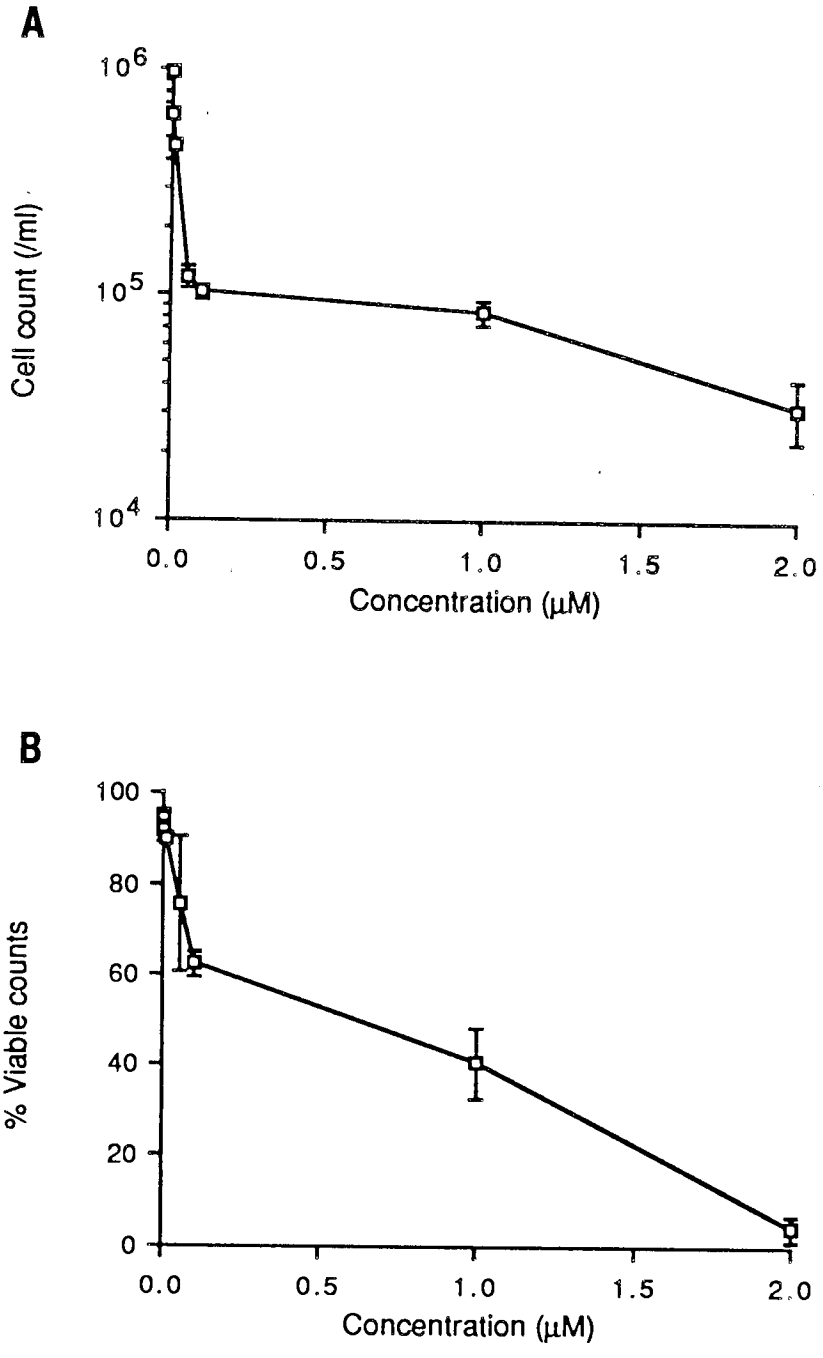


Figure 4.44 The effect of 1uM Adriamycin on HL60 cell growth and viability

HL60 cells were treated with 1uM Adriamycin and cell growth (A) and cell viability (B) were assessed at 24 hour intervals.

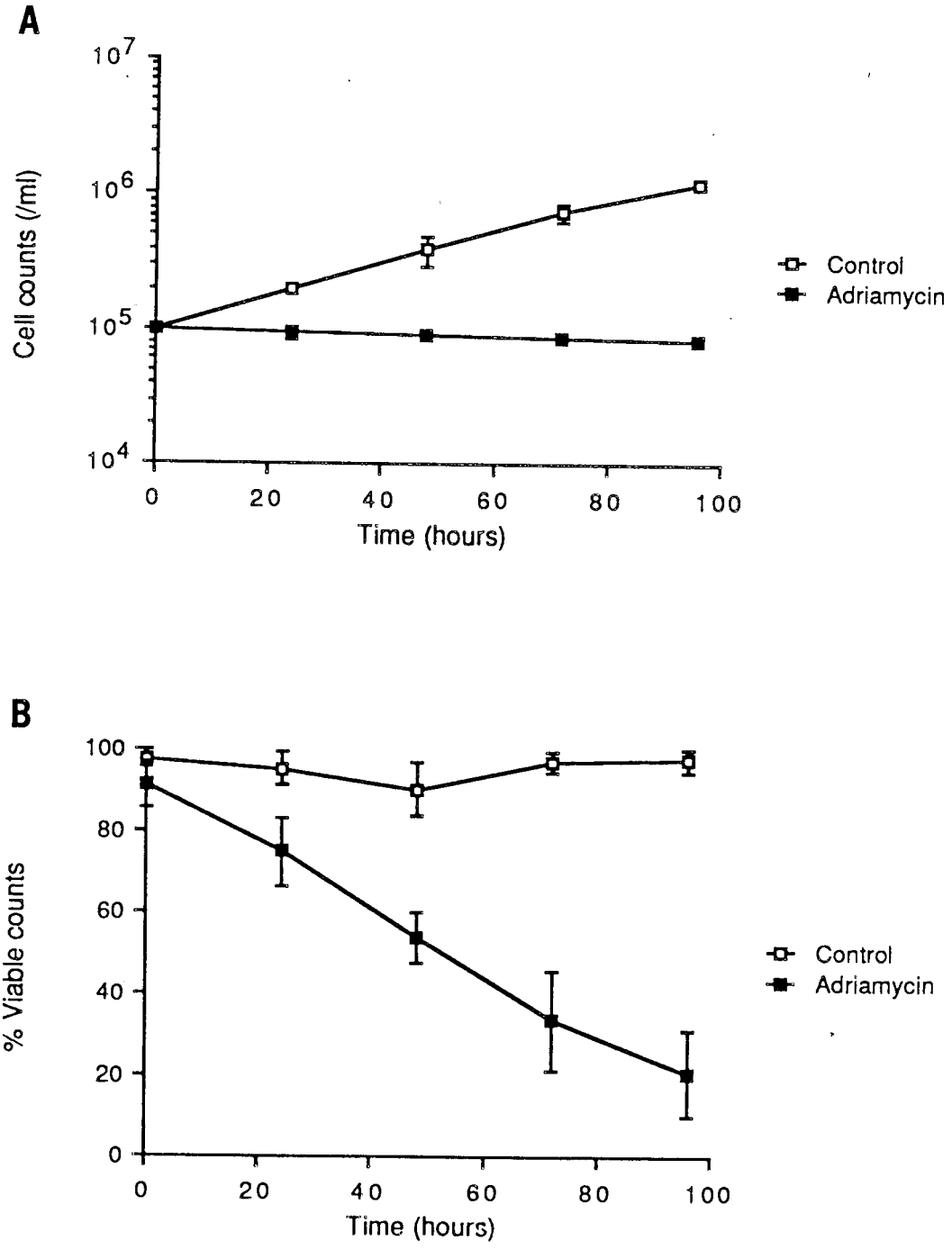
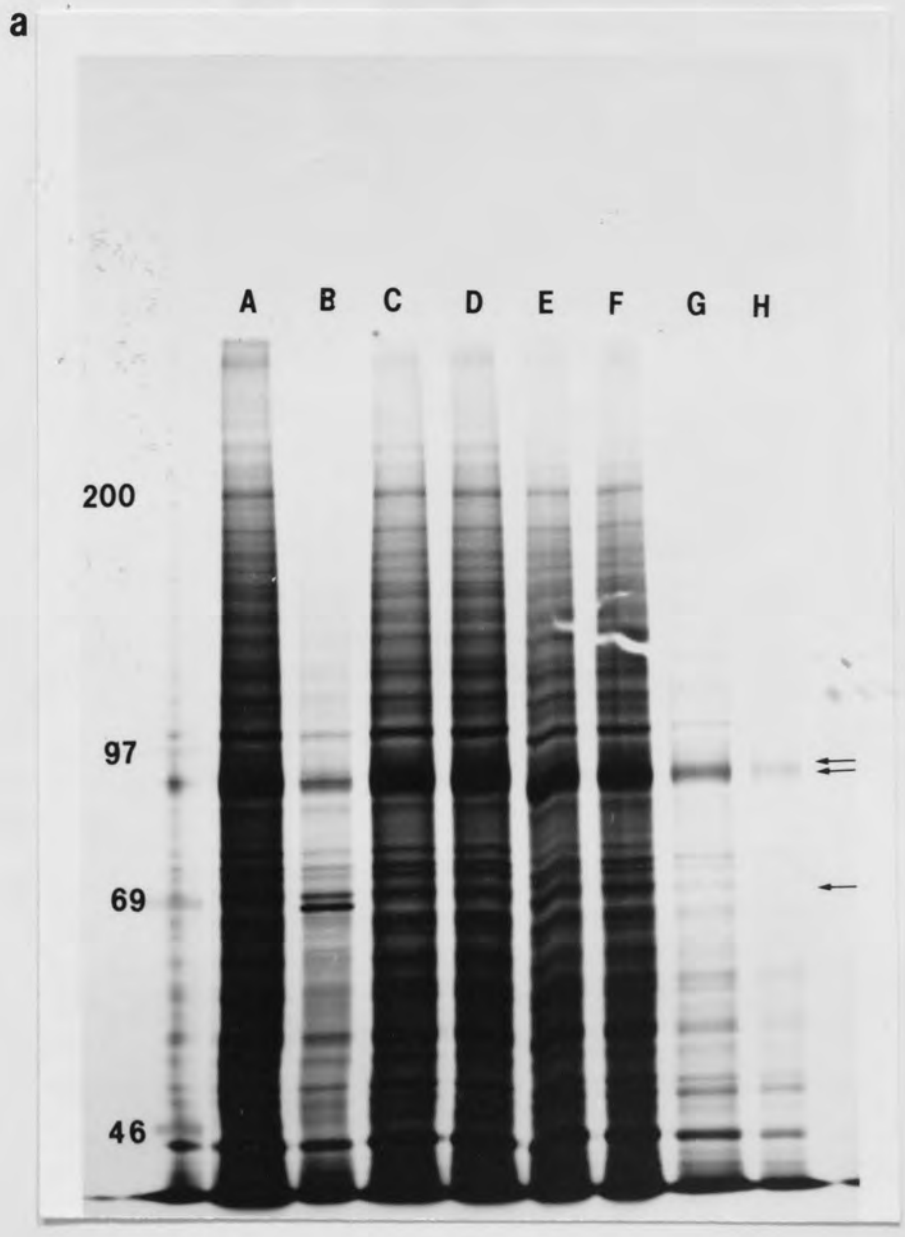


Figure 4.45 The effect of length of exposure to 1μM Adriamycin on protein synthesis in HL60 cells

Fluorograph of equal numbers of ^{35}S methionine labelled cells separated by SDS-PAGE. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 1μM Adriamycin for 2 hours (C); 4 hours (D); 6 hours (E); 8 hours (F); 16 hours (G); 24 hours (H). The results from two experiments are shown.



b

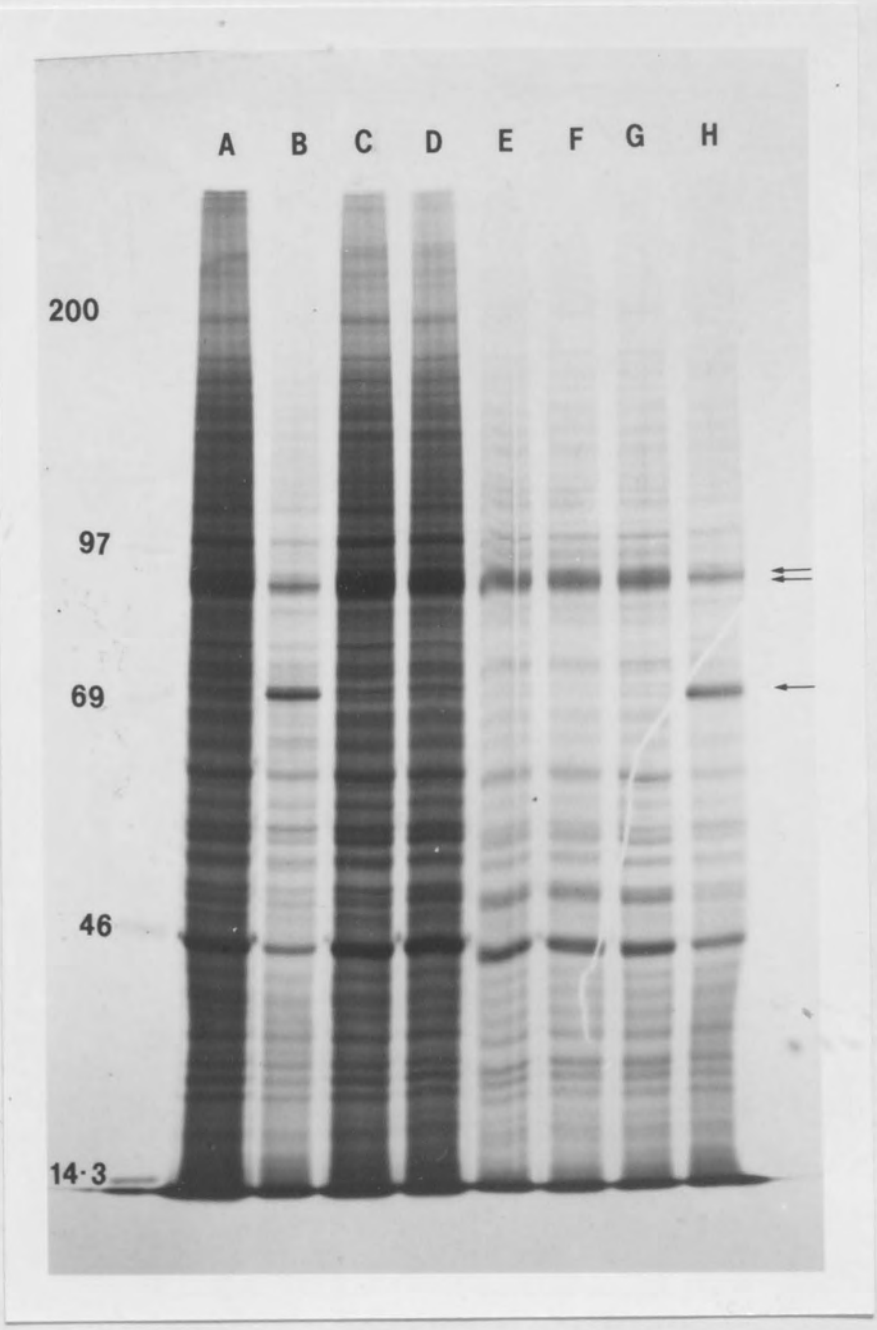


Figure 4.46 The effect of length of exposure to 1μM Adriamycin on amounts of hsp70 in HL60 cells

Immunoblot of HL60 cells probed with 72kD antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 1μM Adriamycin for 2 hours (C); 4 hours (D); 6 hours (E); 8 hours (F); 16 hours (G); 24 hours (H).

Figure 4.47 The effect on HL60 cell viability following a 24 hour incubation in drug-free medium after treatment with 1μM Adriamycin

HL60 cells were treated with 1μM Adriamycin for 2 to 24 hours following which the drug was removed. Viability was assessed by trypan blue exclusion after a further 24 hour incubation in the absence of Adriamycin.

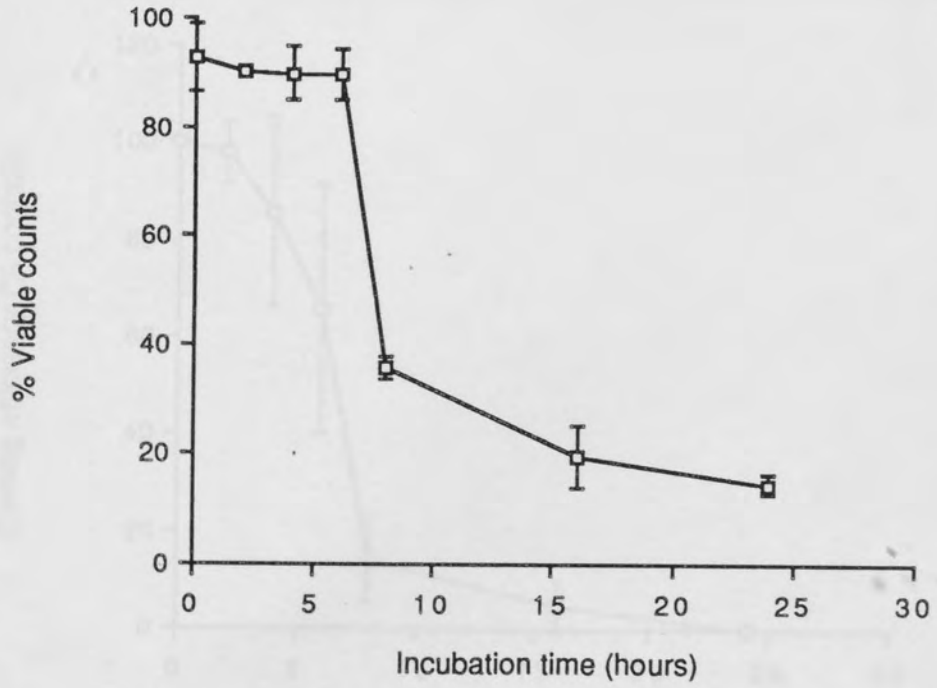


Figure 4.48 The effect of length of exposure to 1μM Adriamycin on the cloning efficiency of HL60 cells

HL60 cells were cultured in suspension in the presence of 1uM Adriamycin. At the times indicated the cells were washed and cloned in drug-free semi-solid medium. Colonies were counted on day 10.

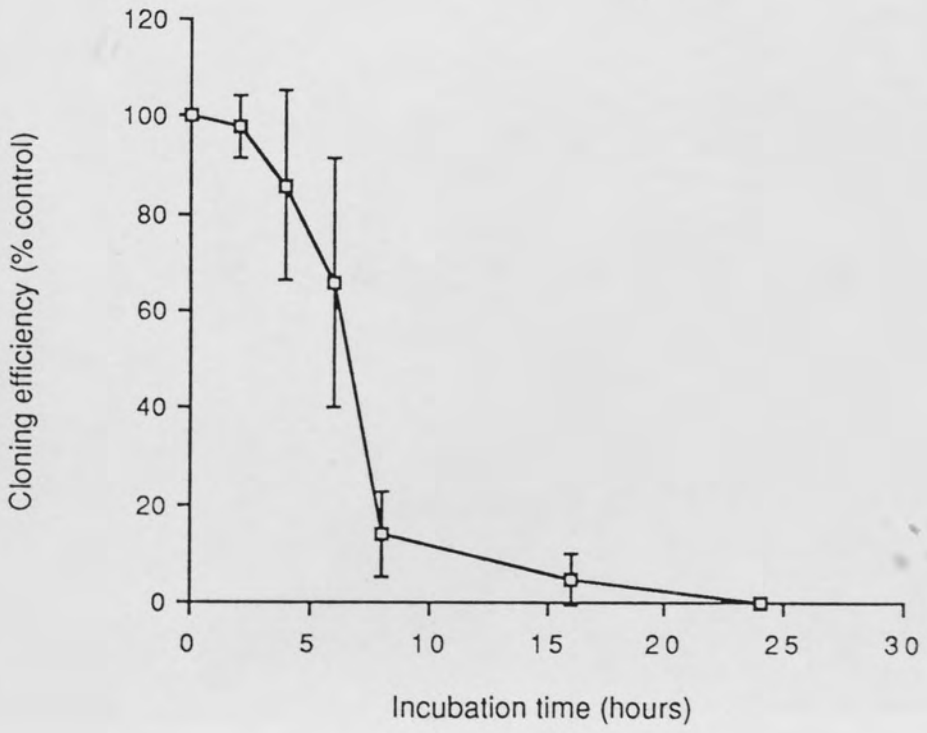


Figure 4.49 The effect of length of exposure to 1μM Adriamycin on amounts of hsp90/grp94 in HL60 cells

Immunoblot of HL60 cells probed with AC88 antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 1μM Adriamycin for 2 hours (C); 4 hours (D); 6 hours (E); 8 hours (F); 16 hours (G); 24 hours (H).

Figure 4.50 The effect of length of exposure to 1μM Adriamycin on amounts of actin in HL60 cells

Immunoblot of HL60 cells probed with actin antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 1μM Adriamycin for 2 hours (C); 4 hours (D); 6 hours (E); 8 hours (F); 16 hours (G); 24 hours (H).

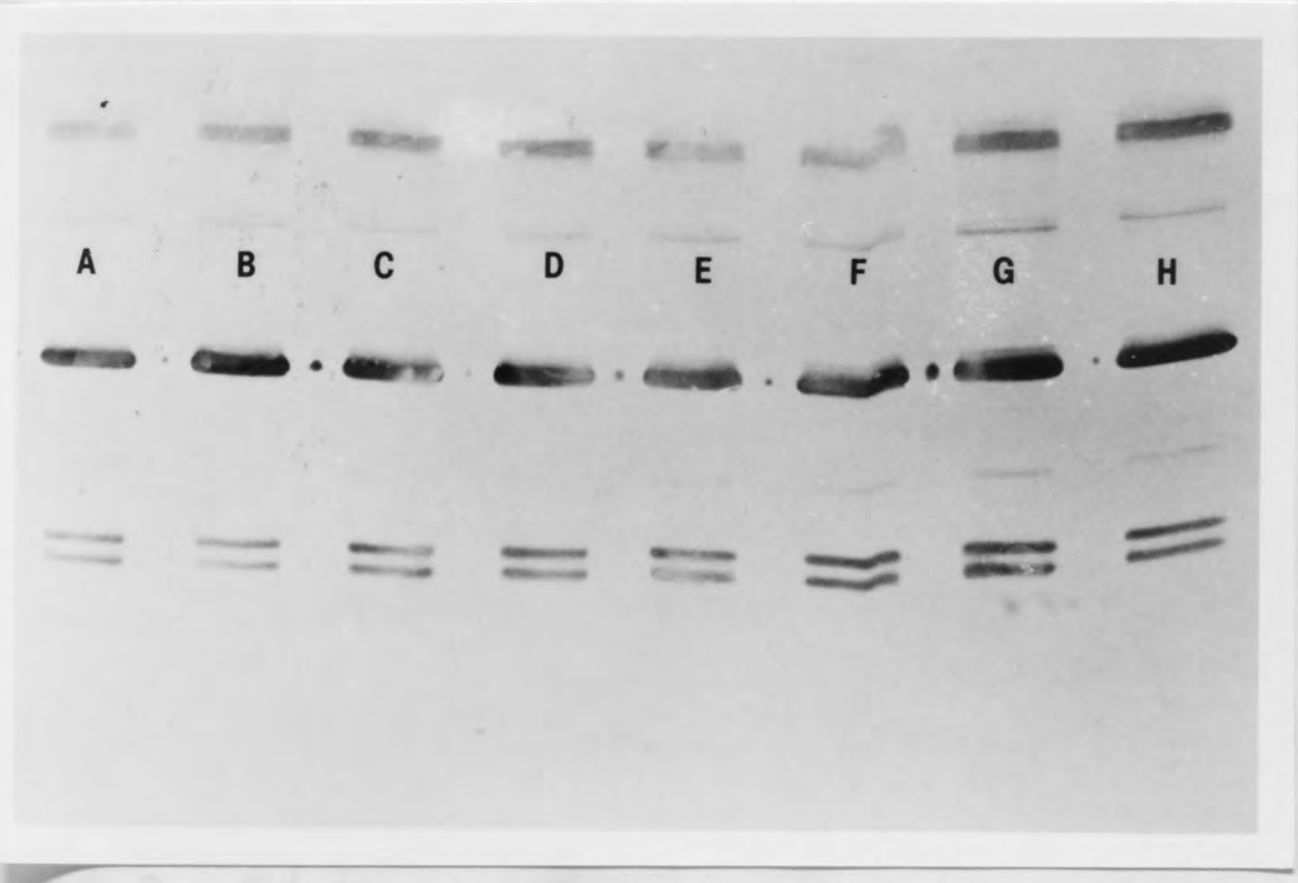
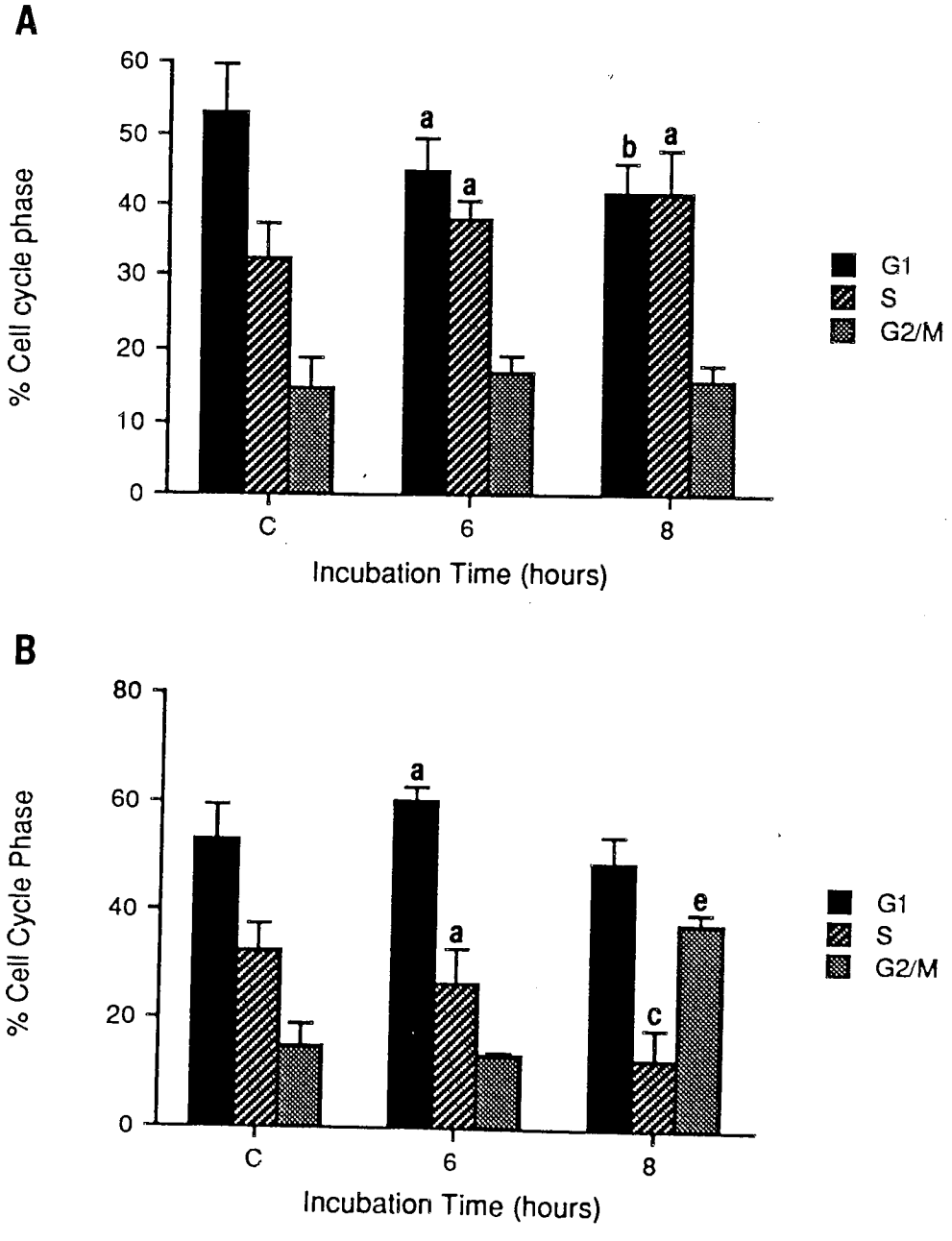


Figure 4.51 The effect of 1uM Adriamycin on HL60 cell cycle

The percentage of HL60 cells in each phase of the cell cycle was determined after treatment with 1 uM Adriamycin for 6 hours and 8 hours (A). Cell cycle analysis was also determined following a 24 hour incubation in the absence of Adriamycin (B). The percentage of cells in each phase of the cell cycle after treatment were compared with control cells and levels of significance calculated using a Students T-test. Unless stated there was no significant difference between control and treated cells. a: $p<0.1$; b: $p<0.05$; c: $p<0.01$; d: $p<0.005$; e: $p<0.001$
Representative histograms of DNA content are shown; cells were harvested immediately following treatment (C); cells were harvested following a 24 hour incubation in the absence of Adriamycin (D).



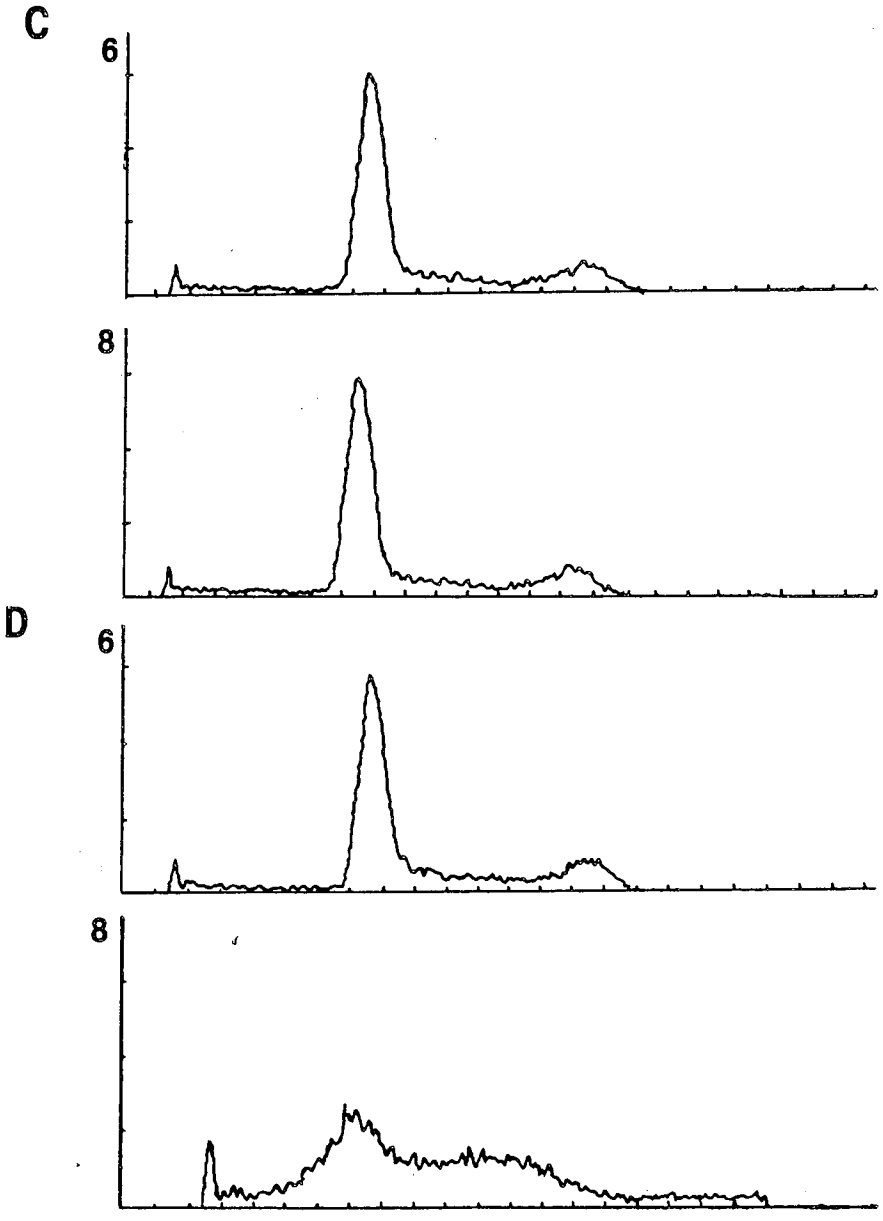
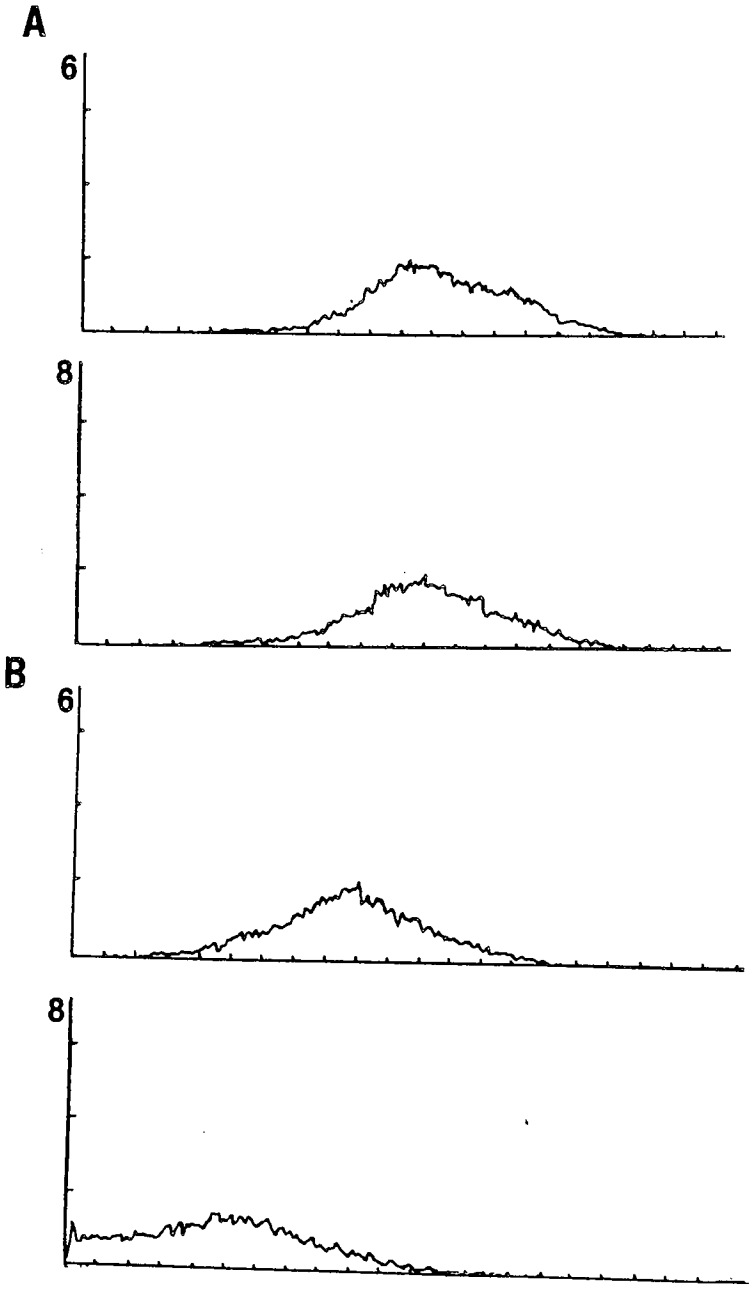

C
6
8
D
6
8

Figure 4.52 The effect of 1uM Adriamycin on HL60 cell size

Histograms of HL60 cell size after treatment with 1uM Adriamycin for 6 hours and 8 hours (A). Cells were also harvested following a 24 hour incubation in drug-free medium (B).



4.5.3 HL60 cells treated with CB3717

CB3717 (N-(4-(N-((2-amino-4-hydroxy-6-quinazolinyl)-methyl)prop-2-ynylamino)benzoyl)-L-glutamic acid) is an antitumor agent that inhibits thymidylate synthetase, the enzyme that catalyses the terminal step in the de novo synthesis of thymidine nucleotides which are required exclusively for DNA synthesis (Jones et al. 1981).

HL60 cells were treated for 96 hours with increasing concentrations of CB3717; cell numbers and viability were monitored at the end of the experiment. Assays for the determination of a differentiated phenotype were also performed.

Figure 4.53, the results of the relationship between viability and cell growth and concentration, show that viability and proliferation were decreased in a concentration-dependent manner. A concentration of 5μM inhibited proliferation and reduced viability to 30% over 96 hours. No differentiation was observed at any concentration. A time course, figure 4.54, using 5uM CB3717, indicated that inhibition of proliferation occurred after the addition of the drug and viability was reduced by 25% over the first 24 hours. 5μM CB3717 was described by Jones et al. (1981), as being the ID_{50} concentration in L1210 murine leukemia cells. This concentration was therefore used for further study to determine any relationship between cellular amounts of hsp70 and a later

loss in HL60 viability after treatment with drugs.

Exponentially growing HL60 cells were treated for up to 30 hours with 5μM CB3717 then samples were labelled with ^{35}S methionine, and harvested for Western blotting. Viability and proliferative potential were assessed as for NMF and Adriamycin.

Figure 4.55, a representative fluorograph, showed no induction of hsp70 after treatment with 5μM CB3717, even after 30 hours incubation. There was decreased synthesis of grp94 after 24 hours but this was also accompanied by a decrease in overall protein synthesis. Probing a Western blot with 72kD antibody revealed an increase in hsp70 amounts after 16 hours incubation (figure 4.56). As with NMF, viability remained greater than 80% at the time cells were harvested and intracellular calcium levels after 8 and 16 hours treatment were less than 200nM (figure 4.57). Viability fell to less than 70% after 24 hours incubation in drug-free media in those samples that had been treated with 5uM CB3717 for 16, 24 and 30 hours (figure 4.58); these samples also showed a reduction in proliferative capacity with the 24 and 30 hour samples unable to form colonies (figure 4.59).

Probing nitrocellulose blots with AC88 (figure 4.60) and the actin antibody (figure 4.61) revealed no changes in amounts of either of these proteins during the time course.

Cell cycle analysis showed no significant change in the numbers of cells within each phase of the cell cycle after 8

or 16 hours incubation with 5μM CB3717 (figure 4.62A). Following the 24 hour incubation in drug-free media, there was an increase in the numbers of cells in S phase, which was accompanied by a decrease in the number of cells in G_1 and G_2M, in those cells that had been treated for 16 hours (figure 4.62B). A change in cell size was observed only when cells that had been treated with 5μM CB3717 for 16 hours were then incubated in drug-free medium for 24 hours (figure 4.63).

Figure 4.53 The effect of CB3717 on HL60 cell growth

Cells were treated with various concentrations of CB3717 and cell growth (A) and cell viability (B) were assessed after 96 hours.

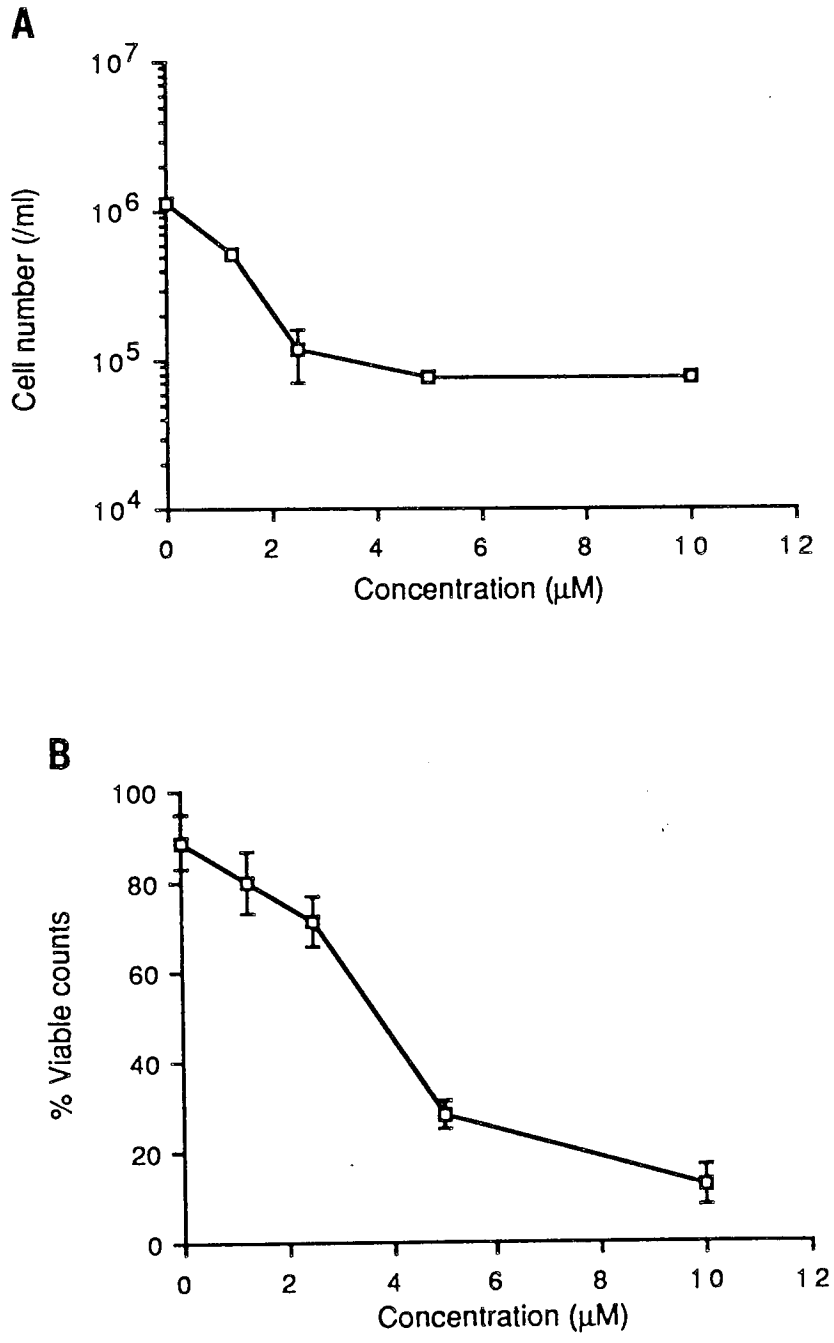


Figure 4.54 The effect of 5μM CB3717 on HL60 cell growth and viability

HL60 cells were treated with 5μM CB3717 and cell growth (A) and cell viability (B) were assessed at 24 hour intervals.

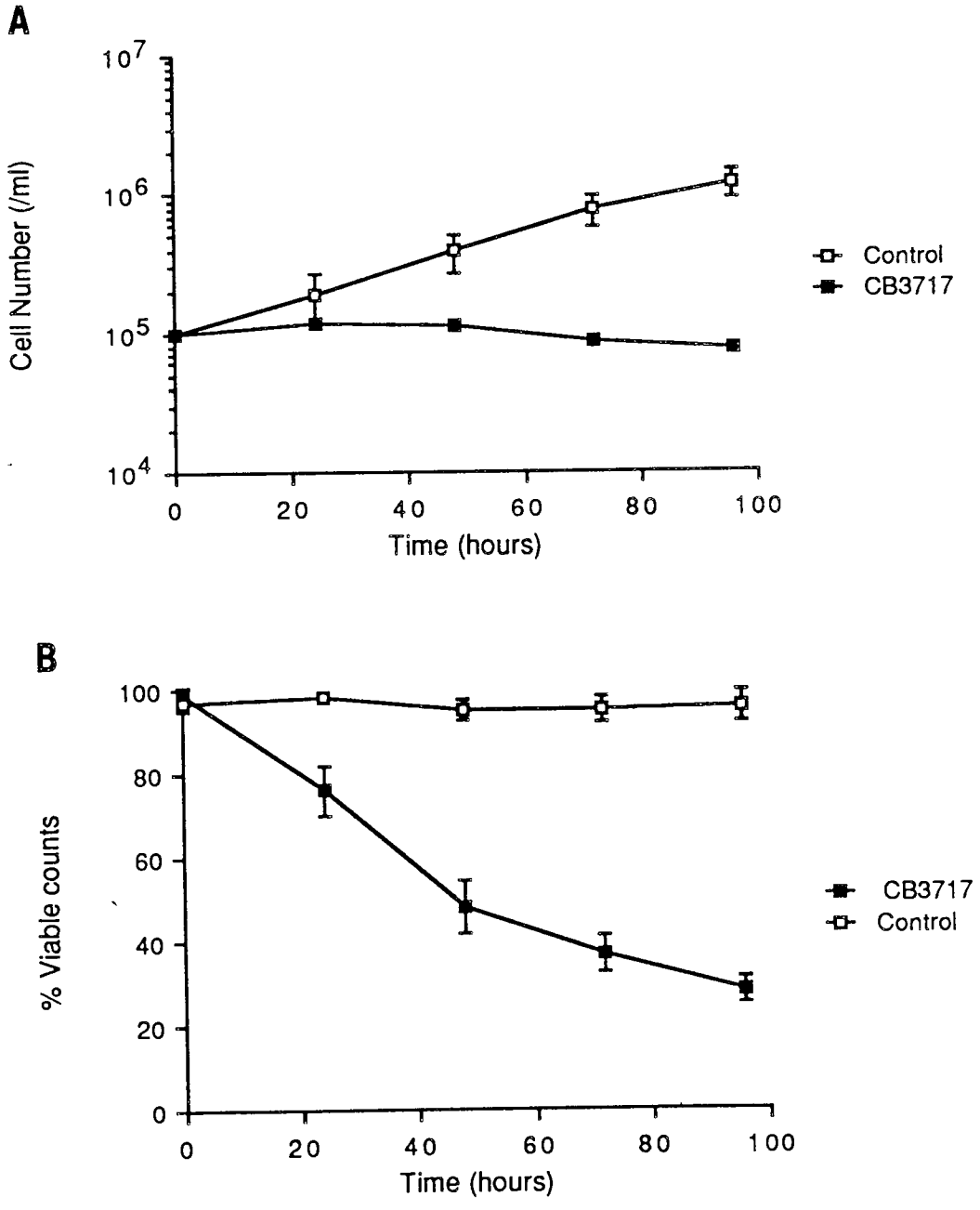


Figure 4.55 The effect of length of exposure to 5μM CB3717 on protein synthesis in HL60 cells

Fluorograph of equal numbers of ^{35}S methionine labelled cells separated by SDS-PAGE. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 5μM CB3717 for 2 hours (C); 4 hours (D); 6 hours (E); 8 hours (F); 16 hours (G); 24 hours (H); 30 hours (I).

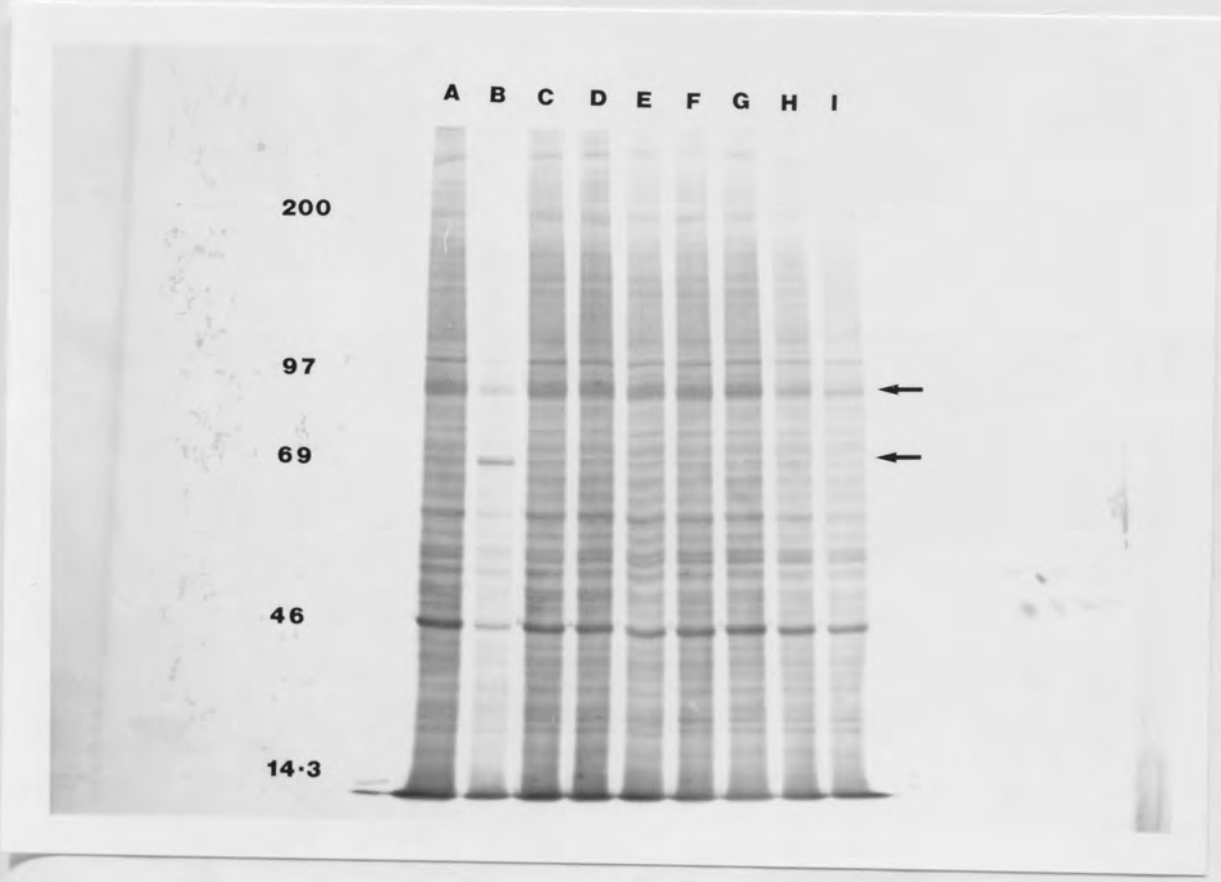


Figure 4.56 The effect of length of exposure to 5μM CB3717 on amounts of hsp70 in HL60 cells

Immunoblot of HL60 cells probed with 72kD antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 5μM CB3717 for 2 hours (C); 4 hours (D); 6 hours (E); 8 hours (F); 16 hours (G); 24 hours (H); 30 hours (I).

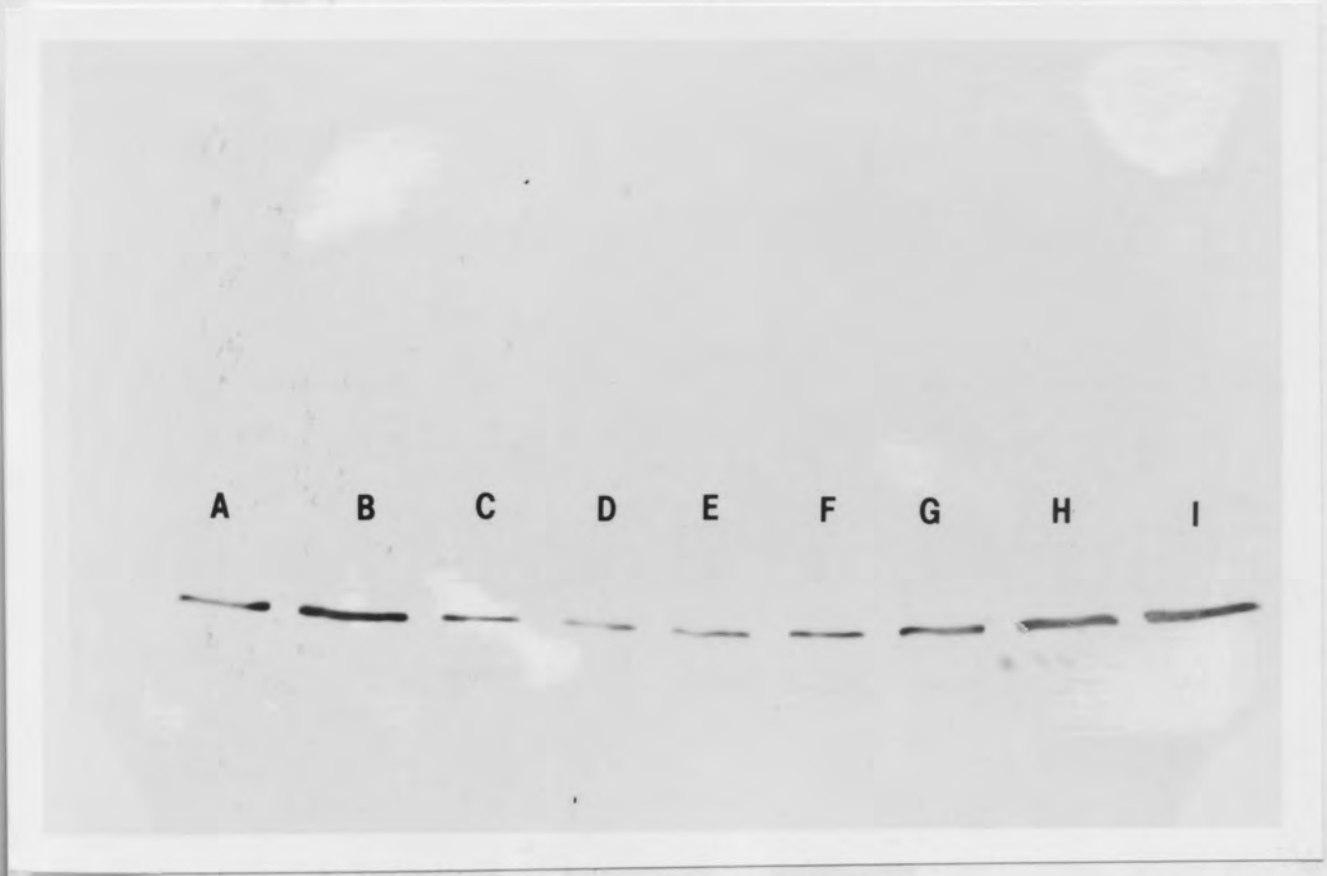


Figure 4.57 Intracellular calcium concentration in HL60 cells treated with 5µM CB3717

Intracellular calcium concentrations were determined by quin 2 loading after 8 and 16 hours incubation with 5µM CB3717 (A). Intracellular calcium concentrations were also determined following a 24 hour incubation in drug-free media (B).

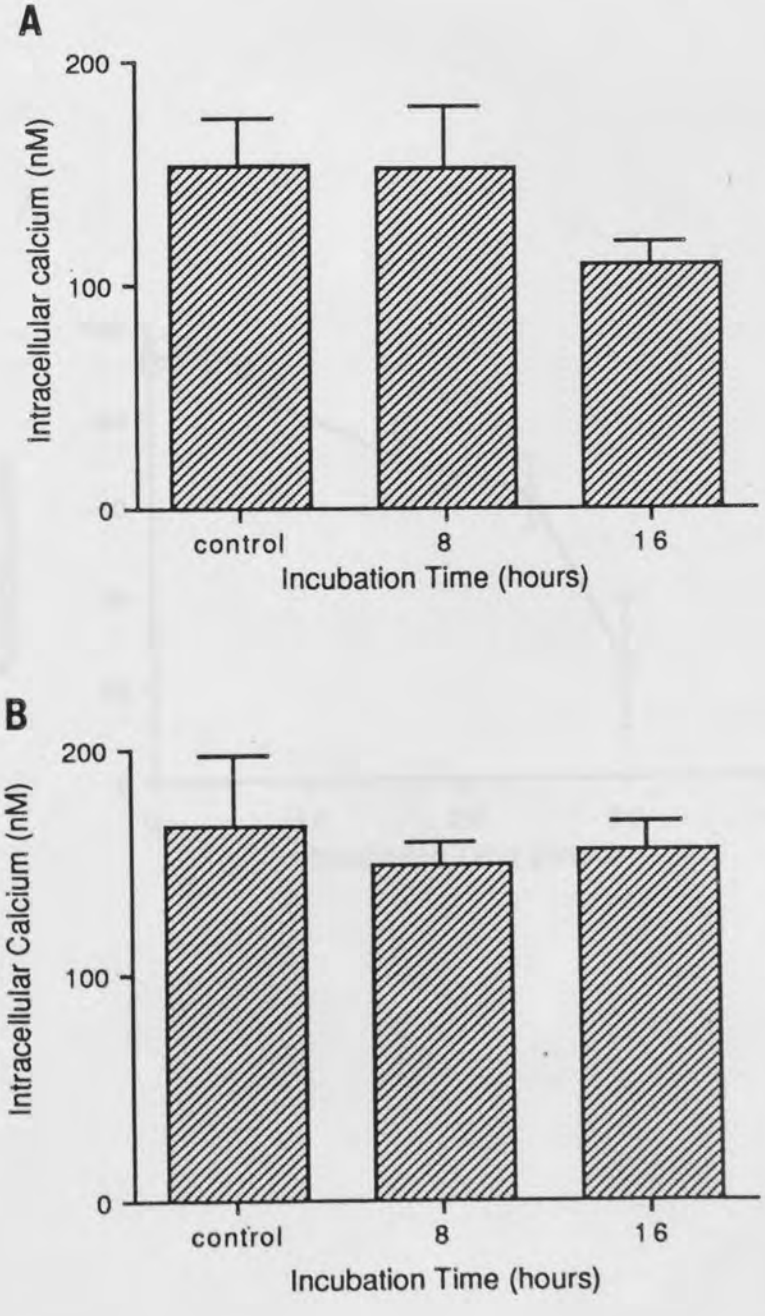


Figure 4.58 The effect on HL60 cell viability following a 24 hour incubation in drug-free medium after treatment with 5μM CB3717

HL60 cells were treated with 5μM CB3717 for 2 to 30 hours following which the drug was removed. Viability was assessed by trypan blue exclusion after a further 24 hour incubation in the absence of CB3717.

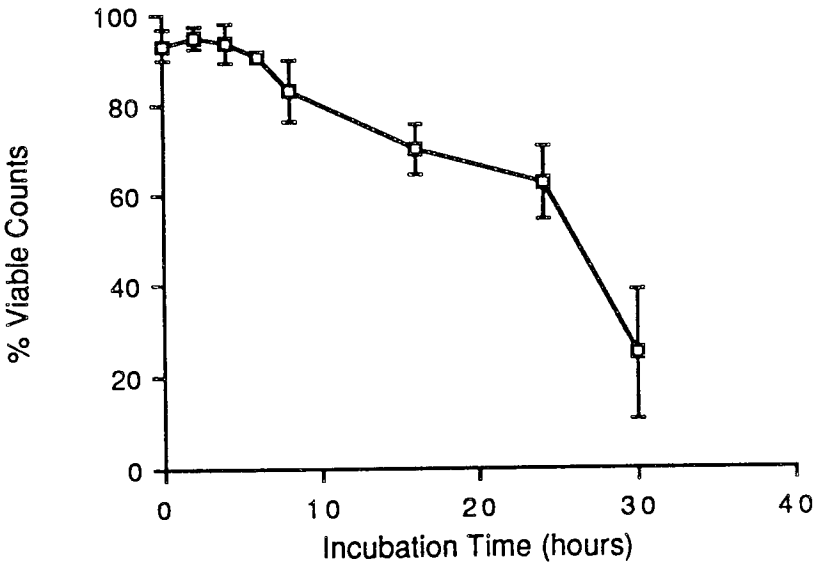


Figure 4.59 The effect of length of exposure to 5μM CB3717 on the cloning efficiency of HL60 cells

HL60 cells were cultured in suspension in the presence of 5uM CB3717. At the times indicated the cells were washed and cloned in drug-free semi-solid medium. Colonies were counted on day 10.

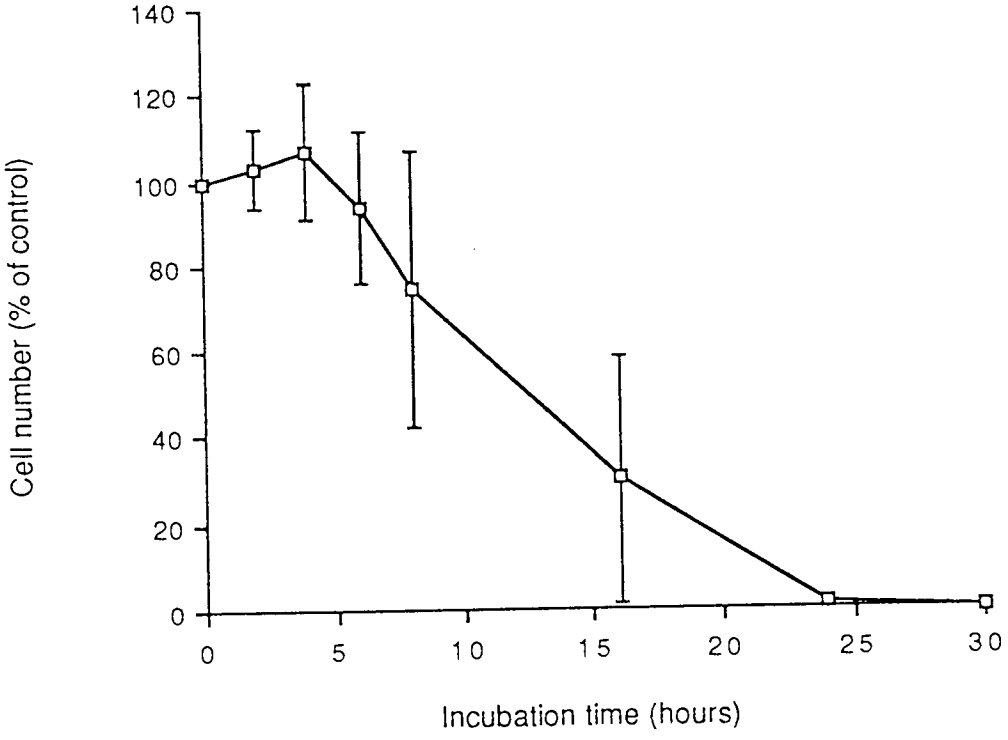


Figure 4.60 The effect of length of exposure to 5μM CB3717 on amounts of hsp90/grp94 in HL60 cells

Immunoblot of HL60 cells probed with AC88 antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 5μM CB3717 for 2 hours (C); 4 hours (D); 6 hours (E); 8 hours (F); 16 hours (G); 24 hours (H); 30 hours (I).

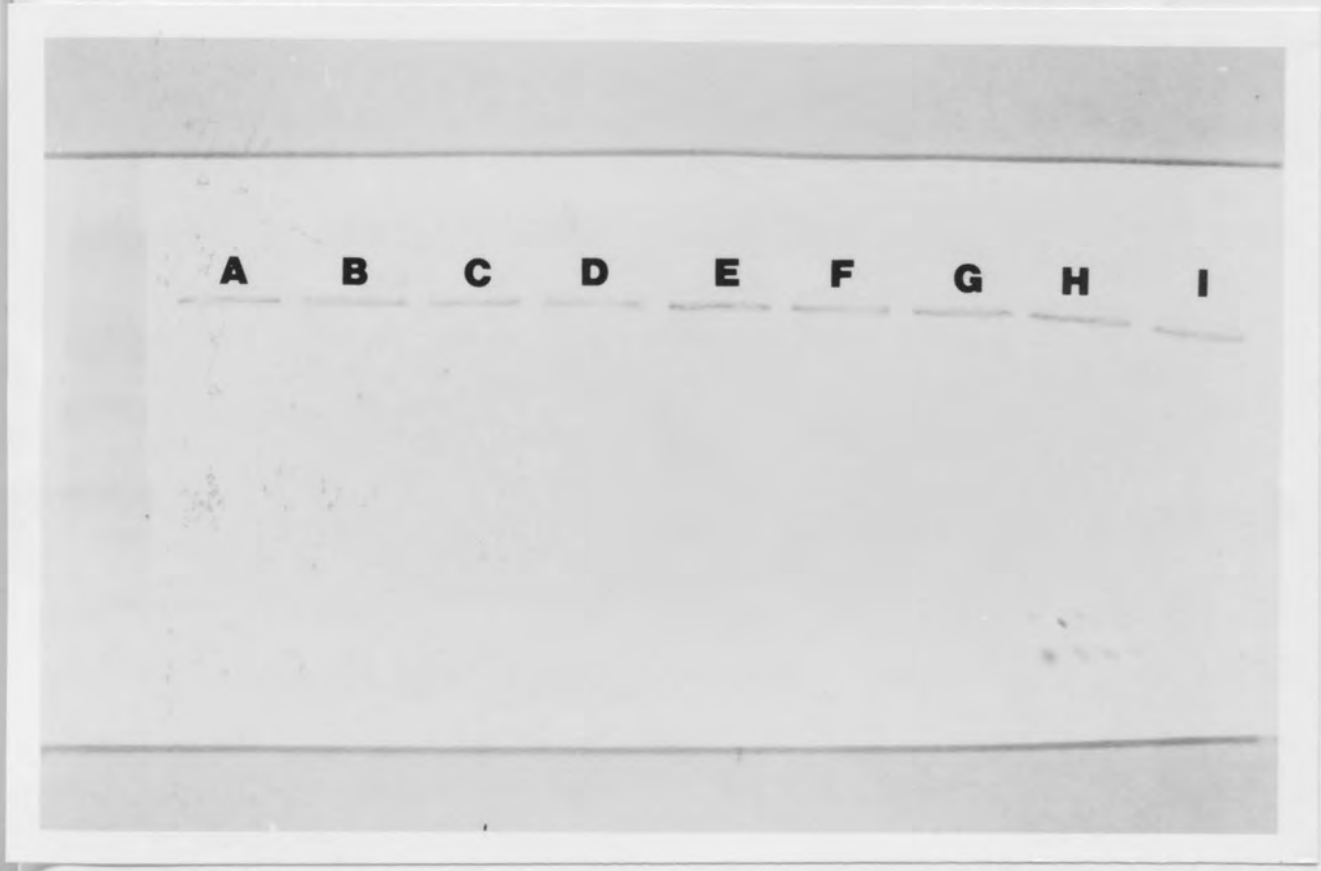
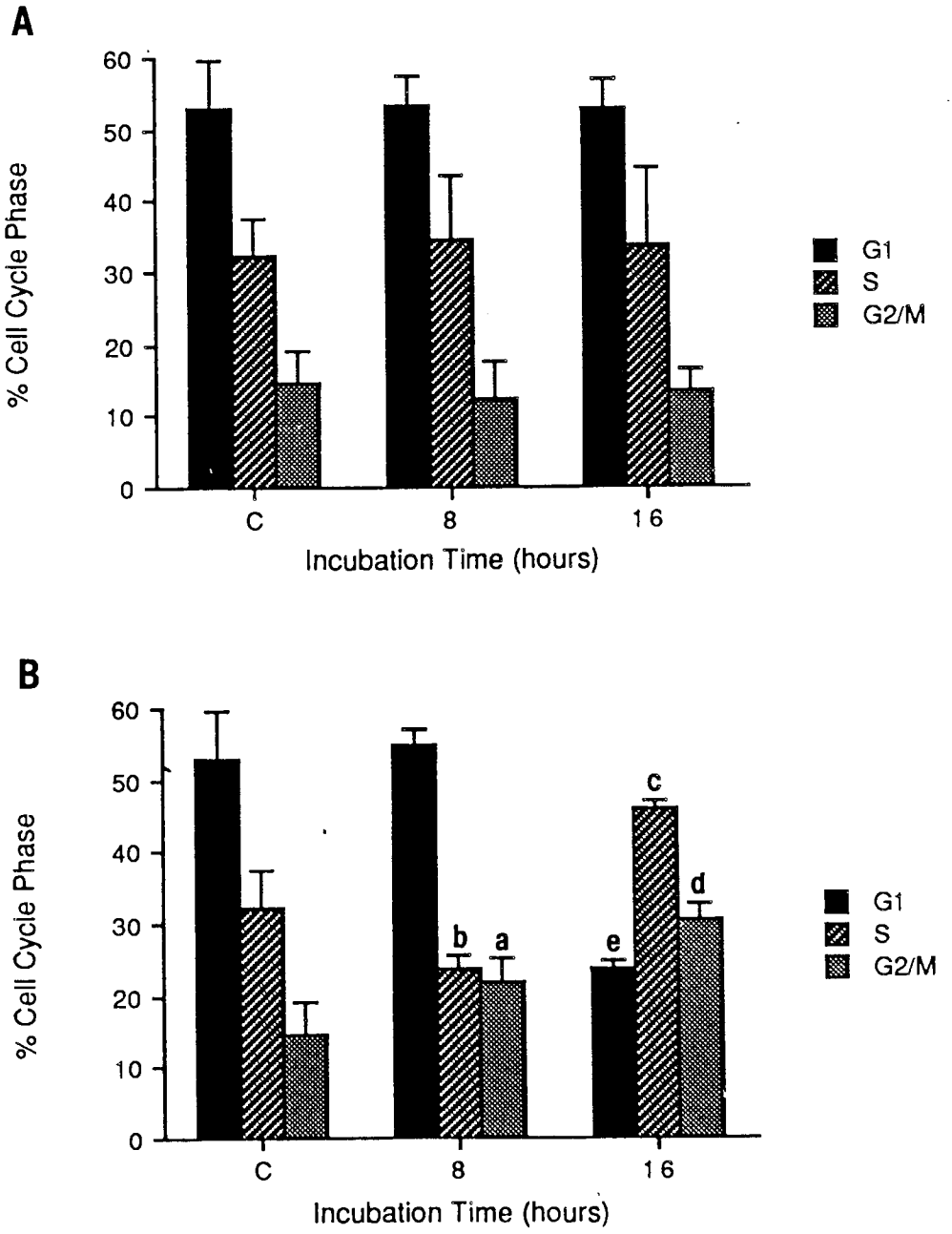


Figure 4.61 The effect of length of exposure to 5μM CB3717 on amounts of actin in HL60 cells

Immunoblot of HL60 cells probed with actin antibody. Cells were untreated (A); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (B); or treated with 5μM CB3717 for 2 hours (C); 4 hours (D); 6 hours (E); 8 hours (F); 16 hours (G); 24 hours (H); 30 hours (I).

Figure 4.62 The effect of 5μM CB3717 on HL60 cell cycle

The percentage of HL60 cells in each phase of the cell cycle was determined after treatment with 5μM CB3717 for 6 hours and 8 hours (A). Cell cycle analysis was also determined following a 24 hour incubation in the absence of CB3717 after treatment (B). The percentage of cells in each phase of the cell cycle after treatment were compared with control cells and levels of significance calculated using a Students T-test. Unless stated there was no significant difference between control and treated cells. a: $p<0.1$; b: $p<0.05$; c: $p<0.01$; d: $p<0.005$; e: $p<0.001$
Representative histograms of DNA content are shown; cells were harvested immediately following treatment (C); cells were harvested following a 24 hour incubation in the absence of CB3717 (D).



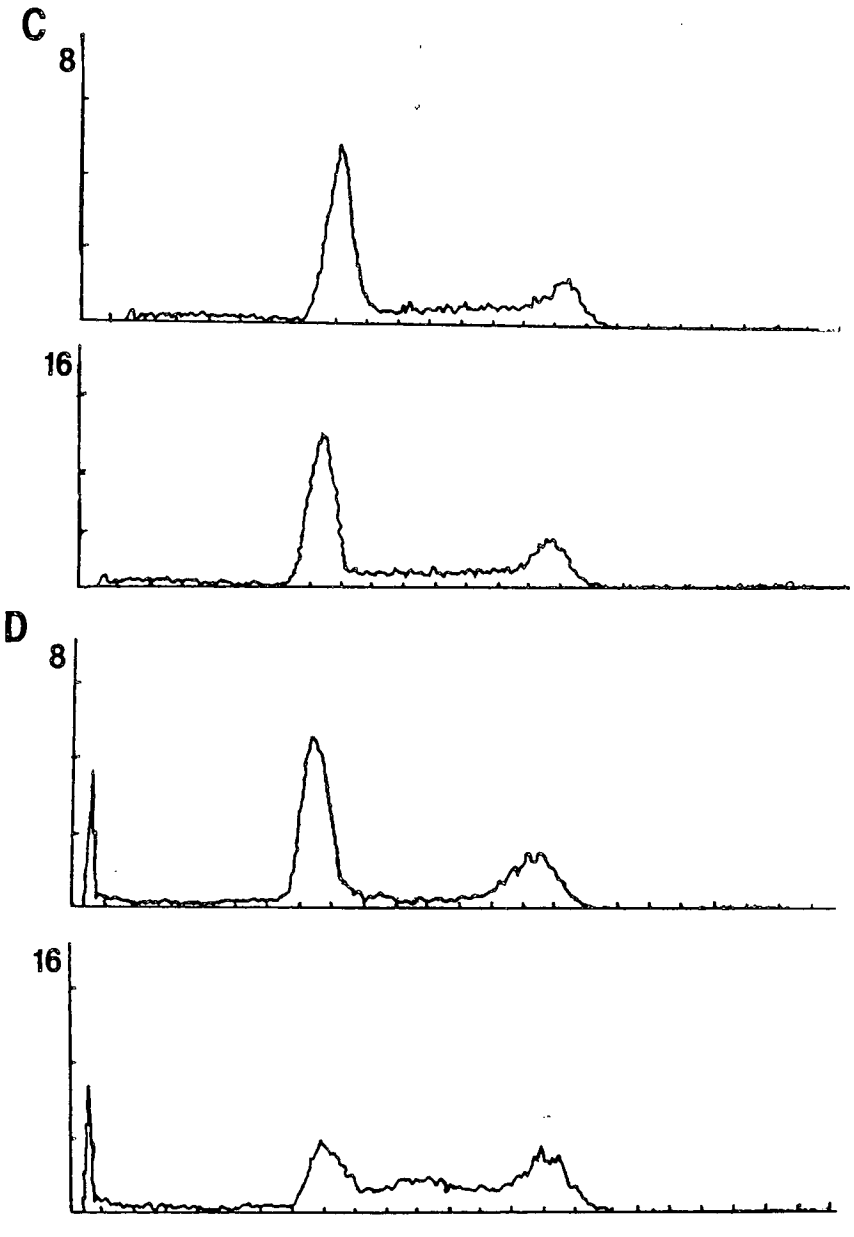
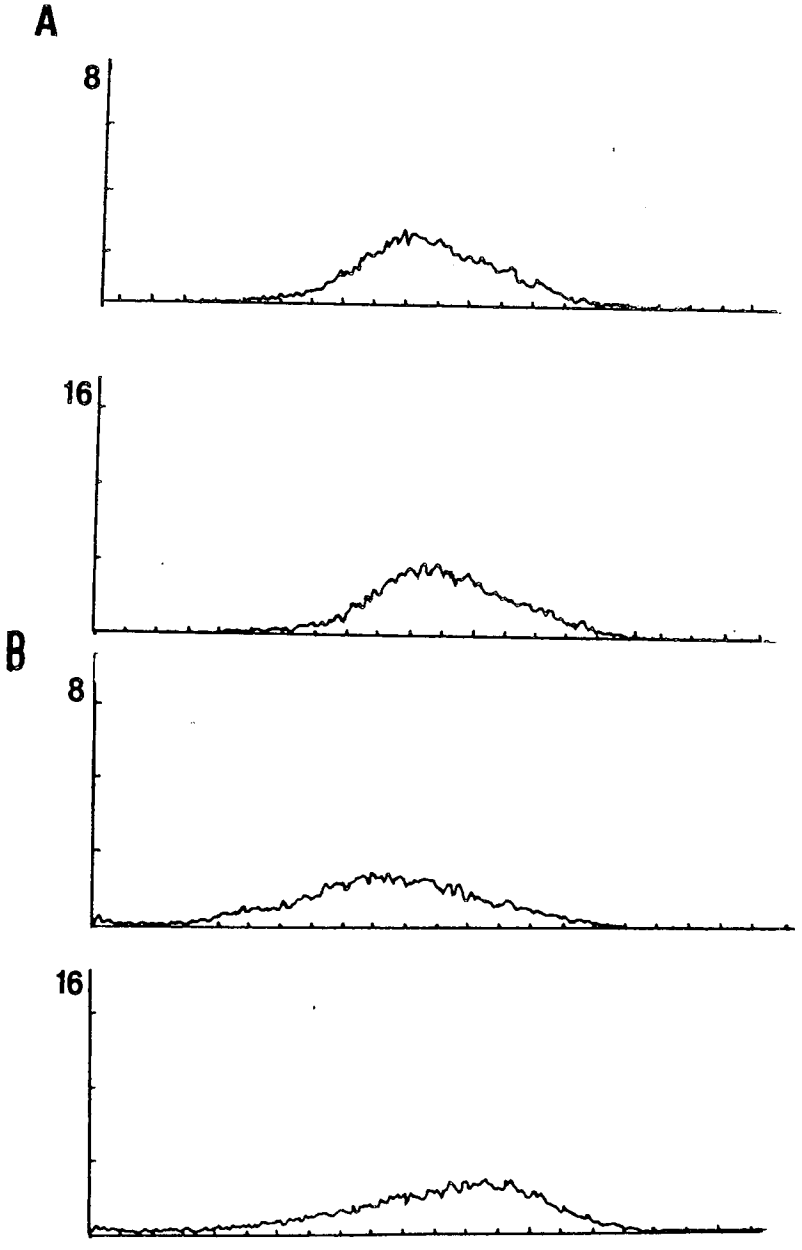

C
8
16
D
8
16

Figure 4.63 The effect of 5μM CB3717 on HL60 cell size

Histograms of HL60 cell size after treatment with 5μM CB3717 for 8 hours and 16 hours (A). Cells were also harvested following a 24 hour incubation in drug-free medium (B).



4.5.4 Discussion

From the results in section 4.5 it can be concluded that the increase in amounts of hsp70 observed when HL60 cells were treated with toxic concentrations of NMF could also be detected when cells were treated with toxic concentrations of a membrane active/DNA damaging drug and an anti-metabolite, and is in agreement with the data discussed in section 4.4, that increased amounts of hsp70 may act as an early marker of a later loss in cell viability. The rise in amounts of hsp70 preceded any loss in cell viability, any changes in intracellular calcium levels and any cell-cycle changes, and occurred only in those cells which went on to die.

The cell cycle events induced by the 3 drugs were in agreement with results by other workers; Bill et al (1989) reported that NMF caused a concentration dependent increase of the proportion of TLX5 cells in the G_1 phase of the cell-cycle; Adriamycin was shown by Burres et al (1988) to block HL60 cell-cycle progression in G_2M in a concentration dependent manner, and the blockage in the S phase of the cell-cycle by CB3717 is typical of anti-metabolites (Pratt and Ruddon 1979).

The viable cells remaining after the 24 hour incubation in the absence of drug, showed a time dependent block in cell cycle. However this block was only observed at the time points which had caused an increase in hsp70. These cells

were unable to proliferate but they remained viable for a further 72 hours (data not shown). In the case of NMF treatment these cells did not have increased amounts of hsp70 when compared to control cells. The fact that the remaining viable cells, that had been treated with NMF for 8 hours, did not have elevated amounts of hsp70 would suggest that hsp70 was not involved in protecting those cells from the toxic effects of the drug, as in thermotolerence, but was elevated in those cells that had died. This hypothesis is supported by the fact that the cells that had been heat shocked then incubated for 24 hours, had amounts of hsp70 that were greater than those in the control cells and the treated cells. This result was somewhat surprising since Mivechi (1989) had shown that hsp70 synthesis and thermotolerence in HL60 cells decayed between 2 and 4 hours after a heat shock of either $45^{o}c$ for 10 minutes, or $42^{o}c$ for 30 minutes. However Fortan et al in 1989 showed that although synthesis of hsp70 and hsp72 had decreased to control values 6 hours after a heat shock, hsps were found to be stable for more than 48 hours. They concluded thermotolerence was correlated with the synthesis rather than the presence of hsps.

The increase in amounts of hsp70 in the absence of any increase in synthesis was surprising in terms of the models that have been proposed for the regulation of stress protein synthesis. A recent publication by Beckmann et al (1990) has suggested a model whereby the concentration of free versus

substrate-bound hsp70 is monitored such that when the equilibrium is shifted towards the substrate-bound forms (for example, after treatment with toxic concentrations of drugs that result in damaged protein), the corresponding reduction in amounts of free hsp70 may induce hsp70 synthesis. This model is supported by several previous observations; hsp70 appears to be autoregulated primarily at the posttranscriptional level (DiDomenico et al 1982), and microinjection of anti-hsp70 resulted in the immediate induction of hsp70 synthesis (Beckmann et al 1990). The results obtained with NMF, Adriamycin and CB3717 should therefore have resulted in the induction of hsp70 synthesis at the later time points if these results were to fit Beckmann's proposed model of regulation. The apparent lack of an increase in synthesis may however be explained by the relatively high levels of constitutive hsp70 synthesis in HL60 cells; examination of the protein synthesis patterns and immunoprecipitations of control HeLa cells, in the studies published by Beckmann et al (1990), reveal no detectable hsp70. High constitutive synthesis of hsp70 may mean that more of the substrate-bound form could be present in the cell before the equilibrium between the free pool and substrate-bound pool reaches the critical concentration required to initiate hsp70 synthesis. The time taken for the shift in equilibrium between bound and unbound hsp70 to result in increases in hsp70 synthesis may be the reason why no synthesis was observed in the concentration experiments

described in section 4.3.

What is interesting about these results is that whilst an increase in amounts of hsp70 was non-toxic for cells that had been heat shocked, cells which had been treated with drugs and had comparable amounts of hsp70, were commited to die. Experiments were therefore designed to investigate possible explanations for this anomaly.

4.6 Localisation and turnover of hsp70

4.6.1 Localisation of hsp70

When cells are heat shocked hsp70 translocates from the cytoplasm to the nucleolus where it is in close association with the pre-ribosomal-containing granular region (Welch et al 1986). It was of interest to determine where hsp70 was localised in HL60 cells after treatment with the three agents under investigation.

Crude cytoplasmic and nuclear fractions of HL60 cells were prepared in control and heat shocked cells and in cells treated with:-

a) 1μM Adriamycin for 8 hours

b) 5μM CB3717 for 16 hours

c) 225mM NMF for 8 hours

The samples were immunoblotted and the nitrocellulose blot probed with the 72kD antibody. It should be noted that the blots of fractionated cells are not quantitative.

From figure 4.64 it can be seen that in the control cells hsp70 was located in the cytoplasmic fraction whereas after a heat shock it was detected in the nuclear fraction, as expected. Following treatment with the different agents hsp70 remained in the cytoplasmic fraction.

When cells are heat shocked there are rearrangements of actin within the cell. Densely packed, parallel running fibres of actin filaments can be seen traversing the nucleus

but these fibres do not appear to extend through the nuclear envelope into the cytoplasm (Welch et al 1985). Similar intranuclear actin rods have been observed in cells exposed to high levels of DMSO or calcium ionophores and it is thought that this occurs as a result of translocation (Sanger et al 1980). It was therefore of interest to see where actin was isolated following incubation with the drugs.

Figure 4.65 an immunoblot of the crude cellular fractionation that had been probed with an antibody against actin, showed that the majority of actin was isolated in the cytoplasmic fraction in control and treated cells.

To ensure that protein translocation per se had not been shut down after drug treatment, immunoblots of the fractionation samples were probed with an antibody against the nuclear protein H2B. Under normal conditions this protein is translocated to the nucleus following its synthesis (Darnell et al 1986). Figure 4.66 shows that histone was detectable in the nuclear fraction only in all the samples.

As already demonstrated in figure 4.64 lane D, hsp70 is translocated to the nucleus after a heat shock. Further studies were conducted to determine whether this translocation to the nucleus was still possible if cells were first treated with either NMF, Adriamycin or CB3717 before being heat shocked.

Cells were treated with the agents as described earlier

and were then heat shocked at 43.5°C for 1 hour followed by an incubation at 37°C for 2 hours. Crude fractions were prepared as before. Figure 4.67 shows the immunoblots that had been probed with 72kD antibody from several experiments due to slight variability in results. In general there was no translocation of hsp70 from the cytoplamic fraction to the nuclear fraction after cells had been treated with drug then heat shocked, but in some cases a degree of translocation was observed.

Figure 4.64 Intracellular localisation of hsp70 in HL60 cells following treatment with toxic concentrations of drugs

Crude cytoplasmic (c), and nuclear fractions (n), of HL60 cells were prepared and the immunoblot probed with 72kD antibody. Cells were untreated: c (A), and n (B); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours): c (C), and n (D); or treated with 1μM Adriamycin for 8 hours: c (E), and n (F); 5μM CB3717 for 16 hours: c (G) and n (H); 225mM NMF for 8 hours c (I), and n (J).

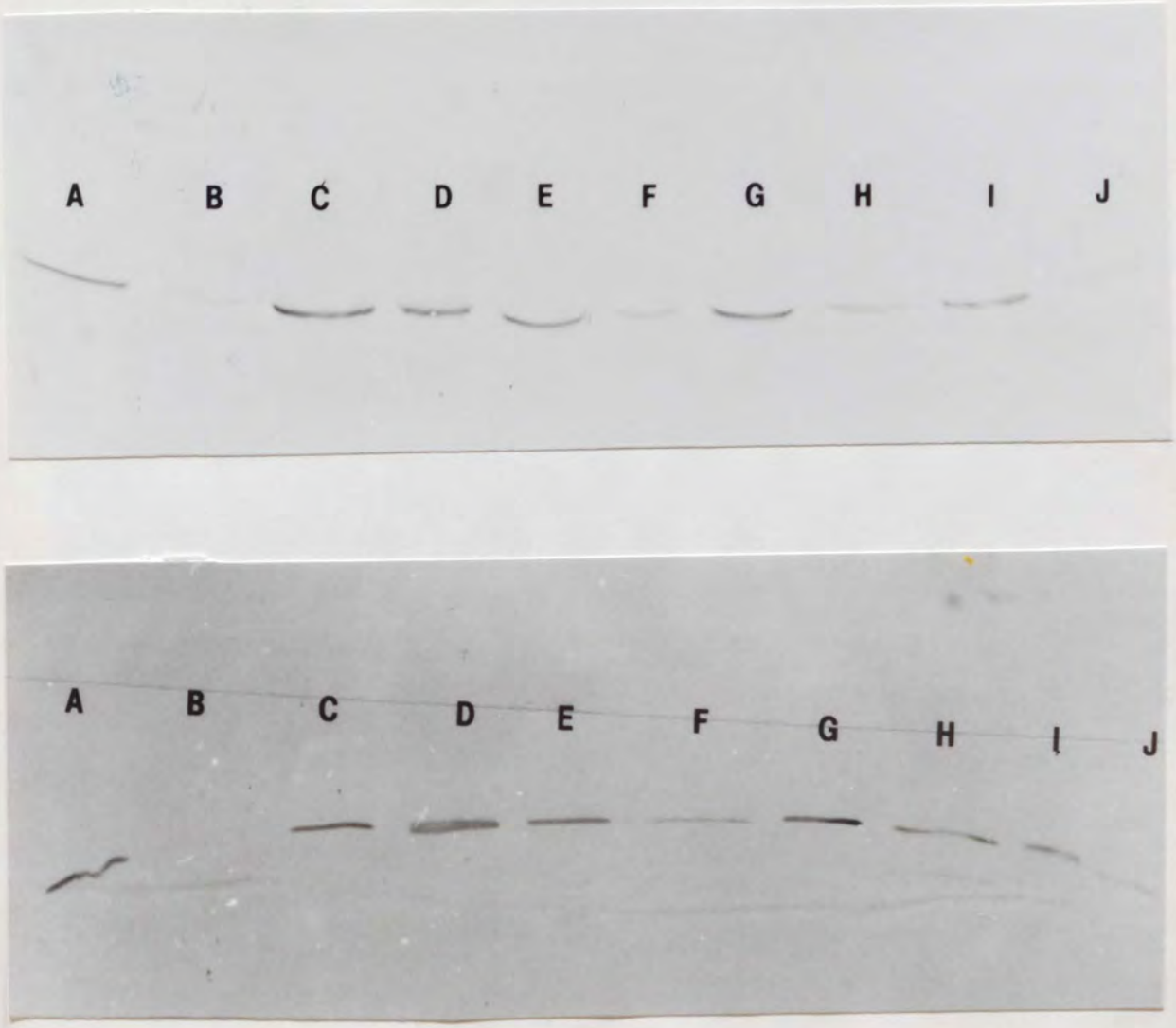


Figure 4.65 Intracellular localisation of actin in HL60 cells following treatment with toxic concentrations of drugs

Crude cytoplasmic (c), and nuclear fractions (n), of HL60 cells were prepared and the immunoblot probed with an anti-actin antibody. Cells were untreated: c (A), and n (B); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours): c (C), and n (D); or treated with 1μM Adriamycin for 8 hours: c (E), and n (F); 5μM CB3717 for 16 hours: c (G), and n (H); 225mM NMF for 8 hours c (I), and n (J).

Figure 4.66 Intracellular localisation of histone H2B in HL60 cells following treatment with toxic concentrations of drugs

Crude cytoplasmic (c), and nuclear fractions (n), of HL60 cells were prepared and the immunoblot probed with an anti-histone antibody. Cells were untreated: c (A), and n (B); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours): c (C), and n (D); or treated with 1μM Adriamycin for 8 hours: c (E), and n (F); 5μM CB3717 for 16 hours: c (G), and n (H); 225mM NMF for 8 hours c (I), and n (J).

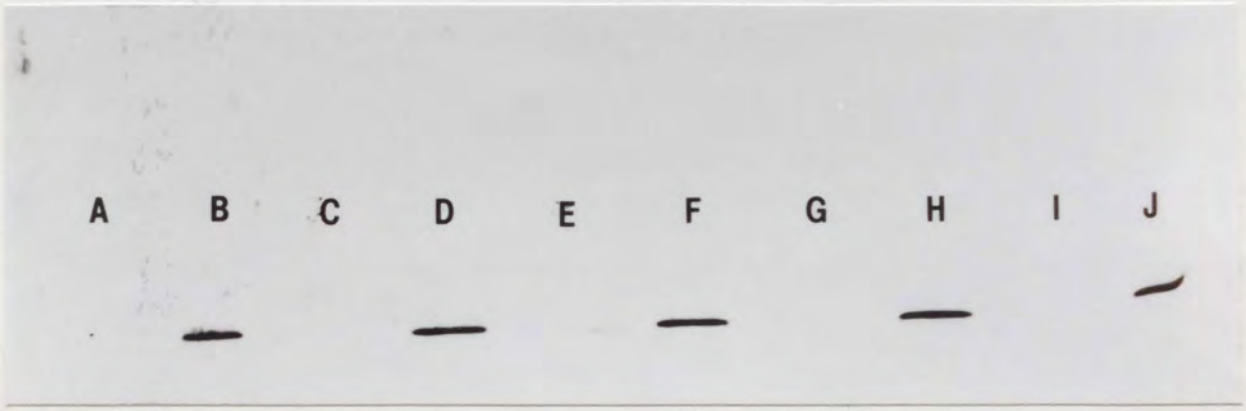
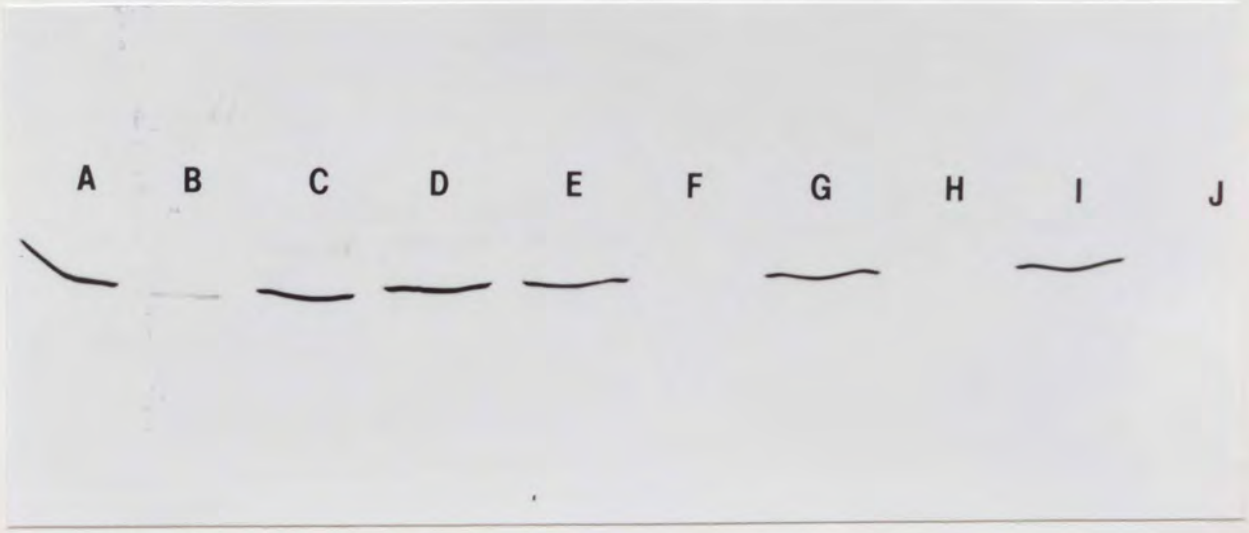
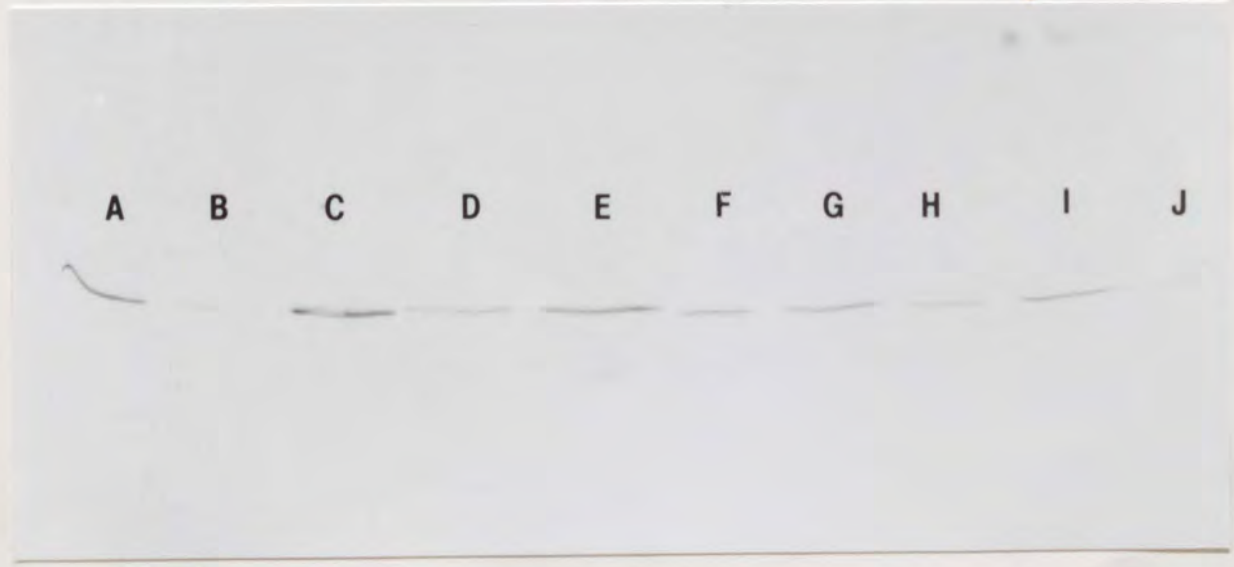
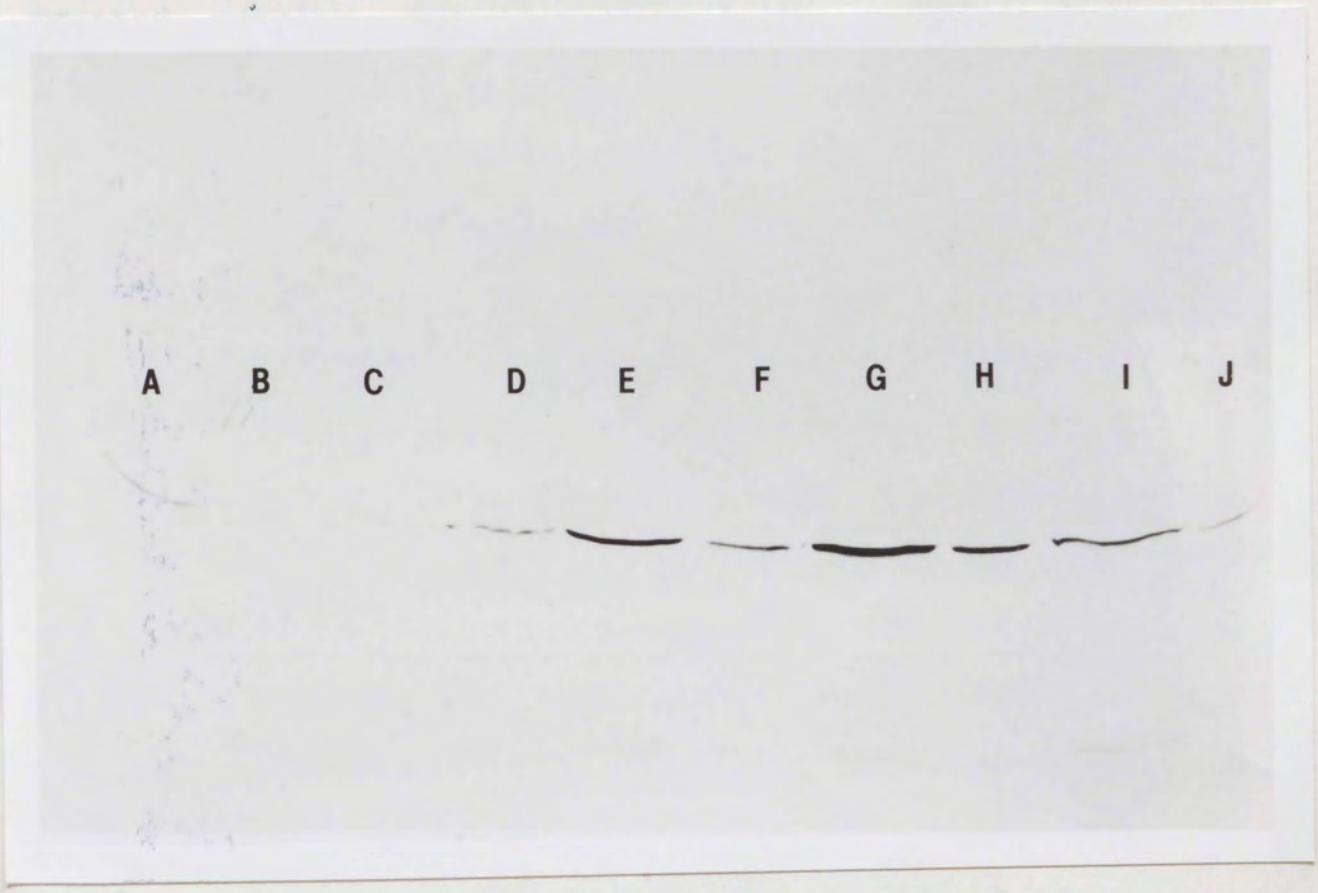


Figure 4.67 Intracellular localisation of hsp70 in HL60 cells after treatment with a toxic concentration of drug followed by a heat shock

Crude cytoplasmic (c), and nuclear (n) fractions of HL60 cells were prepared and the fractions immunoblotted and probed with 72kD antibody. Cells were untreated c (A), and n (B); heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours): c (C), and n (D)n; or treated with 1μM Adriamycin for 8 hours then heat shocked): c (E), and n (F); 5μM CB3717 for 16 hours then heat shocked: c (G), and n (H); 225mM NMF for 8 hours then heat shocked: c (I), and n (J). The results from three experiments are shown.






A
B
C
D
E
F
G
H
I
J

4.6.2 Half-life of hsp70

Hsp70 from pulse-chased control, heat shocked and NMF treated samples was immunoprecipitated and it's decay monitored by autoradiography. Determination of the turnover from monitoring the counts had proved unsuccessful due to the low levels of counts able to be recovered from the polyacrylamide gel.

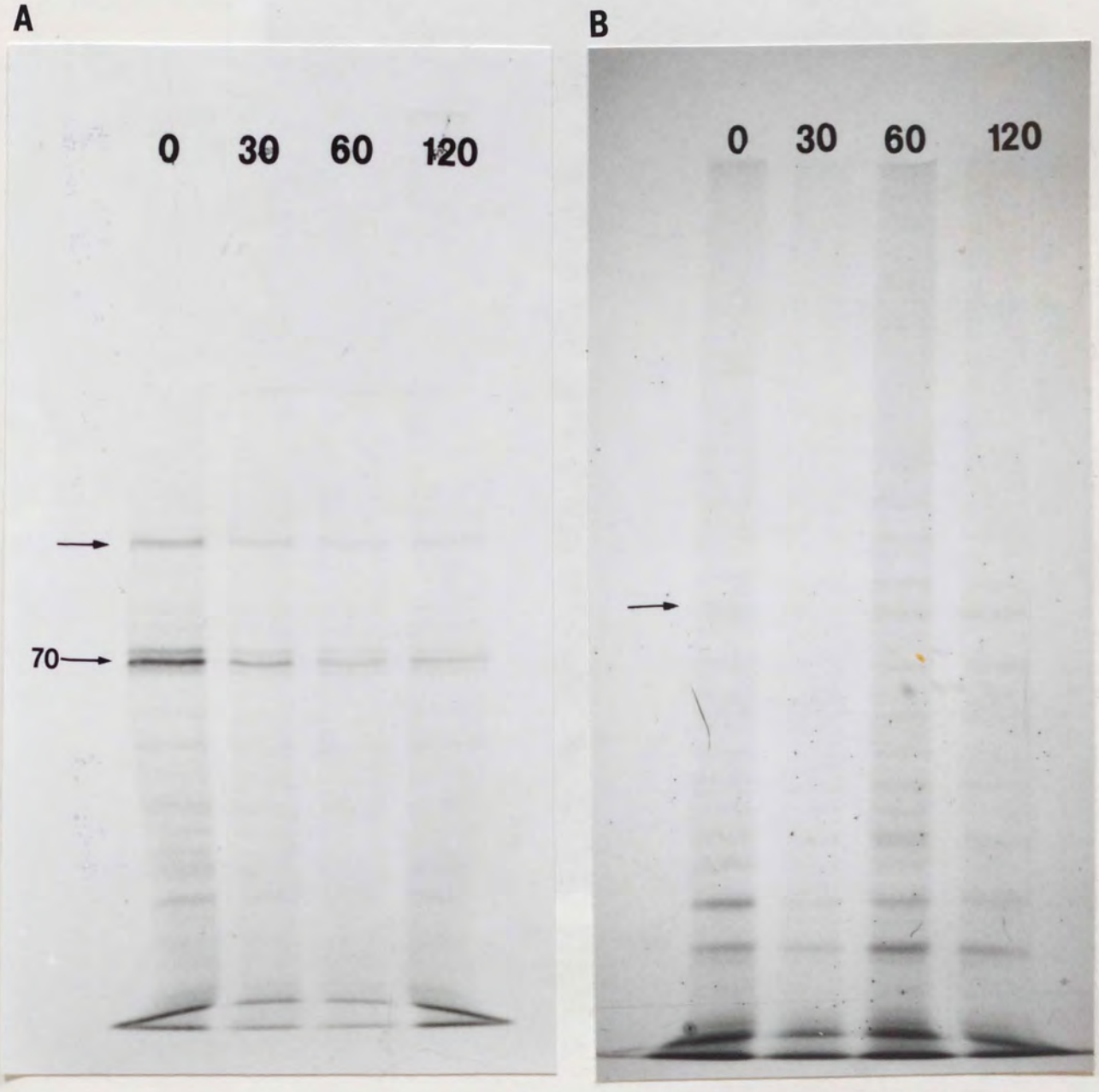
In heat shocked samples the turnover of hsp70 was less than 30 minutes (figure 4.68A) and this was quicker than in NMF (figure 4.68B) samples. The intensity of the bands in the control and NMF samples, even at time 0, was weak due to low levels of hsp70 in the cells and the inefficiency of the process. It is very difficult therefore to draw firm conclusions about the turnover of hsp70 in control cells. It is probably only valid to make comparisons between the three samples.

Of interest was the amounts of protein that coprecipitated with hsp70 in each of the 3 samples. Despite the same conditions of cell lysis and solubilisation, there was more protein complexed to hsp70 in the NMF treated cells than in the heat shocked or control samples. In the control and heat shocked cells, hsp70 co-precipitated with one other major band of higher molecular weight, whereas in the NMF-treated cells several bands were visualised.

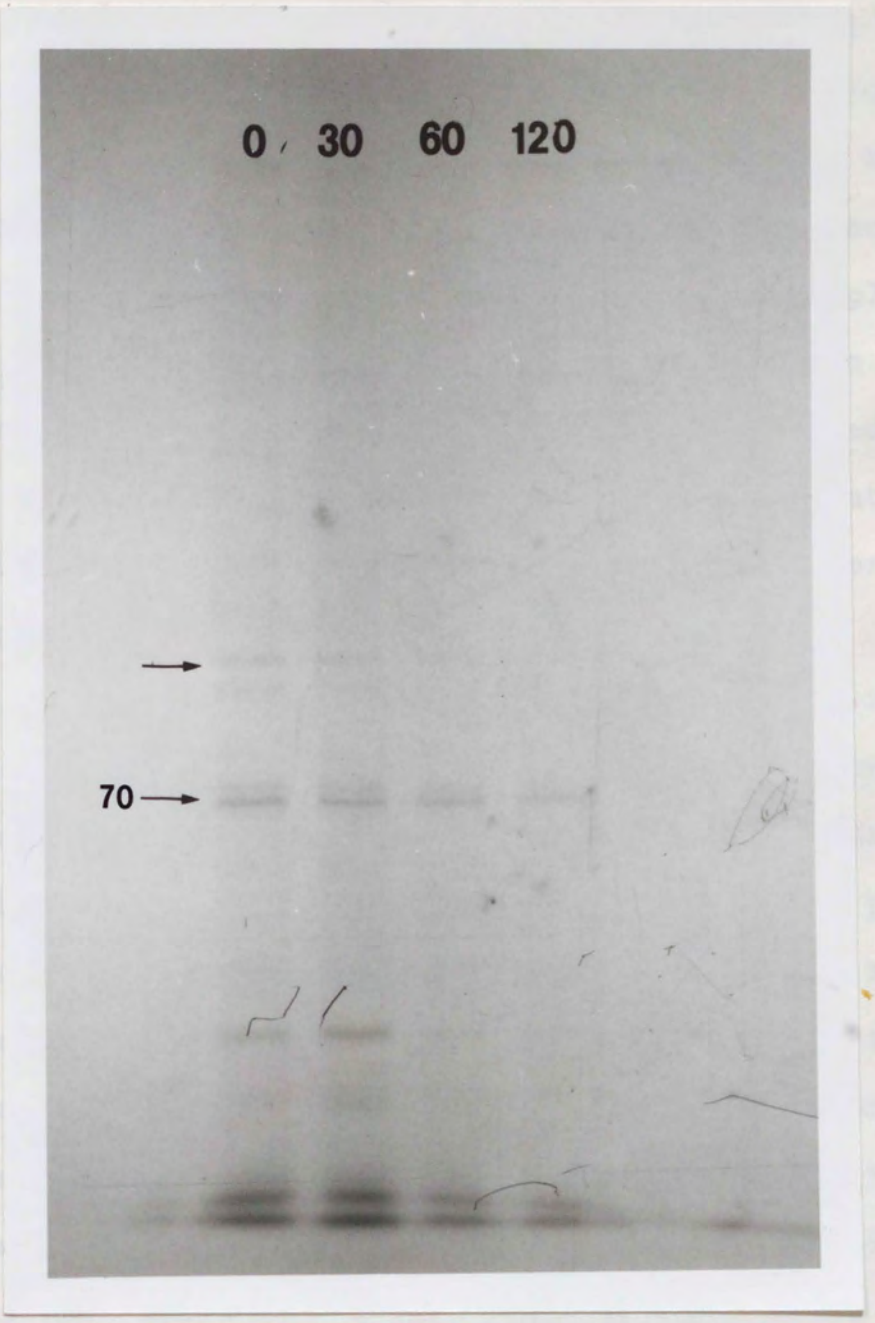
The band corresponding to hsp70 is highlighted by means of an arrow.

Figure 4.68 The effect of heat shock or 225mM NMF for 8 hours on hsp70 protein turnover in HL60 cells

Immunoprecipitation of pulse-chased HL60 cells with 72kD antibody. Cells were heat shocked (43.5°c for 1 hour then incubated at 37°c for 2 hours) (A); treated with 225mM NMF for 8 hours (B); untreated (C). 0, 30, 60, and 120 represent the chase time in minutes.



C



4.6.3 Discussion

When HL60 cells were heat shocked hsp70 was translocated from the cytoplasm to the nucleus. In contrast, when HL60 cells were treated with 225mM NMF for 8 hours, a concentration which caused approximately 60% of the cells to loose viability, the increased amounts of hsp70 were present in the cytoplasm of the cells and the protein was not turned over at the same rate as in the heat shocked cells. Following treatment with Adriamycin or CB3717 at toxic concentrations the increased amounts of hsp70 were also largely localised in the cytoplasm.

When cells are heat shocked, hsp70 plays a role in protecting the cell from further stress and in enhancing the recovery of that cell. Hsp70 binds to the hydrophobic regions that are exposed on denatured nuclear proteins following a heat shock. These hydrophobic regions may well interact to form insoluble aggregates; by binding to these hydrophobic surfaces hsp70 may limit any such interactions and promote disaggregation. From the results of the turnover experiments it would appear as if hsp70 is turned over very rapidly so the interactions in heat shocked cells between hsp70 and denatured proteins are more than likely transient.

This apparent rapid turnover is contrary to the results published by Mizzen and Welch (1986) when the half-life of hsp70 in heat shocked HeLa cells was approximated to be 48 hours. This value was obtained by determining the

counts from a gel slice containing hsp70. However the band was excised from an SDS-PAGE gel onto which had been loaded total cell protein; it would be very difficult to ensure that only the protein of interest had been isolated using this method and that there were no contaminating proteins present. To eliminate this problem it would have been preferable to perform an initial immunoprecipitation so that only the protein of interest was loaded onto the gel. The results obtained in this thesis correlate with the studies by Mivechi (1989) where HL60 cells show a decay in thermotolerance after 4 hours which also suggests a high turnover rate for hsp70.

Studies by Gonda et al (1989) have investigated the degradation of proteins and the signals which may play a role in targeting them for selective degradation. They concluded that in S. cerivisiae an important component of a protein's degradation system is that proteins amino-terminal residue. The resulting code that relates the protein's metabolic stability and the nature of its amino-terminal residue has been called the N-end rule; a similar system has also been defined by the authors in mammalian reticulocytes. From the hsp70 sequence published by Hunt and Morimoto in 1985 the amino-terminal residue is methionine which according to Gonda should imply a half-life of 30 hours for hsp70; this would be in agreement with Mizzens data. However c-myc, which also has methionine as its amino-terminal residue (Colby et al 1983), has been shown by Luscher and Eisenman (1988) to have

a half-life of 20 minutes. These contradictory results highlight the difficulty in calculating half-life values of proteins.

In HL60 cells that had been treated with toxic concentrations of drugs hsp70 was localised in the cytoplasm and did not translocate to the nucleus even when the cells were heat shocked after drug treatment. Toxic drug treatment followed by a heat shock led to greater than 90% of cells loosing viability, as assessed by trypan blue. The inability of hsp70 to translocate after the heat shock and associate with the preribosomes would mean that the cell would be unable to process the denatured proteins produced as a result of the heat shock. Santoro et al (1989) have also demonstrated that hsp70 is localised in the cytoplasmic fraction of K562 cells after treatment with PGA_1 at a concentration that inhibited cell proliferation and induced synthesis of hsp70. This inhibition of proliferation was not however associated with cytotoxicity as once PGA_1 was removed from the medium the cells resumed normal growth and hsp70 synthesis declined.

When parallel fractionation blots were probed with an antibody raised against actin, actin was localised mainly in the cytoplasmic fraction except following a heat shock where significant amounts of actin were also present in the nuclear fraction. As mentioned in section 4.6.1 the presence of actin rods in the nucleus has also been noted following treatment with DMSO. DMSO has been shown to induce

thermotolerence in several cell types (Hahn et al 1985) but as to whether it plays a role analogous to hsp70 in protecting the cells from stress is as yet unclear. It is however interesting that there were no significant amounts of actin in the nuclear fraction following treatment with drugs; the results with the histone antibody had shown that this lack of translocation was not the result of an overall shutdown of protein translocation in these cells.

Hsp70 is thought to interact and facilitate maturation of newly synthesised proteins and these interactions are transient and are dependent upon ATP hydrolysis (Beckmann et al 1990). Under the conditions of drug toxicity it is proposed that hsp70 remains bound to other cellular proteins; this would prevent the nuclear targeting sequence from being exposed and prevent hsp70 from translocating to the nucleus. This proposal is supported by the inability of hsp70 to translocate in the heat shocked cells that were first treated with toxic drugs. From the experiments to determine the half-life of hsp70 following treatment with NMF for 8 hours, it is apparent that the binding of hsp70 to other proteins under these conditions involves very strong interactions since significantly more proteins were coprecipitated with hsp70 after treatment with NMF than were coprecipitated after heat shock. The similarity between proteins that were complexed with hsp70 in control and heat shocked cells was in agreement with the results obtained by Milarski et al (1989a). If, after treatment with drugs that induce

cytotoxicity, hsp70 remained tightly bound to newly synthesised proteins, and to proteins that it is associated with for the purposes of chaperoning, ATP would be unable to bind to hsp70 which would affect the ability of hsp70 to dissociate from complexes; the proteins would not therefore be competent to perform their normal functions. In this way it can be seen how elevated levels of hsp70 may be harmful to cells and consequently lead to cell death. The studies by Milarski et al (1989b) have demonstrated that hsp70 associates specifically with proteins in a cell-cycle dependent manner; hsp70 may therefore also interact with specific proteins during cell death. The role for hsp70 proposed by Chiang et al (1989) in the lysosomal degradation of proteins would also be pertinent to the results presented in section 4.6.2. The presence of damaged proteins in a cell would imply increased lysosomal degradation and the binding of hsp70 is required for the transport of the damaged proteins targeted for degradation.

Further studies were undertaken in an attempt to determine whether the cells died by necrosis or apoptosis after treatment with Adriamycin, CB3717 and NMF (see section 1.4).

4.7 Biochemical and morphological studies on HL60 cell death following treatment with toxic concentrations of drugs

4.7.1 Cleavage of DNA

In apoptotic cells, DNA is thought to be cleaved at internucleosomal sites by an endonuclease enzyme and when the DNA is subjected to agarose gel electrophoresis, it appears "laddered" with fragments of multiples of approximately 180bp. In contrast, necrotic cells show general dissolution of DNA (karyorrhexis). HL60 cells were treated with either 1μM Adriamycin for 8 hours, 5μM CB3717 for 16 hours or 225mM NMF for 8 hours and the presence or absence of DNA cleavage investigated. Samples were also resuspended in drug-free media and left for a further 24 hours before analysis. A sample of HL60 cells was also treated with 1μM methotrexate for 48 hours as this treatment had been shown by Kaufmann (1989), to induce endonucleolytic cleavage.

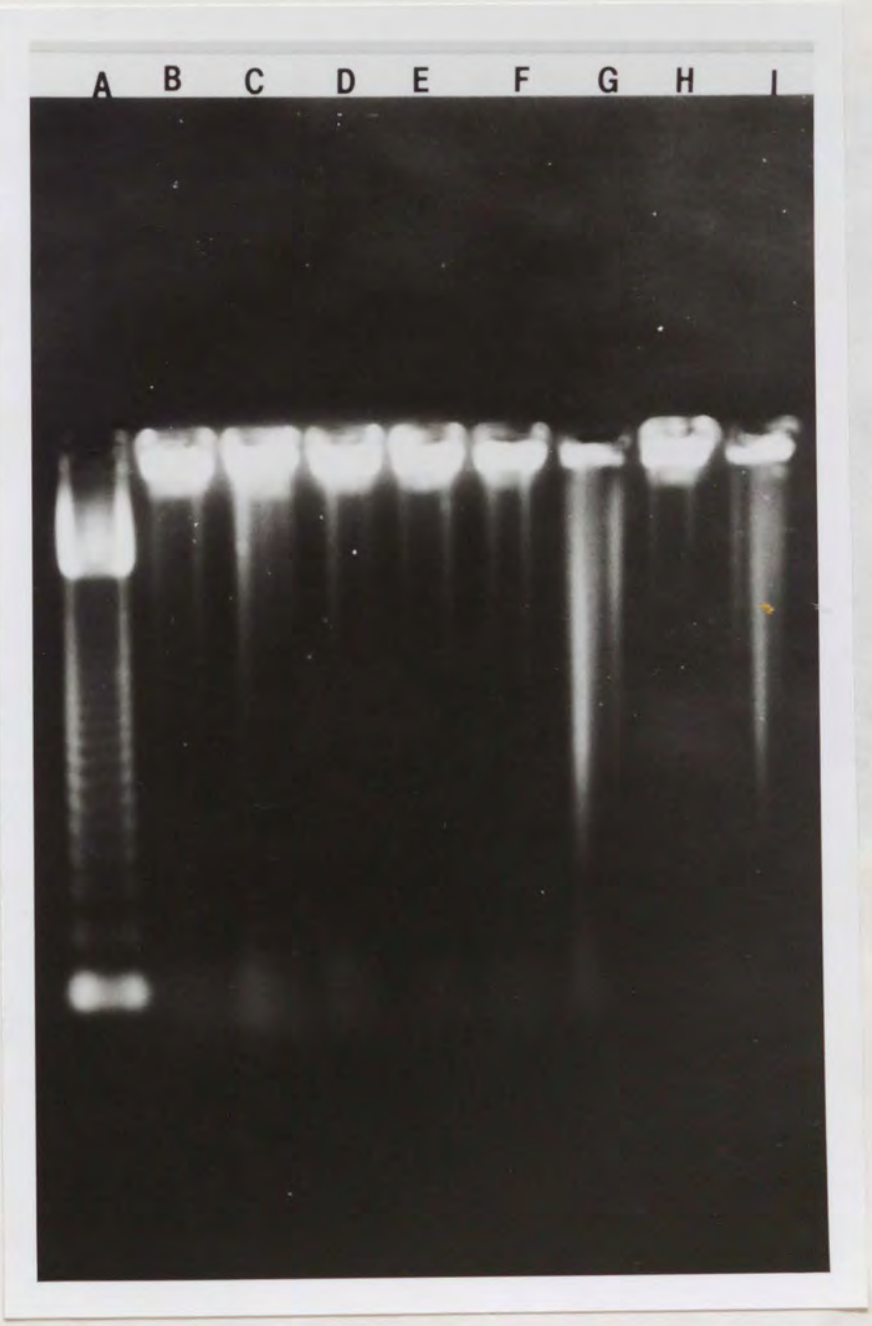
Figure 4.69 B-E, shows the DNA that had been isolated from cells immediately following treatment; no cleavage of DNA was observed in any of the samples. In contrast, following the 24 hour incubation in drug-free media, general dissolution of the DNA was observed but with no evidence of "ladders" in any of the samples (figure 4.69 G-I). However no "ladders" were observed in the methotrexate treated cells (lane F), only karyorrhexis. There was a certain amount of DNA breakdown in the control cells (lane B) demonstrating the

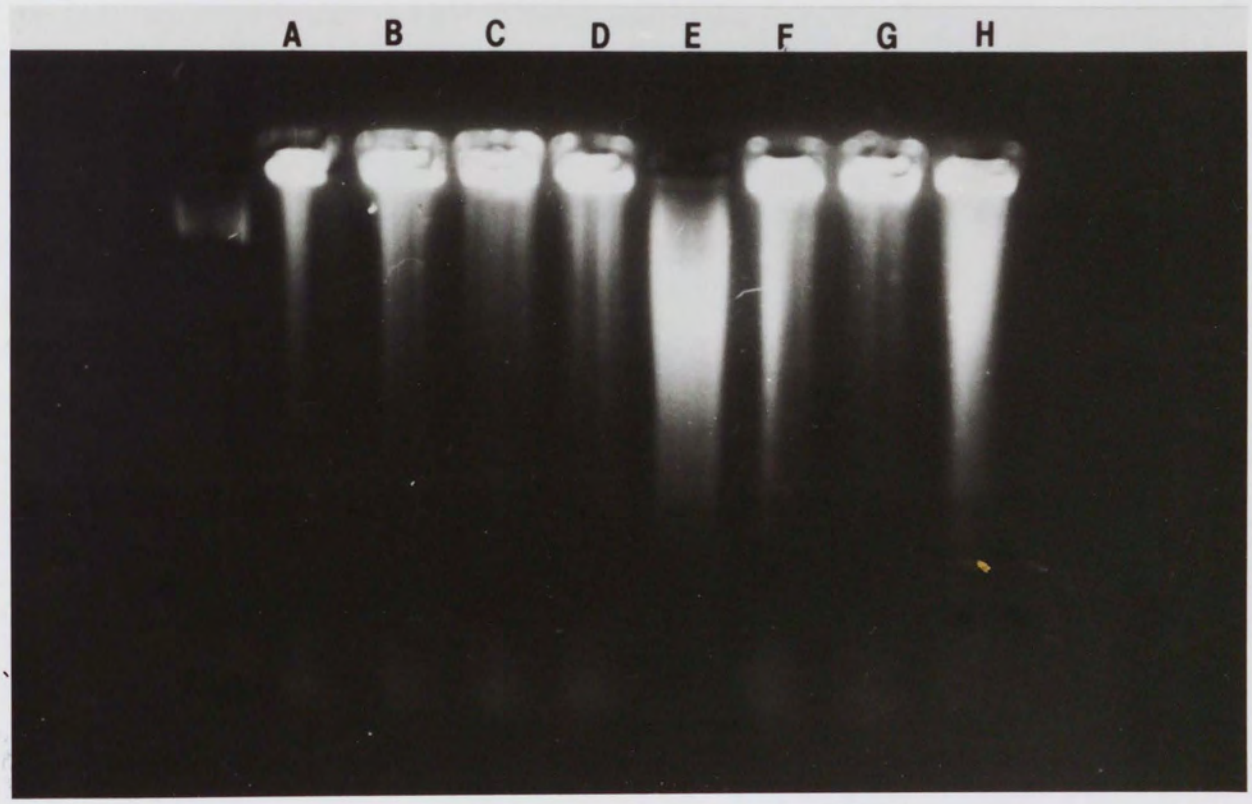
loss in cell viability caused by the wash procedure.

Since this procedure did not allow for the separation of necrotic cells from apoptotic cells it was thought that any evidence of cells that were undergoing apoptosis and producing "ladders" could be masked by those cells undergoing necrosis. In an attempt to overcome this and to provide more evidence of the type of cell death, samples were prepared for scanning and transmission electron microscopy.

Figure 4.69 The effect of toxic concentrations of drugs on DNA integrity

Agarose gel of DNA isolated from control and treated HL60 cells. DNA was isolated immediately following treatment and following a 24 hour incubation in drug-free medium. Markers (A); cells were untreated (B); or treated with 1μM Adriamycin for 8 hours (C); 5μM CB3717 for 16 hours (D); 225mM NMF for 8 hours (E); 1μM methotrexate for 48 hours (F). Cells were also treated as described then incubated for a futher 24 hours; 1μM Adriamycin for 8 hours (G); 5μM CB3717 for 16 hours (H); 225mM NMF for 8 hours (I). The results from two experiments are shown.




A
B
C
D
E
F
G
H

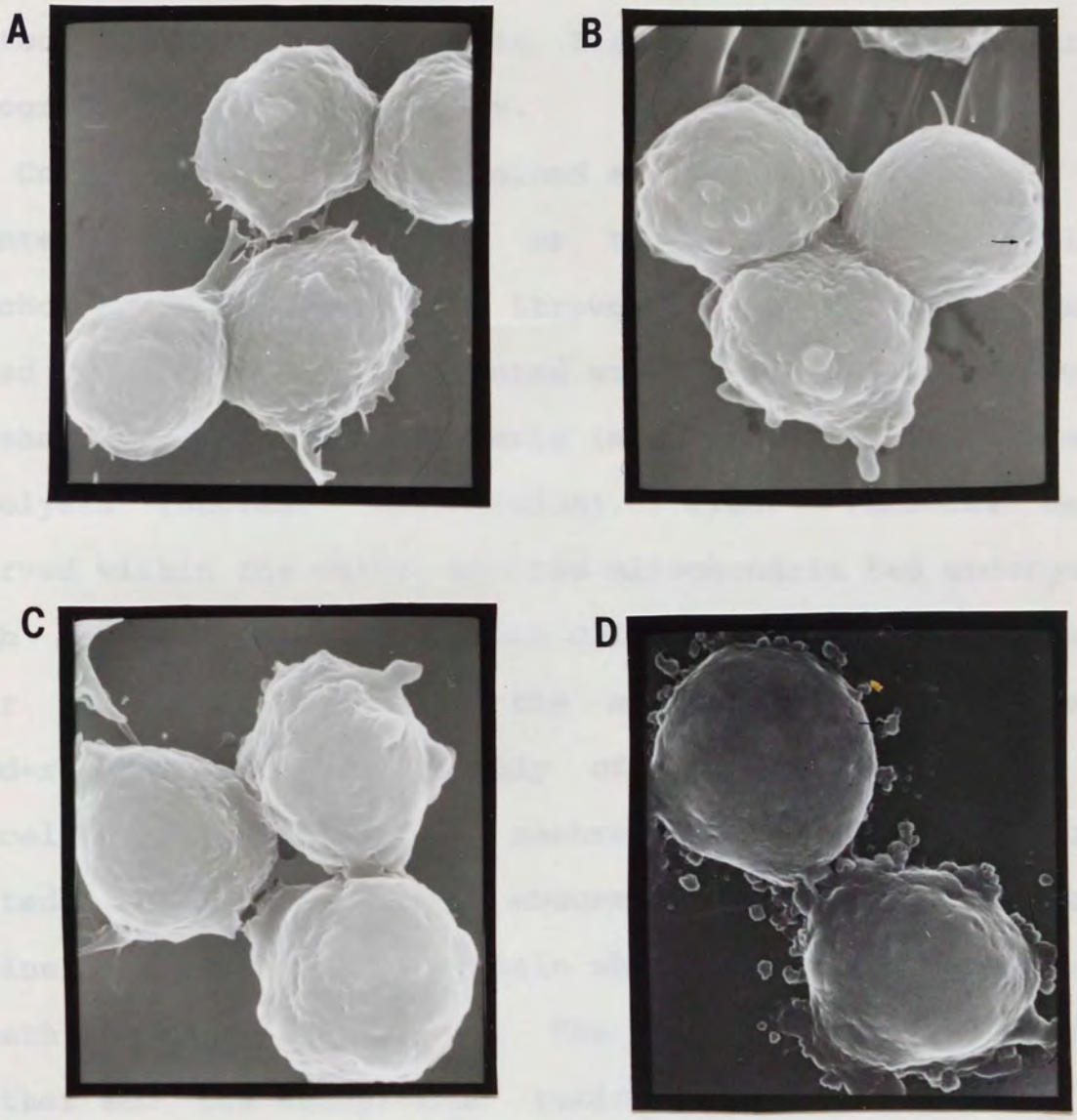
4.7.2 Scanning electron microscopy

HL60 cells were treated with 1uM Adriamycin for 6 and 8 hours, 5uM CB3717 for 8 and 16 hours and 225mM NMF for 4 and 8 hours respectively, then prepared for scanning electron microscopy for assessment of any changes in cell shape and surface morphology. Micrographs of representative control and treated cells are shown in figure 4.70.

Control cells (A) display a slightly blebbed surface Treatment with Adriamycin for 8 hours (B) caused the cells to become more round with the appearance on some cells of "crater-like" pits (arrow). CB3717 (C) caused no apparent change in surface morphology whereas cells treated with NMF for 8 hours (D) had a smooth surface and appeared more rounded but had lost some membrane through blebbing (arrow). Treatment of HL60 cells with these agents at the same concentration for shorter periods of time caused no alteration in cell shape or surface morphology and the cells resembled untreated cells.

Figure 4.70 Scanning electron micrographs of HL60 cells following treatment with Adriamycin, CB3717 or NMF

Cells were untreated (magnification x 6 000) (A); or treated with 1µM Adriamycin for 8 hours (magnification x 5 000) (B); 5µM CB3717 for 16 hours (magnification x 5 000) (C); 225mM NMF for 8 hours (magnification x 5 000) (D).



4.7.3 Transmission electron microscopy

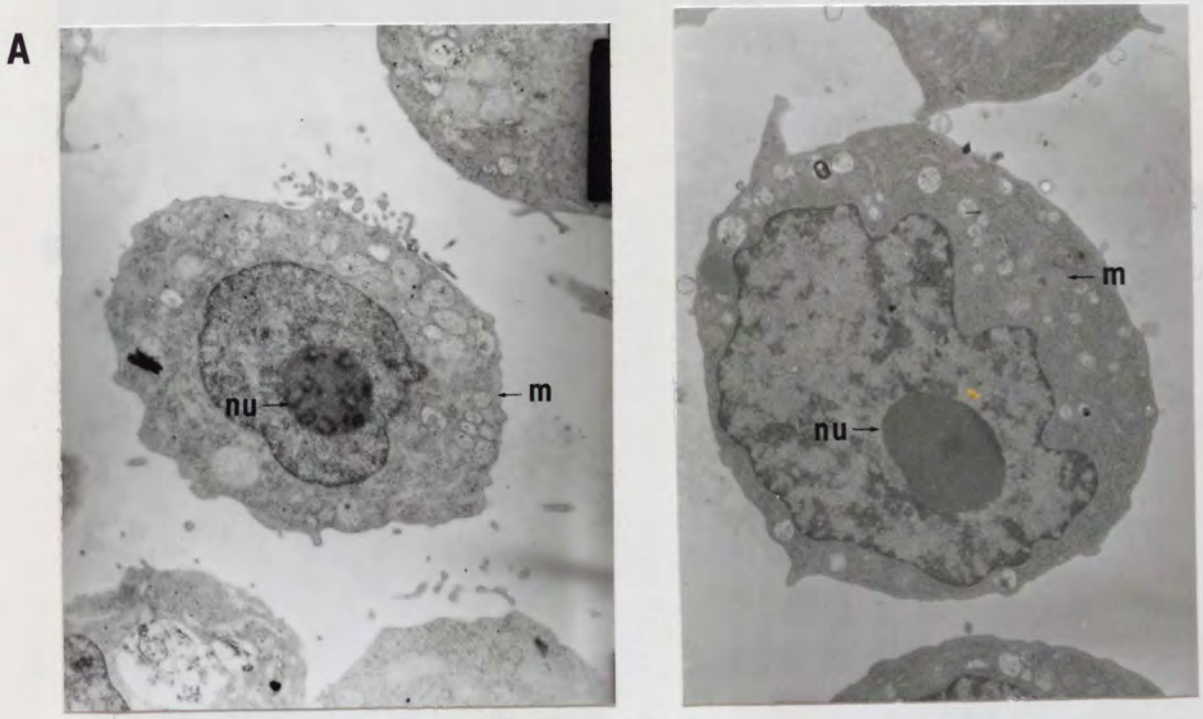
HL60 cells were treated with the three agents as described for scanning electron microscopy then they were fixed, stained with uranyl acetate and lead citrate, and sectioned. Representative micrographs of control and treated cells are shown in figure 4.71 with several micrographs for each treatment.

Control cells (A) contained a single, large round or indented nucleus with one or two prominent nucleoli. Mitochondria were scattered throughout the cell and were varied in number. Cells treated with 225mM NMF for 8 hours (D) showed examples of pyknosis (nuclear condensation) and karyolysis (nuclear dissolution). Clear vacuoles were observed within the cells and the mitochondria had undergone "high amplitude swelling" with dilation of both inner and outer compartments. Within the mitochondria were densely lipid-rich aggregates, probably of matrix lipoproteins. A few cells showed evidence of membrane breakdown. Adriamycin treated cells (B) had an abnormally convoluted nuclear outline and condensed chromatin which formed a dense mass beneath the nuclear membrane. The mitochondria had crowded together and the endoplasmic reticulum appeared dilated as the cytoplasm condensed. The presence of apoptotic bodies containing chromatin and/or intracellular organelles, and vacuoles was also observed. Cells treated with CB3717 (C) had few morphological alterations. The mitochondria and

other organelles were difficult to distinguish from the surrounding cytoplasm and the nucleolus was not apparent. The presence of lipid vacuoles was noted in some cells. Cells treated for 3 hours with NMF, 6 hours with Adriamycin and 8 hours CB3717 showed similar morphological characteristics to control cells.

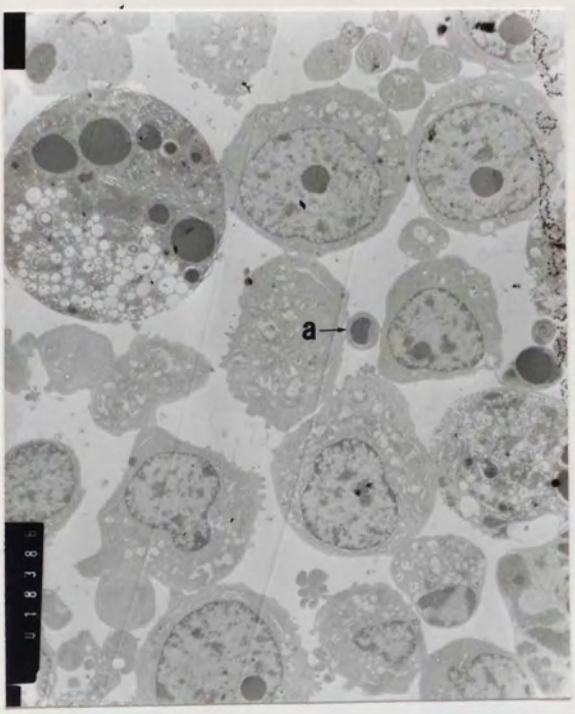
Figure 4.71 Transmission electron micrographs of HL60 cells following treatment with Adriamycin, CB3717 or NMF

Cells were treated as follows:
(A) untreated (magnification x 5 000);
(B) 1µM Adriamycin for 8 hours (magnification (1) x 3 400, (2) x 3 400, (3) x 4 000, (4) x 6 000);
(C) 5µM CB3717 for 16 hours (magnification x 6 000);
(D) 225mM NMF for 8 hours (magnification (1) x 3 400, (2) x 9 100).
Key: a=apoptotic body; c=chromatin; er=endoplasmic reticulum; l=lipid droplet; m=mitochondria; n=nucleus; nu=nucleolus; p=pyknosis; v=vacuole.



B

1



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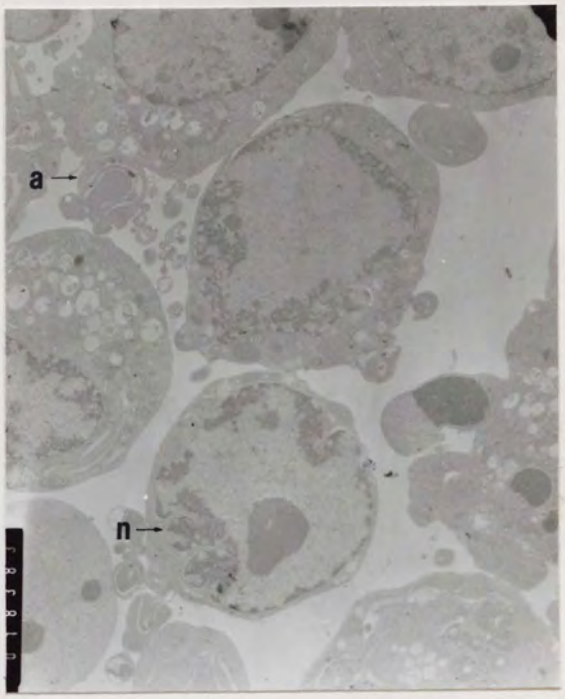

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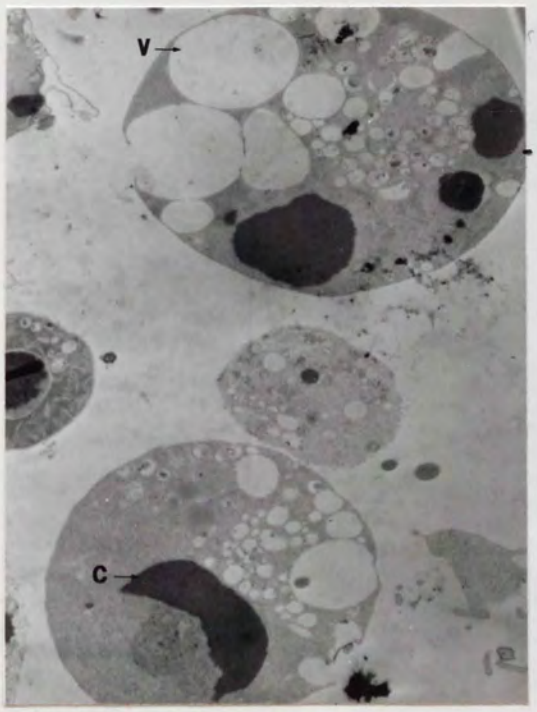

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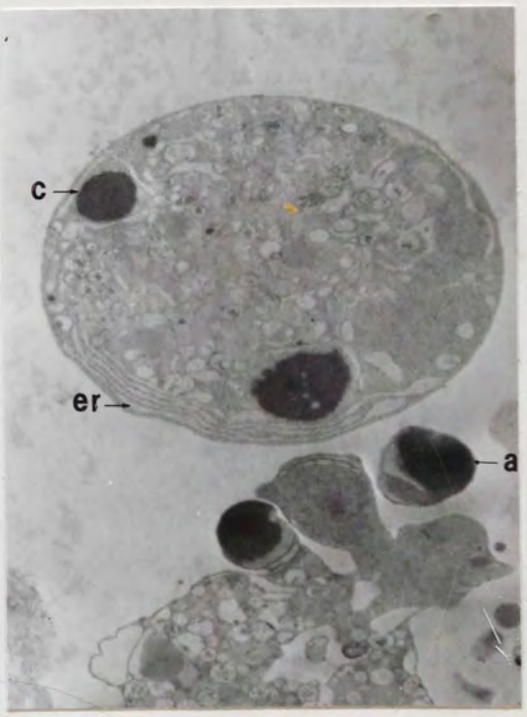
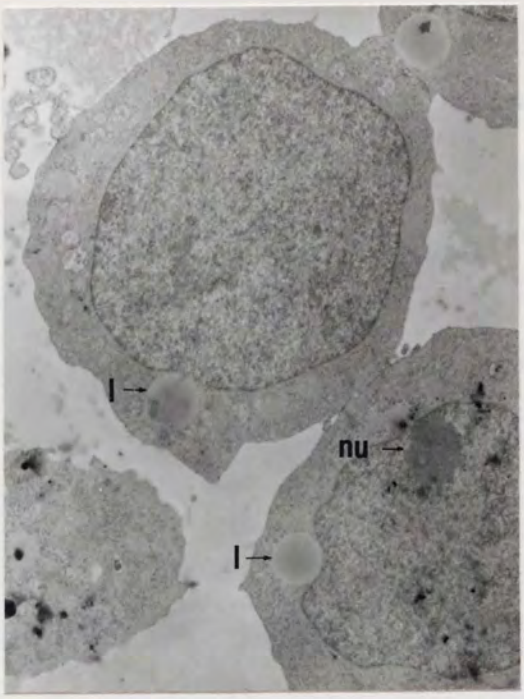
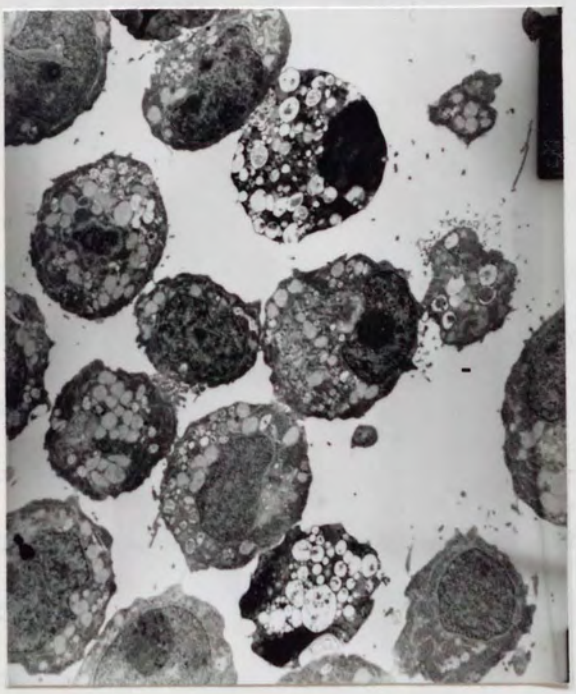


C



D
1



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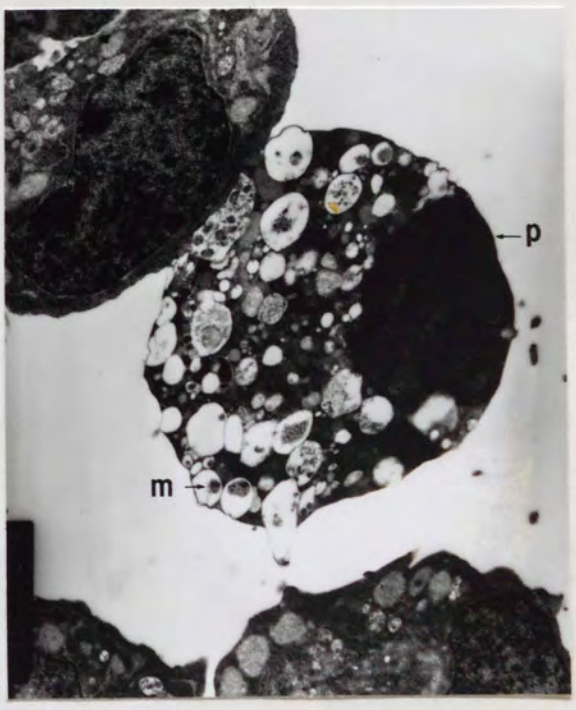

4.7.4 Discussion

After treatment with either NMF, Adriamycin, or CB3717 for periods that brought about a rise in hsp70 amounts, there was no loss of integrity in the cell's DNA. Following the 24 hour incubation in the absence of any drug the DNA had been degraded in a pattern indicative of a necrotic form of cell death. If endonucleolytic cleavage of the DNA had occured, a characteristic of apoptosis, "ladders" of approximatly 180 base pairs would have been observed, although a recent publication by Kaufmann (1989) sheds some doubt on this method as a means of identifing apoptosis (discussed in section 1.4.2).

The mainstay in identification of types of cell death are still morphological methods. From studies of cells that were fixed after treatment with NMF, Adriamycin and CB3717 and processed for scanning and transmission electron microscopy. Morphological changes were only observed in those cells which had increased amounts of hsp70; NMF treated cells showed characteristics that indicated the cells were undergoing necrosis, the Adriamycin treated cells showed characteristics of apoptosis and from the scanning electron micrographs CB3717 appeared to induce necrosis. However care must be taken when interpreting the results obtained from electron micrographs as these results are not in any way quantitative and there not all cells from each treatment showed an altered morphology; artifacts can also occur as a

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CHAPTER FIVE

DISCUSSION

5.1 General discussion

Studies described in this thesis suggest that different members of the hsp70 family are regulated under conditions of differentiation and cytotoxicity in HL60 cells. From the results obtained, the synthesis of the glucose-regulated proteins grp78 and grp94 were modulated during the pre-commitment and commitment stages of HL60 granulocytic differentiation; although these changes in grps were not observed with monocytic differentiation induced by TPA, the protein synthesis and amounts of hsp70 were only measured at one time point so any transient changes in the grps may have occurred at earlier times. In contrast higher concentrations of differentiation-inducing drugs that did not induce differentiation but brought about cytotoxicity, and agents that had no effect on differentiation, at any concentration, caused an early increase in the amounts of hsp70 which was comparable to the amounts obtained following a heat shock. This increase in amounts of hsp70 in drug-treated cells occurred in the absence of any increase in synthesis and was not the result of a stabilisation of all cell proteins.

The specificity of modulation of the stress proteins is thought to involve the localisation of the damage within a cell. In the case of the terminal differentiation of HL60 cells induced by NMF and tunicamycin, the modulation of grps would suggest that the two agents had caused changes in the endoplasmic reticulum.

There was also a later increase in the amounts of hsp70 during NMF-induced differentiation which was probably the result of the sub-toxic concentration of the agents that was required to induce optimum differentiation; this induction of differentiation by a sub-toxic concentration of drug is a characteristic of leukaemic cell differentiation (discussed in section 1.5). However increases in amounts of hsp70 were observed during cytotoxicity and these increased amounts were implicated as being both an early marker and possible cause of a loss in viability. How could increases in amounts of hsp70 be observed during differentiation and cytotoxicity?

The increases cannot be explained by the differences in turnover rate that were observed with heat shocked cells and cells that had been treated with 225mM NMF for 8 hours, since during differentiation the increases in amounts of hsp70 occurred in the absence of any increase in synthesis which would imply an increase in hsp70 half-life.

The localisation of hsp70 may be important in the different processes; during a heat shock, which was non-toxic, hsp70 translocated to the nucleus, but following treatment with concentrations of drug that caused a later loss in viability, hsp70 was localised in the cytoplasm. During the induction of differentiation the increased amounts of hsp70 may be localised in the nucleus hence there was no loss in viability; also the increased amounts of hsp70 were transient.

Another factor to be considered is the cellular proteins

Another factor to be considered is the cellular proteins that the stress proteins hsp70 and grp78 are associated with. As previously discussed, hsp70 has been shown to associate with c-myc, grp78 with the src oncogene product and a role for these associations in maintaining the transformed state of a cell has been presented. The associations that contribute towards the transformed state of the cell are presumably transient such that when cells are induced to differentiate hsp70 dissociates from, for example, c-myc. Since the agents that induce differentiation are used at sub-toxic concentrations they may well induce a certain amount of damage to cellular proteins; hsp70 under these circumstances may have a greater affinity to the damaged proteins rather than the oncogene proteins. The role of hsp70 in interacting with newly synthesised proteins would also suggest that increased amounts of hsp70 would be required during differentiation. Under conditions of cytotoxicity it has been proposed that these normally transient interactions between hsp70 and newly synthesised proteins are stabilised which ultimately leads to cell death.

The hypothesis that the induction of a cellular stress and some type of response by the cell, such as the modulation of particular members of the stress protein family, may lead to differentiation, is attractive since it would explain why so many disparate agents can induce differentiation; in the case of HL60 cells induced to differentiation to granulocyte-like cells it is the modulation of the

glucose-regulated proteins that appears to be important. The inability of drugs to bring about these specific changes may explain why cells may undergo certain changes under conditions of marginal cytotoxicity but do not terminally differentiate. If the drug induces changes in other members of the stress protein family, such as hsp70, cytotoxicity may result. It is not yet clear as to whether the differential regulation of the members of the 70kD stress protein family has a causative role in the processes of differentiation and cytotoxicity, but the early changes that were observed and a review of their known functions would suggest their active participation.

REFERENCES

Abramson S, Miller R G and Phillips R A (1979). The identification in adult bone marrow of pluripotent and restricted stem cells of the myeloid and lymphoid systems. J. Exp. Med. 145: 1567-1579.

Allan D J, Hendry J H and Potten C S (1989). Apoptosis - selectivity, hypersensitivity, and manipulation? Paterson Laboratory Report 13-15.

Ananthan J, Goldberg A L and Voellmy R (1986). Abnormal proteins serve as eukaryotic stress signals and trigger the activation of heat shock genes. Science 232: 522-524.

Andrews G K, Harding M A, Calvet J P and Adamson E D (1987). The heat shock respose in HeLa cells is accompanied by elevated expression of the c-fos proto-oncogene. Mol. Cell Biol. 7: 3452-3458.

Ashburner M and Bonner J J (1979). The induction of gene activity in Drosophilia by heat shock. Cell 17: 241-254.

Banerji S S, Laing K and Morimoto R I (1987). Erythroid lineage-specific expression and inducibility of the major heat shock protein hsp70 during avian embryogenesis. Genes and Development 1: 946-953.

Barnekow A and Gessler M (1986). Activation of the $pp60^{c-src}$ kinase during differentiation of monomyelocytic cells in vitro. EMBO J. 5: 701-705.

Baulieu E E (1987). Steroid hormone antagonists at the receptor level: a role for the heat shock protein MW 90 000 (hsp90). J. Cell. Biochem. 35: 161-174.

Bazill G W, Hayes M, Garland J and Dexter T M (1983). Characterisation and partial purification pf a haemopoietic growth factor in WEHI-3 cell conditioned medium. Biochem. J. 210: 747-759.

Beckmann R P, Mizzen L A and Welch W J (1990). Interaction of hsp70 with newly synthesised proteins: implications for protein folding and assembly. Science 248: 850-854.

Bensaude O and Morange M (1983). Spontaneous high expression of heat shock proteins in mouse embryonal carcinoma cells and ectoderm from day 8 mouse embryo. EMBO 2: 173-177.

Berger N A (1986). Cancer chemotherapy. New strategies for success. J. Clin. Invest. 78:1131-1135.

Bessis M (1973). Living blood cells and their ultrastructure. (Trans. RI Weed), Springer Verlang, Berlin.

Bill C A, Gesher A and Hickman J A (1988). Effects of N-methylformamide on the growth, cell cycle and glutathione ststus of murine TLX5 lymphoma cells. Cancer Res. 48: 3389-3393.

Bonner W M and Laskey R A (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46: 83-86

Bradley T R and Metcalf D (1966). The growth of mouse bone marrow cells *in vitro*. Aust. J. Exp. Biol. Med. Sci. 44: 287-300.

Breitman J R, Collins S J and Selonick S E (1980). Induction of differentiation of the human promyelocytic leukemia cell line HL60 by retinoic acid. Proc. Natl. Acad. Sci. USA 77: 2936-2940.

Brennan J, Abboud G, DiPersio J, Barlow G and Lichtman M (1981). Autostimulation of growth by human myelogenous leukemia cells (HL60). Blood 58: 803-812.

Brugge J, Erikson E and Erikson R L (1981). The specific interaction of the Rous sarcoma virus transforming protein, $pp60^{src}$, with cellular proteins. Cell 25: 363-372.

Bukh A, Martinez-Valdez H, Freedman S J, Freedman M H and Cohen A (1990). The expression of c-*fos*, c-*jun*, and c-*myc* genes is regulated by heat shock in human lymphoid cells. J. Immunology 144: 4835-4840.

Bunce C M, Fisher A G, Toksoz D and Brown G (1983). Isolation and characterisation of dimethylsulphoxide resistant varients from the human promyeloid cell line HL60. Exp. Haematol. 11: 828-833.

Burdon R H and Cutmore C M M (1982). Human heat shock gene expression and the modulation of plasma membrane Na^+, K^+-ATPase activity. FEBS Letters 140: 45-48.

Burgess A W and Metcalf D (1980a). Characterisation of a serum factor stimulating the differentiation of myelomonocytic leukemia cells. Int. J. Cancer 26: 647-645.

Burgess A W, Metcalf D, Russel S H M and Nichola N A (1980b). Granulocyte-macrophage, megakaryocyte, eosinophil and erythroid colony-stimulating factors profuced by mouse spleen cells. Biochem. J. 185: 301-314.

Burres N S, Myers M and Sartorelli A C (1988). Evidence of multifactorial mechanisms in an Adriamycin-resistant HL60 promyelocytic leukemia cell line. Cancer Biochem. Biophys. 10: 47-57.

Buttyan R, Zakeri Z, Lockshin R and Wolgemuth D (1988). Cascade induction of c-fos, c-myc, and heat shock 70K transcripts during regression of the rat ventral prostate gland. Mol. Endocrinol. 2: 650-657.

Cajone F, Saliina M and Bernelli-Zazzera A (1988). c-myc gene expression in heat-adapted and heat-shocked cells. Cell Biol. Inter. Reports 12: 549-553.

Caracciolo D, Clark S and Rovera G (1989). Differential activity of recombinant colony-stimulating factors in supporting proliferation of human peripheral blood and bone marrow myeloid progenitors in culture. Br. J. Haematol. 73: 306-311.

Carr J I, Huang T H, Buzin C H and Itakura K (1986). Induction of heat shock gene expression without heat shock by hepatocarcinogens and during hepatic regeneration in rat liver. Cancer Res. 46: 5106-5111.

Castagna M, Takai Y, Kaibuchi K, Suno K, Kikkawa U and Nishizuka Y (1982). Direct activation of calcium-activated, phospholipid dependent protein kinase by tumor promoting phorbol esters. J. Biol. Chem. 257: 7847-7851.

Chang S C, Erwin A E and Lee A S (1989). Glucose-regulated protein (GRP94 and GRP78) genes share common regulatory domains and are coordinately regulated by common trans-acting factors. Mol. Cell Biol. 9: 2153-2162.

Chappell T G, Welch W J, Schlossman D M, Palter K B, Schlesinger M J and Rothman J E (1986). Uncoating ATPase is a member of the 70kD family of stress proteins. Cell 45: 3-13.

Chiang H L, Terlecky S R, Plant C P and Dice J R (1989). A role for a 70-kilodalton heat shock protein in lysosomal degradation of intracellular proteins. Science 246: 382-385.

Chow K-C and Ross W E (1987). Topoisomerase-specific drug sensitivity in relation to cell cycle progression. Mol. Cell Biol. 7: 3119-3123.

Christman S K, Mendelsohn N, Hersog D and Schneiderman N (1983). Effect of 5-aza-cytidine on differentiation and DNA metabolism in human promyelocytic leukemia (HL60) cells. Cancer Res. 43: 763-769.

Cline M J amd Golde D W (1979). Cellular interactions in haematopoiesis. Nature 277: 177-181.

Colby W W, Chen E Y, Smith D H and Levinson A D (1983). Identification and nucleotide sequence of a human locus homologous to the v-myc oncogene of avian myelocytomamosis virus MC29. Nature 301: 722-725.

Collins S J, Gallo R C and Gallagher R E (1977). Continuous growth and differentiation of human myeloid leukemic cells in suspension culture. Nature 270: 347-349.

Collins S J, Ruscetti F W, Gallager R E and Gallo R C (1978). Terminal differentiation of human promyelocytic leukemia cells induced by dimethylsulphoxide and other polar compounds. Proc. Natl. Acad. Sci. 75: 2458-2462.

Dalton W T, Ahearn M J, McCreadie K B, Freireich E J, Stass E A and Trujillo J M (1988). HL60 cell line was derived from a patient with FAB-M2 and not FAB-M3. Blood 71: 242-250.

Dang C V and Lee W M F (1989). Nuclear and nucleolar targeting sequences of c-erb-A, c-myb, N-myc, p53, hsp70 and HIV tat proteins. J. Bio. Chem. 264: 18019-18023.

Darnell J, Lodish H and Baltimore D (1986). Molecular Cell Biology. Scientific American Books.

Dexter T M, Allen T D and Lajtha L G (1977). Conditions controlling the proliferation of haemopoietic stem cells in vitro. J. Cell Physiol. 91: 335-344.

Dexter T M and Spooncer E (1987). Growth and differentiation of the haemopoietic system. Ann. Rev. Cell Biol. 3: 423-441

DiDomenico B J, Bugaisky G E and Lindquist S (1982). The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. Cell 31: 593-603.

Dony C, Kessel M, and Gruss P (1985). Post-transcriptional control of c-myc and p53 expression during differentiation of the embryonal carcinoma cell line F9. Nature 317: 636-639.

Drummond I A S, McClute S A, Poenie M, Tsien R Y and Steinhardt (1986). Large changes in intracellular pH and calcium observed during heat shock are not responsible for the induction of heat shock proteins in Drosophilis melanogaster. Mol. Cell. Biol. 6: 1767-1775.

Ellis R J and Hemmingsen S M (1989). Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures. TIBS 14: 339-342.

Faassen A E, O'Leary J L, Rodysill K J, Bergh N and Hallgren H M (1989). Diminished heat shock protein synthesis following mitogen stimulation of lymphocytes from aged donors. Exp. Cell Res. 183: 326-334.

Farrelly F W and Finkelstein D B (1984). Complete sequence of the heat shock inducible HSP90 gene of Saccharomyces cerivisiae. J. Biol. Chem. 9: 5745-5751.

Ferris D K, Harel-Bellan A, Morimoto R I, Welch W J and Farrar W L (1988). Mitogen and lymphokine stimulation of heat shock prpteins in T lymphocytes. Proc. Natl. Acad. Sci. 85: 3850-3854.

Fibach E, Peled T, Treves A, Kornberg A and Rachmilewitz E A (1982). Modulation of the maturation of human leukemic promyelocytes (HL60) to granulocytes or macrophages. Leuk. Res. 6: 781-790.

Finlay C A, Hinds P W, Tan T H Eliyahu D, Oren M and Levine A J (1988). Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol. Cell Biol. 8: 531-539.

Fornace A J, Alamo I, Hollander M C and Lamoreaux E (1989a). Induction of heat shock protein transcripts by various stresses in Chinese hamster cells. Exp. Cell Res. 182: 61-74.

Fornace A J, Alamo I, Hollander M C and Lamoreaux E (1989b). Ubiquitin mRNA is a major stress-inducible transcript in mammalian cells. Nucleic Acids Res. 17: 1215-1230.

Fortan L, Cornelissen F, De Neve W, Beeckmans S, Van den Berge D, Storme G and Kanarek L (1989). Not the presence but the synthesis of hsps correlates with thermotolerance in a fibrosarcoma MO_4 cell-line. Anticancer Res. 9: 1207-1212.

Gallagher R E, Collins S J, Trujillo J, McCredie K, Ahearn M, Tsai S, Metzgar R, Aulakh G, Ting R, Ruscetti F and Gallo R (1979). Characterisation of the continuous, differentiating myeloid cell line (HL60) from a patient with acute promyelocytic leukemia. Blood 54: 713-733.

Gesher A, Gibson N W, Hickman J A, Langdon S P, Ross D and Atassi G (1982). N-methylformamide antitumor activity and metabolism in mice. Br. J. Cancer 45: 843-850.

Goldwasser E (1976). Erythropoietin. Blut 33: 135-140.

Gonda D K, Bachmair A, Wunning I, Tobias J W, Lane W S and Varshavsky A (1989). Universality and structure of the N-end rule. J. Biol. Chem. 264: 16700-16712.

Gonzalez M F, Shiraishi K, Hisanaga K, Sagar S M, Mandabach M and Sharp F R (1989). Heat shock proteins as markers of neural injury. Mol. Brain Res. 6: 93-100.

Graf T, Ade N and Beug H (1978). Temperature-sensitive mutant of avian erythroblastosis virus suggests a block of differentiation as a mechanism of leukemogenesis. Nature 275: 496-501.

Graham F L and Whitamore G F (1970). Studies in mouse L-cells on the incorporation of 1-B-D-arabinofuranosylcytosine into DNA and on inhibition of DNA polymerase by 1-B-D-arabinofuranosylcytosine-5'-triphosphate. Cancer Res. 30: 2636-2644.

Gray J W and Coffino P (1979). Cell cycle analysis by flow cytometry. In: Methods in enzymology, Vol LVIII. (Eds. Jakoby W B and Pastan I H), 233-248, Academic Press, London.

Griffen J, Munroe D, Major P and Kufe D (1981). Induction of differentiation of human myeloid cells by inhibition of DNA synthesis. Exp. Haem. 10: 774-781.

Haces A, Breitman T R and Driscoll J S (1987). Chemical differentiating agents. Differentiation of HL60 cells by hexamethylenebis[acetamide] analogues. J. Med. Chem. 30: 405-409.

Hahn G M, Shiu E C, Goldstein L and Lee G C (1985). Mechanistic implications of the induction of thermotolerance in Chinese hamster cells by organic solvents. Cancer Res. 45: 4138-4143.

Hensold J O and Housman D E (1988). Decreased expression of the stress protein hsp70 is an early event in murine erythroleukemic differentiation. Mol. Cell Biol. 8: 2219-2223.

Hensold J O, Hunt C R, Calderwood S K, Housman D E and Kingston R E (1990). DNA binding of heat shock transcription factor to the heat shock element is insufficient for transcriptional activation in murine erythroleukemia cells. Mol. Cell Biol. 10: 1600-1608.

Hickey E, Brandon S E, Smale G, Lloyd D and Weber L A (1989). Sequence and regulation of a gene encoding a human 89-kilodalton heat shock protein. Mol. Cell Biol. 9: 2615-2626.

Hubner K, Isobe M, Croce C M, Golde D W, Kaufman S E and Gasson J C (1985). The human gene encoding GM-CSF is at 5q21-q23, the chromosome region deleyed in the 5q- anomaly. Science 230: 1282-1285.

Hughes L (1987). PhD. thesis.

Hunt C and Morimoto R I (1985). Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. Proc. Natl. Acad. Sci. 82: 6455-6459.

Ireland R C and Berger E M (1982). Synthesis of low molecular weight heat shock peptides stimulated by ecdysterone in a cultuered Drosophilia cell line. Proc. Natl. Acad. Sci. 79: 855-859.

Jones T R, Calvert A H, Jackman A L, Brown S J, Jones M and Harrap K R (1981). A potent antitumor quinazoline inhibitor of thymidylate synthetase: synthesis, biological properties and therapeutic results in mice. Europ. J. Cancer 17: 11-19.

Kaufmann S H (1989). Induction of endonucleolytic cleavage in acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res. 49: 5870-5878.

Kennedy A R and Symons M C R (1987). "Water structure" verses "radical scavenger" theories as explanations for the suppressive effects of DMSO and related compounds on radiation-induced transformation in vitro. Carcinogenesis 8: 683-688.

Kingston R E, Schuetz T J and Larin Z (1987). Heat inducible human factor that binds to a human hsp70 promoter. Mol. Cell Biol. 7: 1530-1534.

Koch G L E, Smith M, Macer D, Webster P and Mortara R (1986). Endoplasmic reticulum contains a common abundant calcium-binding glycoprotein, endoplasmin. J. Cell Sci. 86: 217-232.

Koury M J and Boudurant M C (1990). Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science 248: 378-381.

Laemmli U (1970). Cleavage of structural proteins during assembly of the head of the bacteriophage T4. Nature, 227: 680-685.

Land H, Parada L F and Weinberg R A (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two co-operating oncogenes. Nature 304: 596-598.

Langdon S P and Hickman J A (1987). Correlation between the molecular weight and potency of polar compounds which induce the differentiation of HL60 human promyelocytic leukemia cells. Cancer Res., 47: 140-144.

Langdon S P, Richards F M and Hickman J A (1988). Investigations of the relationship between cell proliferation and differentiation of HL60 cells induced to differentiate by N-methylformamide. Leuk. Res., 12: 211-214.

Larson J S, Schuetz T J and Kingston R E (1988). Activation *in vitro* of sequence-specific DNA binding by a human regulatory factor. Nature 335: 372-375.

Laskey R A and Mills A D (1975). Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by flurography. Eur. J. Biochem. 56: 335-341.

Laster S M, Wood J G and Gooding L R (1988). Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis. J. Immunology 141: 2629-2634.

Lindquist S and Craig E A (1988). The heat shock proteins. Ann. Rev. Genetics 22: 631-677.

Lord B I, Mori K J and Wright E G (1977). A stimulator of stem cell proliferation in regenerating bone marrow. Biomed. Express Paris 27: 223-226.

Lotan R, Francis G E, Freeman C S and Waxman S (1990). Differentiation Therapy (meeting report). Cancer Res. 50: 3453-3464.

Lotem J and Sachs L (1977). Genetic dissection of the control of normal differentiation in myeloid leukemia cells. Proc. Natl. Acad. Sci. USA. 74: 5554-5559.

Lozzio B B, and Lozzio C B (1975). Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 45: 321-334.

Lusher B and Eisenman R N (1988). c-*myc* and c-*myb* protein degradation: effect of metabolic inhibitors and heat shock. Mol. Cell Biol. 8: 2514-2512.

Martin S J, Bradley J G and Cotter T G (1990). HL60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. Cli. Exp. Immunol. 79: 448-453.

McConkey D J, Nicotera P, Hartzell P, Bellmo G, Wyllie A H and Orrenius S (1989). Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca^{2+} concentration. Arch. Biochem. Biophys. 269: 365-370.

Mendel D B and Orti E (1988). Isoform composition and stoichiometry of the 90-kDa heat shock protein associated with glucocorticoid receptors. J. Biol. Chem. 263: 6695-6702.

Metcalf D and Foster R (1967). Bone marrow colony-stimulating activity of serum from mice with viral-induced leukemia. J. Natl. Cancer Inst. 39: 1235-1245.

Metcalf D and Burgess A (1983). Clonal analysis of progenitor cell committment to granulocyte or macrophage production. J. Cell Physiol. 111: 275-283.

Milarski K L and Morimoto R I (1986). Expression of human hsp70 during the synthetic phase of the cell cycle. Proc. Natl. Acad. Sci. 83: 9517-9512.

Milarski K L and Morimoto R I (1989a). Mutational analysis of the human hsp70 protein: distinct domains for nucleolar localisation and adenosine triphosphate binding. J. Cell Biol. 109: 1947-1962.

Milarski K L, Welch W J and Morimoto R I (1989b). Cell-cycle dependent association of hsp70 with specific cellular proteins. J. Cell Biol. 108: 413-423.

Mivechi N F (1989). Heat sensitivity, thermotolerance, and profile of heat shock protein synthesis of human myelogenous leukemias. Cancer Res. 49: 1954-1958.

Mizzen L A, Chang C C, Garrels J I and Welch W L (1989). Identification, characterisation, and purification of two mammalian stress proteins present in mitochondria, grp75, a member of the hsp70 family and hsp58, a homolog of the bacteria groEL protein. J. Biol. Chem. 264: 20664-20675.

Mizzen L A and Welch W J (1988). Characterisation of the thermotolerant cell. J. Cell Biol. 106: 1105-1116.

Moore M A S, Williams N and Metcalf D (1972). Purification and characterisation of the in vitro colony-forming cells in monkey haemopoietic tissue. J. Cell. Physiol. 79: 283-292.

Morimoto R I and Milarski K L (1990). Expression and function of vertebrate hsp70 genes. Stress Proteins in Biology and Medicine. Cold Spring Harbour Press.

Mosser D D, Theodorakis N G and Morimoto (1988). Coordinate changes in heat shock element-binding activity and hsp70 gene transcription rates in human cells. Mol. Cell Biol. 8: 4736-4744.

Munro S and Pelham H R B (1986). An hsp70-like protein in the ER; identity with the 78kD glucose regulated protein and immunoglobulin heavy chain binding protein. Cell 46: 291-300.

Munro S and Pelham H R B (1987). A C-terminal signal prevents secretion of luminal ER proteins. Cell 48: 899-907.

Munroe D, Sugiura M, Griffen J and Kufe D (1984). Effect of ara-A on differentiation and proliferation of HL60 cells. Leuk. Res. 8: 355-361.

Murakami H, Pain D and Blobel G (1988). 70kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. J. Cell Biol. 107: 2051-2057.

Nakayasu M, Terada M, Tamura G and Sugimura T (1980). Induction of differentiation of human and murine myeloid leukemia cells in culture by tunicamycin. Proc. Natl. Acad. Sci. USA, 77: 409-413.

Nakene P K (1968). Simultaneous localisation of multiple tissue antigens using the peroxidase-labelled antibody method: a study on pituitary glands of the rat. J. Histochem. Cytochem. 16: 557-560.

Newburger P E, Baker R D, Hansen S L, Duncan R A and Greenburger J S (1981). Functionally deficient differentiation of HL60 promyelocytic leukemia cells induced by phorbol myristate acetate. Cancer Res. 41: 1861-1865.

Nichola N A, Metcalf D, Matsumoto M and Johnston G R (1979). Separation of functionally distinct human granulocyte-macrophage colony-stimulating factors. Blood 54: 614-627.

Nichola N A, Metcalf D, Matsumoto M and Johnston G R (1983). Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells. J. Biol. Chem. 258: 9017-9023.

Nishida E, Koyasu S, Sakai H and Yahara I (1986). Calmodulin-regulated binding of the 90kD heat shock protein to actin filaments. J. Biol. Chem. 261: 16033-16036.

Norten P M, Isenberg D A and Latchman D S (1989). Elevated levels of the 90kD heat shock protein in a proportion of SLE patients with active disease. J. Autoimmunity 2: 187-195.

O'Garra A, Warren D J, Holman M, Popham A M et al **(1986).** Interleukin 4 (B cell growth factor II/eosinophil differentiation factor) is a mitogen and differentiation factor for preactivated murine B lymphocytes. Proc. Natl. Acad. Sci. USA 83: 5228-5232.

Okazaki T, Mochizuki T, Tashima M, Sawada H and Uchino H (1986). Role of intracellular calcium ion in human promyelocytic leukemia HL60 cell differentiation. Cancer Res. 46: 6059-6063.

Olsson I and Olofsson T (1981). Induction of differentiation in a human promyelocytic leukemic cell line (HL60). Production of granule proteins. Exp. Cell Res. 131: 225-230.

Oppermann H, Levinson W and Bishop J M (1981). A cellular protein that associates with a transforming protein of Rous sarcoma virus is also a heat shock protein. Proc. Natl. Acad. Sci. 78: 1067-1071.

Orrenius S, McConkey D J, Bellomo G and Nicotera P (1989). Role of Ca^{2+} in toxic cell killing. TIPS 10: 281-285.

Pantazis P, Lazarou S and Papadopoulus (1981). Isozymes of lactate dehydrogenase in human leukemic cells treated with inducers of differentiation. J. Cell Biol. 90: 396-401.

Pelham H R B (1982). A regulatory upstream promoter element in the Drosophilia hsp70 heat shock gene. Cell 30: 517-528.

Pelham H R B (1984). HSP70 accelerates the recovery of nucleolar morphology after heat shock. EMBO J. 3: 3095-3100.

Pelham H R B (1986). Speculations on the functions of the major heat shock and glucose regulated proteins. Cell 46: 959-961.

Pelham H R B (1988). Coming in from the cold. Nature 332: 776-777.

Perkins S, Andreotti P, Sinka S, Wu M and Unis A (1984). Human myeloid leukemic cell (HL60) autostimulator: relationship to colony-stimulating factor. Cancer Res. 44: 5160-5175.

Peterson R and Lindquist S (1988). The Drosophilia hsp70 message is rapidly degraded at normal temperatures and stabilised by heat shock. Gene 72: 161-168.

Pfiel W (1987). Is thermally denatured protein unfolded? The example of alpha lactalbumin. Biochim. Biophys. Acta 911: 114-116.

Pierce G B (1970). Differentiation of normal and malignant cells. Fed. Pro. 29: 1248-1252.

Pluznick D H and Sachs L (1986). The cloning of normal mast cells in tissue culture. J. Cell. Comp. Physiol. 66: 319-324.

Potten C S (1987). Perspectives on Mammalian Cell Death. Oxford University Press. Oxford.

Potter V R (1969). Recent trends in cancer biochemistry: The importance of studies on fetal tissue. Can. Cancer Conf. 8: 9-15.

Pratt W B and Ruddon R W (1979). The anticancer drugs. Oxford University Press.

Pullen G and Hoskin C (1985). Differentiated HL60 promyelocytic leukemia cells have a deficient myeloperoxidase/halide killing system. Clin. Exp. Immunol. 62: 304-309.

Rebbe N F, Hickman W S, Ley T J, Stafford D W and Hickman S (1989). Nucleotide sequence and regulation of a human 90-kDa heat shock protein gene. J. Biol. Chem. 264: 15006-15011.

Resendez E, Wooden S K, and Lee A S (1988). Identification of highly conserved regulatory domains and protein-binding sites in the promoters of the rat and human genes encoding the stress-inducible 78-kilodalton glucose-regulated protein. Mol. Cell Biol. 8: 4579-4584.

Reuben R C, Khanna P L, Gazitt Y, Breslow R, Rifkind R A and Marks P A (1987). Inducers of erythroleukemic differentiation. Relationship of structure to activity among polar-planar compounds. J. Biol. Chem. 253: 4214-4218.

Riabowol K T, Mizzen L A and Welch W J (1988). Heat shock is lethal to fibroblasts microinjected with antibodies against hsp70. Science 242: 433-436.

Richards F M (1987). PhD. thesis.

Richards F M, Watson A and Hickman J A (1988). Investigation of the effects of heat shock and agents which induce heat shock response on induction of differentiation of HL60 cells. Cancer Res. 48: 6715-6720.

Ritossa F M (1962). A new puffing pattern induced by heat shock and DNP in Drosophilia. Experentia 18: 571-580.

Rivett A J (1986). Regulation of intracellular protein turnover: covalent modification as a mechanism of marking proteins for degradation. Current Topics in Cellular Regulation, 28: 291-337. Academic Press Inc., New York.

Rose D W, Welch W J, Kramer G and Hardesty B (1989). Possible involvement of the 90kD heat shock protein in the regulation of protein synthesis. J. Biol. Chem. 264: 6239-6244.

Rovera G, Santoli D and Damsky G (1979). Human promyelocytic leukemia cells differentiate into macrophage-like cells when treated with phorbol diesters. Proc. Natl. Acad. Sci. USA, 76: 2729-2734.

Rowley P T, Ohlsson-Wilhelm B M, Farley B A and LaBella S (1981). Inducers of erythroid differentiation in K562 human leukemia cells. Exp. Hematol., 9: 32-37.

Ruddon R W (1987). Cancer Biology. Oxford University Press.

Sachs L (1986). Growth, differentiation and reversal of malignancy. Scientific American 246: 30-37.

Sanchez Y and Lindquist S L (1990). HSP104 required for induced thermotolerance. Science 248: 1112-1115.

Sanger J W, Sanger J M, Kreis T E and Jockusch B M (1980). Reversible translocation of cytoplasmic actin into the nucleus caused by dimethyl sulfoxide. Proc. Natl. Acad. Sci. USA. 77: 5268-5272.

Santoro M G, Garaci E and Amici C (1989). Prostaglandins with antiproliferative activity induce the synthesis of a heat shock protein in human cells. Proc. Natl. Acad. Sci. USA. 86: 8407-8411.

Sartorelli A C (1985). Malignant cell differentiation as a potential therapeutic approach. Br. J. Cancer 52: 293-302.

Savill J S, Wyllie A H, Henson J E, Walport M J, Henson P M and Haslett C (1989). Macrophage phagocytosis of aging neutrophils in inflammation. J. Clin. Invest. 83: 865-875.

Schaefer E L, Morimoto R I, Theodorakis N G and Seidenfeld J (1988). Chemical specificity for induction of stress response genes by DNA-damaging drugs in human adenocarcinoma cells. Carcinogenesis 9: 1733-1738.

Schofield R (1979). The pluripotent stem cell. Clin. Haematol. 8: 21-237.

Schwartz E L, and Sartorelli A C (1982). Structure-activity relationships for the induction of differentiation of HL60 human acute promyelocytic leukemia cells by anthracyclines. Cancer Res. 42: 2651-2657.

Searle J, Kerr J F R and Bishop C J (1982). Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathol. Annu. 17: 229-259.

Shaala A, Dhaliwal H, Bishop S and Ling H (1979). Ingestion of dye-oponized yeasts are a simple way of detecting phagocytes in lymphocyte preparations. Cytophilic binding of immunoglobulins by ingesting cells. J. Immunol. Meth., 27: 175-187.

Singh S K and Yu J (1984). Accumulation of a heat shock-like protein during differentiation of human erythroid cell line K562. Nature 309: 631-633.

Sorger R K, Lewis M J and Pelham H R B (1987). Heat shock is regulated differently in yeast and and HeLa cells. Nature 329: 81-84.

Stevenson M A, Calderwood S K and Hahn G M (1986). Rapid increases in inositol triphosphate and intracellular Ca^{2+} after heat shock. Biochem. Biophys. Res. Comm. 137: 826-833.

Stoeckle M Y, Sugano S S, Hampe A, Vashistha A, Pellman D and Hanafusa H (1988). 78-kilodalton glucose-regulated protein is induced in Rous Sarcoma virus-transformed cells is independently of glucose deprivation. Mol. Cell Biol. 8: 2675-2680.

Supino R, Gibelli N and Zunino F (1986). Induction of differentiation of Friend murine erythroleukemia cells by poly-L-lysine and daunorubicin-poly-L-lysine conjugate. J.N.C.I. 77: 453-457.

Tabilio A, Pelicci P G, Vinci G, Mannoni P, Civin C I, Vainchenker W, Testa U, Lipinski M, Rochant H and Breton-Gorius J (1983). Myeloid and megakaryocytic properties of K-562 cell lines. Cancer Res. 43: 4569-4574.

Tanaka H, Abe E, Miyaura C, Shiina Y and Suda T (1983). 1-alpha,25-dihydroxyvitamin D_3 induces differentiation of human promyelocytic leukemia cells (HL60) into monocyte-macrophages, but not into granulocytes. Biochem. Biophys. Res. Comm. 117: 86-92.

Tarella C, Ferrero D, Gallo E, Pagliardi G L and Ruscetti F W (1982). Induction of differentiation of HL60 cells by dimethyl sulphoxide: evidence for a stochastic model not linked to the cell division cycle. Cancer Res. 42: 445-449.

Theodorakis N G, Zand D J, Kotzbauer P T, Williams G T and Morimoto R I (1989). Haemin-induced transcriptional activation of the hsp70 gene during erythroid maturation in K562 cells is due to a heat shock factor-mediated stress response. Mol. Cell Biol. 9: 3166-3173.

Till J E and McCulloch E A (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14: 215-222.

Ting J and Lee S A (1988). Human gene encoding the 78 000 dalton glucose-regulated protein and its pseudogene: structure, conservation, and regulation. DNA 7: 275-286.

Towbin H, Stachin T and Gorden J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA., 76: 4350-4354.

Tritton T R and Yee G (1982). The anticancer agent Adriamycin can be actively cytotoxic without entering cells. Science 217: 248-250.

Tsien R Y, Pozzan T and Rink T J (1982). Calcuim homeostasis in intact lymphocytes: cytoplasmic free calcuim monitered with a new, intracellularly trapped flurescent indicator. J. Cell Biol. 94: 325-334.

Velazquez J M, Sonodo S, Bugaisky G and Lindquist S (1983). Is the major Drosophilia heat shock protein present in cells that have not been heat shocked? J. Cell Biol. 96: 286-290.

Watowich S S and Morimoto R I (1988). Complex regulation of heat shock- and glucose- responsive genes in human cells. Mol. Cell. Biol. 8: 393-405.

Walker A I, Hunt T, Jackson R J and Anderson C W (1985). Double-stranded DNA induces the phosphorylation of several heat shock proteins including thr 90 000 mol wt heat-shock protein in animal cell extracts. EMBO J. 4: 139-145.

Werb (1984). Phagocytic cells: Chemotaxis and effector function of macrophages and granulocytes. In Basic and Clinical Immunology. Ed. Sites D P, Stobo J D, Fundenburg H H, Wells J V. 109-124.

Welch W J and Feramisco J R (1985). Disruption of the three cytoskeletal networks in mammalian cells does not affect translocation, translation or protein translocation changes induced by heat shock. Mol. Cell. Biol. 5: 1571-1581.

Welch W J and Suhan J P (1986). Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. J. Cell Biol. 103: 2035-2052.

Williams G T, Smith C A, Spooncer E, Dexter T M and Taylor D R (1990). Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature 343: 76-79.

Wolf D and Rotter V (1985). Major deletions in the gene encoding p53 tumor antigen cause lack of p53 expression in HL60 cells. Proc. Natl. Acad. Sci. USA 82: 790-794.

Wolman S R, Lanfrancone L, Dalla-Favera R, Ripley S and Henderson A S (1985). Oncogene mobility in a human leukemia cell line, HL60. Cancer Genet. Cytogenet. 17: 133-135.

Wu B J and Morimoto R I (1985). Transcription of the human hsp70 gene is induced by serum stimulation. Proc. Natl. Acad. Sci. 82: 6070-6074.

Wu B J and Morimoto R I (1986). Human hsp70 promoter contains at least two distinct regulatory domains. Proc. Natl. Acad. Sci. USA 83: 629-633.

Wyllie A H, Kerr J F R and Currie A R (1980). Cell death: the significance of apoptosis. Inter. Rev. Cytol. 68: 251-307.

Wyllie A H and Morris R G (1982). Hormone-induced cell death. Purification and properties of thymocytes undergoing apoptosis after glucocorticoid treatment. American J. Pathol. 109: 78-87.

Wyllie A H, Morris R G, Smith A L and Dunlop D (1984). Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J. Pathol. 142: 67-77.

Yam L, Li C and Crosby W (1971). Cytochemical identification of monocytes and granulocytes. Am. J. Clin. Pathol., 55: 283-290.

Yen A and Albright K L (1984). Evidence for cell cycle phase-specific initiation of a program of HL60 cell myeloid differentiation mediated by inducer uptake. Cancer Res. 44: 2511-2515.

Yen A, Freeman L, Powers V, Van Sant R and Fishbaugh J (1986). Cell cycle dependence of calmodulin levels during HL60 proliferation and myeloid differentiation. No changes during precommitment. Exp. Cell Res. 165: 139-151.

Yost H J and Lindquist S (1988). Translation of unspliced transcripts after heat shock. Science 242: 1544-1547.

Yufu Y, Nishimura J, Takahira H, Ideguchi H and Nawata H (1989). Down-regulation of a Mr 90 000 heat shock cognate protein during granulocytic differentiation in HL60 human leukemia cells. Cancer Res. 49: 2405-2408.