MOLECULAR MECHANISMS INVOLVED IN THE CONTROL OF NEUTROPHIL APOPTOSIS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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I declare that this thesis entitled:

'Molecular mechanisms involved in the control of neutrophil apoptosis'

is entirely my own work, except where collaboration is indicated in the text.

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ABSTRACT

Neutrophils are important constituents of the immune system and their primary function is to seek out and destroy invading microbial pathogens. The lifespan of the mature neutrophil is very short (less than one day) as they constitutively undergo apoptosis. There is a constant production and release of vast numbers of neutrophils from the bone marrow into the bloodstream to maintain a steady number of neutrophils in the circulation.

The rapid entry into apoptosis experienced by the neutrophil can be delayed by treatment with certain cytokines (i.e. GM-CSF, IL-1) *in vitro*, and similarly during inflammation *in vivo*, such that the lifespan is extended from hours to several days. The mechanisms by which the neutrophil apoptosis can be delayed are largely undefined. Understanding the control of neutrophil apoptosis may enable manipulation of neutrophil survival, which would be of potential benefit in many conditions. Extending neutrophil lifespan by delaying apoptosis may benefit immuno-compromised patients (i.e. during cytotoxic treatment for cancer) by boosting their ability to fight infections. On the other hand, decreasing neutrophil lifespan by accelerating apoptosis could benefit patients with inflammatory diseases (such as rheumatoid arthritis) by deleting these potentially harmful cells.

In this thesis I have investigated the mechanisms by which neutrophil apoptosis can be delayed, and shown that many agents which delay neutrophil apoptosis enhance gene expression, whilst inhibition of protein synthesis accelerates entry into apoptosis. These observations suggest the neutrophil requires the constant synthesis of one or more proteins to delay entry into apoptosis. The fatty acid butyrate is also shown to delay entry into apoptosis in these cells, by increasing protein synthesis. The action of butyrate is shown to be associated with inhibition of histone deacetylases, suggesting histone hyperacetylation is able to induce expression of the proteins required for neutrophil survival. I also show GM-CSF alters the rate of acetyl-group turnover on neutrophil histones.

The maintenance of surface expression of CD16 (FcγRIIIb) is associated with delayed apoptosis, and is shown here to be achieved by maintaining the ability to mobilise internal stores of preformed CD16 to the cell surface. This is shown for both GM-CSF which primes the neutrophil and activates shedding of CD16 from the cell surface, and for butyrate, which neither primes nor activates CD16 shedding.

The role of superantigens in controlling neutrophil apoptosis is also examined. I have found that although there is no obvious explanation for a direct action of superantigens on neutrophils, they do affect some functions. However, they have no ability to directly alter the rate of neutrophil apoptosis. Apoptosis can be delayed by superantigens *in vitro* via stimulation of cytokine secretion from contaminating mononuclear cells, even when the contamination is less than 3 %, demonstrating the potency of these cytokines (such as IFN-γ and IL-1β) in delaying neutrophil apoptosis.

The Bcl-2 family of proteins are intimately associated with the control of apoptosis in many cell types and organisms, and I have shown that neutrophils constitutively express the pro-apoptotic protein Bax, but not the anti-apoptotic proteins Bcl-2 and Bcl-X. However, I have demonstrated the constitutive expression of the anti-apoptotic protein Mcl-1 in bloodstream neutrophils. Furthermore, I have shown an up-regulation of Mcl-1 expression by all agents tested which delay neutrophil apoptosis. I have also demonstrated a correlation between neutrophil survival and Mcl-1 protein levels, suggesting that Mcl-1 protein levels may be the key determinant for neutrophil survival.

ABBREVIATIONS

ActD	Actinomycin D
ATP	Adenosine triphosphate
BH	Bcl-2 homology
bp	base pairs
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine
C5a	Complement fragment 5a
$[Ca^{2+}]_i$	Intracellular free calcium
cAMP	cyclic adenosine monophosphate
CGD	Chronic granulomatous disease
CD	Cluster of differentiation
CHX	Cycloheximide
cIAP	cellular inhibitior of apoptosis protein
CR	Complement receptor
CTP	Cytosine triphosphate
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra-acetic acid disodium salt
EGTA	Ethyleneglycol-*bis*-(β-aminoethyl)-N,N,N',N',-tetraacetic acid
FcγR	Fc receptor type γ
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-L-methionyl-L-leucyl-L-phenylalanine
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte Macrophage colony-stimulating factor
GSH	Glutathione
GTP	Guanidine triphosphate
Hepes	N-2-Hydroxyethylpiperazine-N'2-ethanesulphonic acid
HMBA	Hexamethylene-*bis*-acetamide
hnRNP	heteronuclear ribonuclear protein
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IL	Interleukin
IP_3	Inositol 1,4,5-triposphate
LFA-1	Lymphocyte function-associated molecule

LPS	Lipopolysaccharide
LTB_4	Leukotriene B_4
Mac-1	Membrane attack complex (CD11b/CD18 complex)
M-CSF	Macrophage colony-stimulating factor
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NIM	Neutrophil isolation medium
NK	Natural Killer (cell)
NO	Nitric oxide
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PAO	Phenyl arsine oxide
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PI	Propidium iodide
PKC	Protein kinase C
PMA	Phorbol-12-myristate acetate
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
SD	Standard deviation
SEA, SEB	*Staphylococcal* enterotoxin A or B
S.F.	Synovial fluid
SDS	Sodium dodecyl sulphate
SSC	Sodium chloride/ sodium citrate buffer
TCA	Trichloroacetic acid
TGF-β	Transforming growth factor-β
TNF	Tumour necrosis factor
Tris	Tris(hydroxymethyl)methylamine
TSA	Trichostatin A
TSST-1	Toxic Shock Syndrome Toxin-1
UV	Ultraviolet

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CHAPTER 1 GENERAL INTRODUCTION

1.1 Overview

The neutrophils are the first cells of the immune system recruited in response to bacterial and fungal infections. It is therefore essential to the well-being of the host that there is a ready supply of these dispensable cells to deal with infections if and when they arise. Neutrophils are well adapted as a front line of defence, as they are normally abundant in the circulation, are able to quickly infiltrate an infected site, and are well armed with a variety of anti-pathogenic systems. They are also pre-programmed to make the ultimate sacrifice: to commit suicide if they are not needed to fight infection, or if they have completed their role in an immune response to allow the resolution of inflammation. This predisposition to commit suicide can, however, give rise to problems: if production of neutrophils is interrupted then the number of circulatory neutrophils falls quickly, leaving the host susceptible to infections. Conversely, an inflammatory response may be needlessly prolonged by extended neutrophil survival, leading to tissue damage.

1.2 Neutrophil Physiology

The neutrophil is the must abundant of all white blood cells constituting 40 - 65% of the total white blood cell pool. It is one of three types of polymorphonuclear leukocytes, with neutrophil preparations normally containing eosinophils (1-7%) and basophils (<1%). The number of neutrophils in the circulation is normally in the range of 3-5 x 10^6/ml blood, but this can rise dramatically in cases of infection (Edwards, 1994). Polymorphonuclear leukocytes have a distinctive multilobed nucleus, a very granular cytoplasm, and are spherical in the circulation with a diameter of about 10 μm. Electron microscopy shows little or no endoplasmic reticulum or golgi apparatus (Bainton *et al*, 1971), which led to the suggestion that neutrophils were terminally

differentiated, with little or no need for protein synthesis (Cline, 1966). The neutrophil also appears devoid of mitochondria, and relies on glycolysis for the production of energy. This O_2-independent mechanism for energy production allows the neutrophil to function in the low oxygen tensions that often exist in inflammatory sites (Edwards, 1994).

The lifespan of the mature neutrophil is quite short, estimated to be between 8-20 h before constitutively undergoing programmed cell death or apoptosis. This lifespan is longer in the tissues, but is difficult to determine accurately. There is obviously a vast number of neutrophils produced by the bone marrow to maintain the circulating neutrophil pool, with turnover rates for human neutrophils estimated to range from 5 x 10^8 to 3.4 x 10^9 /kg/day (Nelson, 1994).

1.3 Phagocytopoiesis

The mature circulating neutrophil is the result of a long developmental process in the bone marrow, termed phagocytopoiesis (for review see Souid, 1996), which is a particular developmental line in the whole process of blood cell development termed haematopoiesis. All blood cells develop from pluripotent stem cells, with neutrophils developing from the myeloid stem cell which can produce erythroid, megakaryocyte, monocyte and neutrophil lineages. This whole haematopoietic system is controlled by cytokines (such as IL-1, IL-3, IL-6, G-CSF, GM-CSF, and M-CSF), with different cytokine combinations promoting the development of each type of blood cell.

The neutrophil develops in the bone marrow from myeloid stem cells, which differentiate into type I and then type II myeloblasts, promyelocytes, and neutrophil myelocytes which are all capable of mitosis. The neutrophil metamyelocyte is the first stage of the non-mitotic differentiation into mature neutrophils, which then becomes a band neutrophil, and finally a mature neutrophil. The whole process from myeloid

stem cell to circulating neutrophil takes around 14 days, with the mitotic development taking ~6 days, and the non-mitotic maturation taking 8 days. There is thought to be a 9-10 day reserve of mature and maturing neutrophils in the bone marrow, that is estimated to contain at least 13 times the number of neutrophils in the blood (Boggs, 1967).

Having been released to the bloodstream the mature neutrophil will live for up to 20 h before becoming apoptotic and removed by phagocytic cells, unless recruited to the tissues where the lifespan may be extended by a few days.

1.4 Neutrophil Host Defence

The primary role of the neutrophil is to destroy invading micro-organisms before they have the chance to multiply and spread throughout the body or infect tissues. To be able to do this, the neutrophil must quickly gain access to the site of the infection, and then proceed to destroy the invading pathogen.

1.4.1 Neutrophil recruitment

The first process necessary for efficient microbial killing is the recruitment of neutrophils to the site of infection. This involves the neutrophil migrating toward the site by following a path either of chemoattractants released from the host cells at the site of infection, or bacterial breakdown products (such as fMLP) for which the neutrophil possesses cell surface receptors. In order to migrate towards these attractants, the neutrophil must first attach to the capillary walls near the infection site, a process called margination. This attachment (reviewed by Butcher, 1991; Hogg, 1992) is mediated firstly by the translocation of selectin receptors to the surface of the endothelial cells as a result of stimulation by agents, such as thrombin or histamine. L-selectin on the neutrophil cell surface is expressed constitutively and mediates a loose

interaction between venule wall and neutrophil within minutes of the tissue injury. The neutrophil then 'rolls' along the venule and will only continue to do so if the local environment contains other factors indicative of infection. For firmer attachment neutrophil integrin receptors (LFA-1, Mac-1, gp150,95) are up-regulated which mediate a stronger, sustainable attachment to the endothelial cells via binding to the appropriate endothelial counterpart (e.g. ICAM-1) (see Springer, 1990 for review). The neutrophils then squeeze through the endothelial walls by a process called diapedesis, and then follow the concentration gradient of chemoattractants such as fMLP, C5a, platelet-activating factor, leukotriene B_4 (Baggiolini *et al*, 1991) and IL-8 (Baggiolini *et al.* 1989). Once a critical number of receptors are occupied the cell moves directionally, towards the highest concentration of the chemoattractants. This allows the neutrophils to follow the trail of attractants to the appropriate site of tissue inflammation, where the neutrophil begins to attack the invading organism.

1.4.2 Phagocytosis and killing

Upon arrival at the inflammatory site the neutrophils begin the process of phagocytosing and killing the bacteria. Bacteria may be recognised by neutrophil surface receptors to microbial sugar molecules, but phagocytosis of many bacteria is more efficient if they are coated with serum opsonins. The opsonised bacteria coated with antibody or complement fragments bind to the Fc receptors (FcγRI, FcγRII and FcγRIIIb) and complement receptors (CR1 and CR3), respectively (for review see Cohen, 1994). Having phagocytosed the bacteria, the phagosome fuses with granules to form a phagolysosome in which the bacteria is killed (for review see Smith, 1994). Killing of the bacteria involves both the release of anti-bacterial and anti-fungal proteins from neutrophil granules, and also the production of reactive oxygen intermediates. These intermediates are produced by the NADPH complex which is assembled and activated on the phagolysosome, and they are extremely toxic. The killing is usually contained within the phagolysosome, thereby preventing the leakage

of these highly toxic molecules into the surrounding tissues. However, under certain inflammatory conditions, such as those found in some autoimmune diseases, the pathogenic killing mechanism can be directed against the host tissue. In diseases such as glomerulonephritis, antibodies and immune complexes are deposited onto host tissues. The neutrophil is unable to phagocytose such tissues and will undergo a process termed frustrated phagocytosis (Edwards & Hallet, 1997). The granule enzymes and reactive oxygen intermediates are then released outside the neutrophil and are just as effective at killing host cells as they are at destroying pathogens (Edwards & Hallet, 1997). There are many diseases in which symptoms and tissue injury may be partly mediated by neutrophils and the inappropriate release of toxic molecules, including rheumatoid arthritis, gout, inflammatory bowel disease and asthma (see Malech and Gallin, 1987 for review).

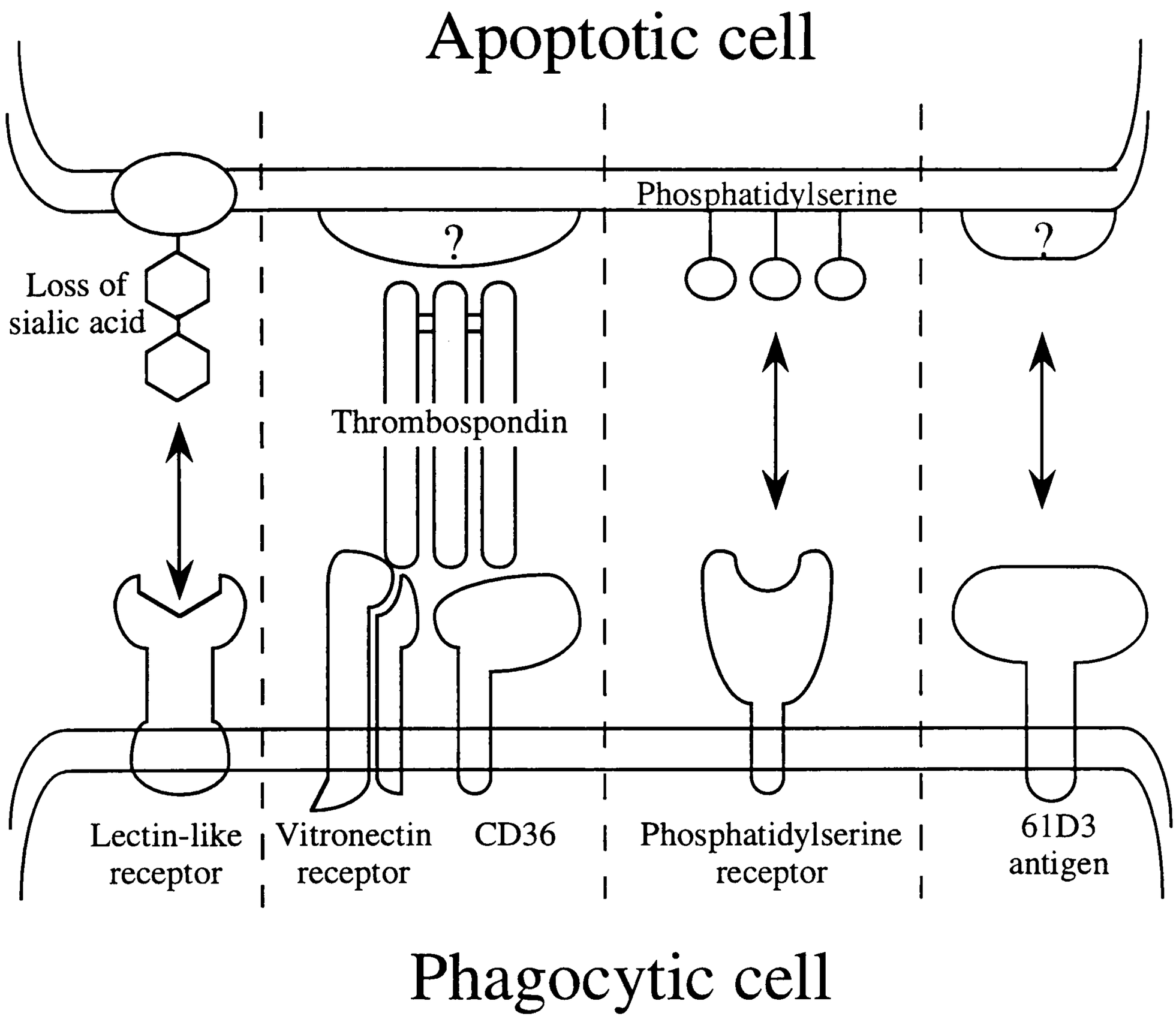
1.4.3 Resolution of inflammation

After an infection has been successfully cleared from a site of inflammation, the huge influx of neutrophils must be stopped and then the recruited neutrophils dispersed or removed to allow the inflammation to be resolved. The cessation of neutrophil influx to an inflamed site occurs remarkably early in the course of inflammation. In an inflammatory model in which acute inflammation develops over 48 h and resolves after 7-9 days, the influx of neutrophils occurred for only 24 h (Haslett, 1992). This early termination of neutrophil recruitment seems likely to be the first step in the resolution of inflammation, but the mechanism(s) of this termination remains unknown. Termination of influx may involve chemotactic factor inhibitors, neutrophil desensitisation, recruited neutrophils preventing further recruitment, decrease in chemotactic factors, or blockage of neutrophil migration by surrounding tissue (Haslett 1992).

The neutrophils at the site of inflammation will commit suicide after completing their role in the immune response. This cellular suicide is termed apoptosis (Kerr *et al*, 1972), and it has recently been shown that bacterial ingestion, tumour necrosis factor-α (TNF-α) and heat (all of which may be experienced by neutrophils at an inflammatory site) will all trigger apoptosis in these activated neutrophils (Watson *et al*, 1996). These apoptotic neutrophils can then be removed from the site of inflammation by surrounding phagocytes rather than becoming necrotic. Neutrophil necrosis would cause further inflammation as cytotoxic molecules usually safely contained within the neutrophil would be released as membrane integrity is lost. The phagocytosis of apoptotic neutrophils occurs before the loss of membrane integrity, thereby preventing the prolongation of inflammation.

Apoptotic cells can be removed by macrophages (Newman *et al*, 1982) or other neighbouring cells such as liver endothelial cells (Dini *et al*, 1995) or fibroblasts (Hall *et al*, 1994). This recognition and phagocytosis of apoptotic neutrophils does not involve antibody or complement and occurs via a different mechanism to that involved in the phagocytosis of foreign material, thereby avoiding further tissue damage (Meagher *et al*, 1992). There has been extensive research into the recognition system involved in this process, with the identification of a number of receptors on phagocytic cells and cell surface changes in apoptotic cells (reviewed by Hart *et al*, 1996; Haslett *et al*, 1994; Savill *et al*, 1993). The recognition systems known to date are shown in Figure 1.1. The loss of sialic acid from surface carbohydrates on the apoptotic cell allows recognition via lectin-like receptors on the phagocytic cell. Thrombospondin can act as a bridge between an as yet unidentified site on the apoptotic cell and the vitronectin receptor and CD36 (a macrophage surface receptor) on the phagocyte. Exposure of phosphatidylserine on the apoptotic cell is recognized by a phosphatidylserine receptor which may be a class A scavenger receptor such as CD68, but it appears other receptors are also involved. Finally, the antigen to mAb 61D3

Figure 1.1 Recognition of apoptotic cells for phagocytosis



The recognition of apoptotic cells by a neighbouring phagocyte may be mediated by a number of mechanisms. The mechanisms known to date are shown in this figure, which is adapted from Savill *et al*, 1993. For details and references see main text.

(Flora and Gregory, 1994) which is an uncharacterised protein found in mononuclear phagocytes, is involved in recognition of apoptotic cells.

Without the apoptosis of inflammatory neutrophils, it seems likely that resolution of inflammation would be severely delayed, and chronic inflammation and permanent tissue damage and scarring may result. Indeed, resolution of inflammation is enhanced by promoting neutrophil apoptosis *in vivo* (Cox, 1996).

1.5 Apoptosis

Cell death can occur in two distinct manners, necrosis or apoptosis. Necrosis is the term used to describe the death of a cell as a result of severe trauma in which the trauma is rapidly followed by loss of membrane integrity. Apoptosis is a more controlled form of cell death in which the dying cell executes a process whereby key cellular components are disabled and then marks itself for phagocytosis by neighbouring cells with no loss of membrane integrity. This form of cell death was termed apoptosis (Kerr *et al*, 1972) from a Greek term referring to the falling of leaves from trees, or petals from flowers. The spontaneous loss of cells from animal cell populations had been known for many years before the work of Kerr and colleagues, but was not considered a form of cell death separate to necrosis. It was whilst studying the morphology of these spontaneously dying cells that it became apparent that this was a type of cell death distinct from necrosis.

Apoptosis is a descriptive term given to cells exhibiting a distinct set of morphological features, and includes programmed cell death (PCD) and specialized cell death (Fesus *et al* 1996). PCD is the elimination of specific cells predictable in space and time during the development of tissues and organs; specialised cell death occurs in specific tissues where the death program is suspended allowing the completion of a unique function such as occurs in red blood cells. PCD and specialized cell death are therefore

both death via apoptosis, but apoptosis may also be triggered by non-physiologic agents.

1.5.1 Occurrence

Apoptosis is essential during the development and function of multicellular organisms (Umansky, 1996). Tissue development requires the elimination of specific cells such as excess neurons in the developing nervous system (Raff *et al*, 1993), and in many other tissues (Bright & Khar, 1994). The predictability of apoptosis in PCD is clearly illustrated in the normal development of the nematode *Caenorhabditis elegans* (Hengartner & Horvitz, 1994a) in which there is always elimination of the same 131 cells. The normal tissue turnover in adult somatic cells is also dependent on apoptosis (Carson & Ribeiro, 1993).

Many processes in the immune system require apoptosis, such as the development of T lymphocytes in the thymus where many autoreactive and non-reactive T cells are produced, and then deleted via apoptosis such that only beneficial T lymphocytes reach maturity (Williams, 1994). A similar removal of B lymphocytes is also likely (Tsubata *et al*, 1994), though the evidence for this phenomenon is not as strong as that for T lymphocytes. The death of tumour cells induced by Tumour Necrosis Factor (TNF) is via apoptosis (Laster *et al*, 1988). T lymphocyte mediated cytotoxicity, natural killer cell mediated cytotoxicity and antibody-dependent cellular cytotoxicity all induce apoptosis in the target cell (Williams, 1994). As discussed earlier, apoptosis is also essential for the resolution of inflammation.

The withdrawal of growth factors from many growth factor dependent cell types *in vitro* leads to apoptotic death (Schwartzman & Cidlowski, 1993). Injury to cells, not so severe as to cause necrosis but leaving the cell beyond repair, also results in death via apoptosis to protect the surrounding tissues. The death induced by many

chemotherapeutic chemicals and irradiation is also via apoptosis (Hannun, 1997). It is clear that apoptosis is an essential process in the life of a multicellular organism, and is seen in the death of cells in many tissues and under a wide variety of circumstances.

1.5.2 Cellular changes characteristic of apoptosis

The morphological changes characteristic of apoptosis, as first described by Kerr and colleagues, are compaction of chromatin against the nuclear membrane, cell shrinkage, detachment from surrounding cells, and nuclear and cytoplasmic budding to form membrane bound apoptotic bodies which are then rapidly phagocytosed. There are numerous recent reviews on this subject (e.g. Cummings *et al*, 1997) which discuss the morphological changes associated with apoptosis detected using light microscopy and electron microscopy. The first observable event is the condensation of chromatin into uniformly dense masses against the nuclear envelope, which occurs at the same time as detachment of the cell from its surrounding tissue. There is also a loss of specialized surface structures, decrease in cell volume and increase in cell density, compaction of organelles, and often convolution of the nuclear and cell outline. Nuclear structure may change further into several discrete parts, while cytoplasmic changes may include clustering of ribosomes and cytoskeletal filaments, and the production of vacuoles. The surface of the cell will then form many blebs and protuberances which seal themselves off from the cell to form apoptotic bodies.

In 1980, Wyllie was the first to show that there is fragmentation of DNA into fragments with sizes that are multiples of ~200 bp, as found when chromatin is cleaved between nucleosomes (Wyllie, 1980). This has been considered the biochemical hallmark of apoptosis, with electrophoresis of DNA from apoptotic cells giving a characteristic ladder. However, this internucleosomal chromatin cleavage is not found in all cells undergoing apoptosis (Ueda & Shah, 1994). There is an initial cleavage of chromatin into 300 kb and 50 kb fragments (Brown *et al*, 1993;

Oberhammer *et al*, 1993b) which appears to be universal in apoptotic cells. Chromatin condensation is not dependent on the cleavage by endonucleases (Oberhammer *et al* , 1993a), and follows a separate metabolic pathway (Sun *et al*, 1994). Therefore, internucleosomal chromatin fragmentation is neither universal nor necessary for the completion of the apoptotic program.

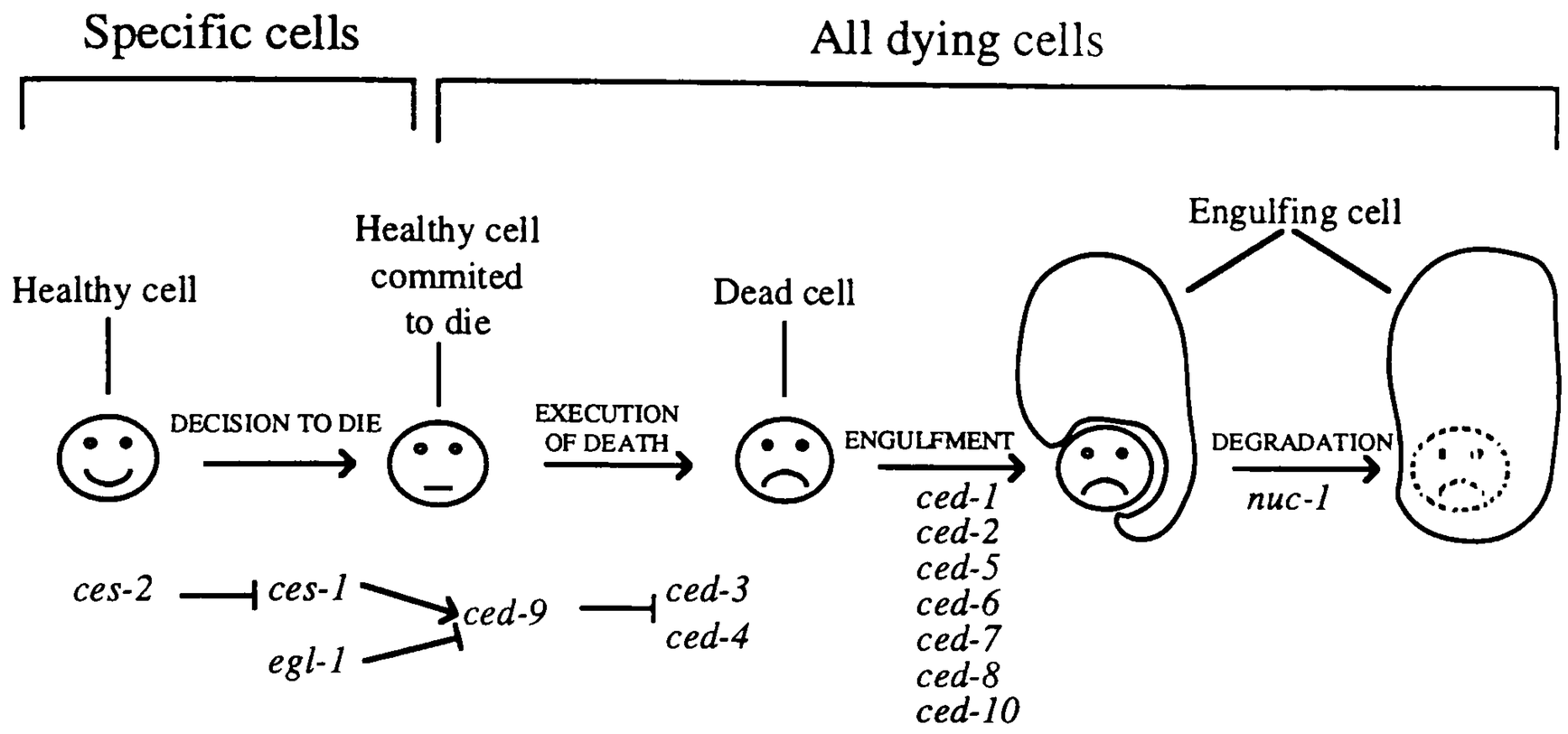
1.5.3 Control of apoptosis

Too little or too much apoptosis could have catastrophic effects on the organism as a whole, so the process of apoptosis must be well controlled. Only unwanted cells should be destroyed by apoptosis and once targeted as a cell to be deleted, the execution of the apoptotic program should be flawless, to ensure targeted cells are indeed removed. The mechanisms in place to control apoptosis are only now being uncovered, and are proving to be quite an elaborate interplay of pro-apoptotic and anti-apoptotic factors. The organism which is perhaps best understood with regard to the control and execution of apoptosis is *C. elegans* (reviewed by Hengartner & Horvitz, 1994a). The developmental progression of each cell in *C. elegans* is known (Sulsten & Horvitz 1977), including the identity of each cell that is programmed to die via apoptosis during development.

1.5.3.1 Control of apoptosis in *C. elegans*

This relatively simple organism provides an excellent model to delineate the genetics of apoptosis. Figure 1.2 summarises the roles of the fourteen genes that are known to be involved in the process of programmed cell death. These genes operate to control the overall process of apoptosis, ranging from selection of cells to be deleted, through activation of the apoptotic program, and absorption of the apoptotic cell by neighbouring tissues. The genes *ces-1* and *ces-2* (cell death specification)(Sulston *et al*, 1983) and *egl-1* (egg laying)(Desari & Horvitz, 1989) control the designation of

Figure 1.2 Genes involved in apoptosis in *C. elegans*



ces-1 allows specific cells to die.
ces-2 prevents specific cell death.
egl-1 allows death in the male of cells needed for egg laying.

ced-9 sufficient and necessary to protect from cell death.
ced-3, *ced-4* } necessary for cell death.

ced-1,2,5,6,7,8,10 involved in engulfment.

nuc-1 encodes a nuclease needed for DNA degredation of the dead cell

⊣ indicates positive regulation, ⟶ indicates negative regulation.
See main text for details. Adapted from Hengartner & Horvitz (1994).

specific cells for apoptosis. The remaining eleven genes are involved in the death of all cells in *C. elegans* which die via apoptosis. *nuc-1* encodes for a nuclease, without which the DNA of the engulfed cell is left intact. There are seven genes essential to the recognition and engulfment of apoptotic cells which are normally engulfed within hours of induction of the apoptotic program. Loss of function of one of these genes leaves apoptotic cells unengulfed even after the cell has finally died. Finally, there are three genes (*ced-3*, *ced-4* and *ced-9*) which are needed for the control and execution of the death process itself. *ced-9* protects cells from apoptosis; a gain of function mutation or overexpression of the *ced-9* gene prevents the apoptosis of cells normally expected to die (Hengartner *et al*, 1992; Hengartner & Horvitz, 1994b). Conversely, inactivation of *ced-9* results in the death of many extra cells via apoptosis (Hengartner *et al*, 1992). *ced-3* and *ced-4* are both required for apoptosis: nearly all cells that normally die via apoptosis survive if either gene is inactivated (Ellis & Horvitz, 1986).

Mammalian homologues to each of the gene products central to the initiation and execution of apoptosis (*ced-3*, *ced-4* and *ced-9*) have now been identified. *ced-9* encodes for a protein with homology to a mammalian gene first identified in a human B cell lymphoma (Tsujimoto *et al*, 1984) termed *bcl-2* (B cell lymphoma). This homology was definitively shown in 1994 (Hengartner & Horvitz, 1994b) after many groups had demonstrated that the *bcl-2* gene product shared many functional similarities with the *ced-9* gene product (for example Vaux *et al*, 1992). However *bcl-2* was just the first of what is still an expanding family of mammalian genes encoding proteins with homology to Ced-9. This family of Ced-9 homologues adds another level of complexity to the control of apoptosis in mammals, as some members of the family protect from entry into apoptosis, whilst others promote apoptosis (see section 1.5.3.2).

Ced-3 is homologous to the mammalian protease ICE (interleukin 1-β converting enzyme), first described in 1992 (Cerretti *et al*, 1992 and Thornberry *et al*, 1992).

Again, there is another level of complexity in mammalian systems; ICE is just one of a growing family of cysteine proteases identified in mammals (see section 1.5.3.5) now called caspases (cysteinyl aspartate-specific proteinases, Nicholson & Thornberry, 1997).

A Ced-4 homologue in mammals proved elusive until very recently, when Apaf-1 (apoptotic protease activating factor) (Lui *et al*, 1996) was shown to share homology with Ced-4 (Zou *et al*, 1997). A second mammalian molecule with homology to Ced-4 has also been identified, called MRIT (MACH-related inducer of toxicity; *mrit* also means "death" in Sanskrit) (Han *et al*, 1997). Although homology to Ced-4 is only weak, MRIT appears to perform the function of Ced-4 in mammalian cells (see section 1.5.3.3).

Two papers published early in 1997 show that Ced-4 can interact with both Ced-3 and Ced-9 (Chinnaiyan *et al*, 1997a; Wu *et al*, 1997). Furthermore, Ced-4 is able to bind simultaneously to both Ced-3 and Ced-9, as demonstrated by co-immunoprecipitation experiments. Ced-4 will activate Ced-3 (and therefore promote apoptosis), unless Ced-9 is present: binding of Ced-9 to Ced-4 prevents Ced-4 from activating Ced-3. These events take place on intracellular membranes (likely to be mitochondrial) and the perinuclear region, and requires the transmembrane domain of Ced-9 for localisation to these regions. The activation of Ced-3 by Ced-4 has now been shown to be dependent on a putative ATP/GTP-binding motif (Seshagiri & Miller, 1997). Binding of ATP by Ced-4 has also been shown, and is not seen when the putative ATP/GTP-binding motif is deleted from the Ced-4 molecule (Chinnaiyan *et al*, 1997b).

The molecular mechanisms controlling the execution of apoptosis in *C. elegans* are now reasonable well characterised, with activation of Ced-3 being the critical event committing the cell to an apoptotic death. Ced-3 activation is catalysed by Ced-4 with evidence mounting that this is an ATP dependent process. Ced-9 prevents this

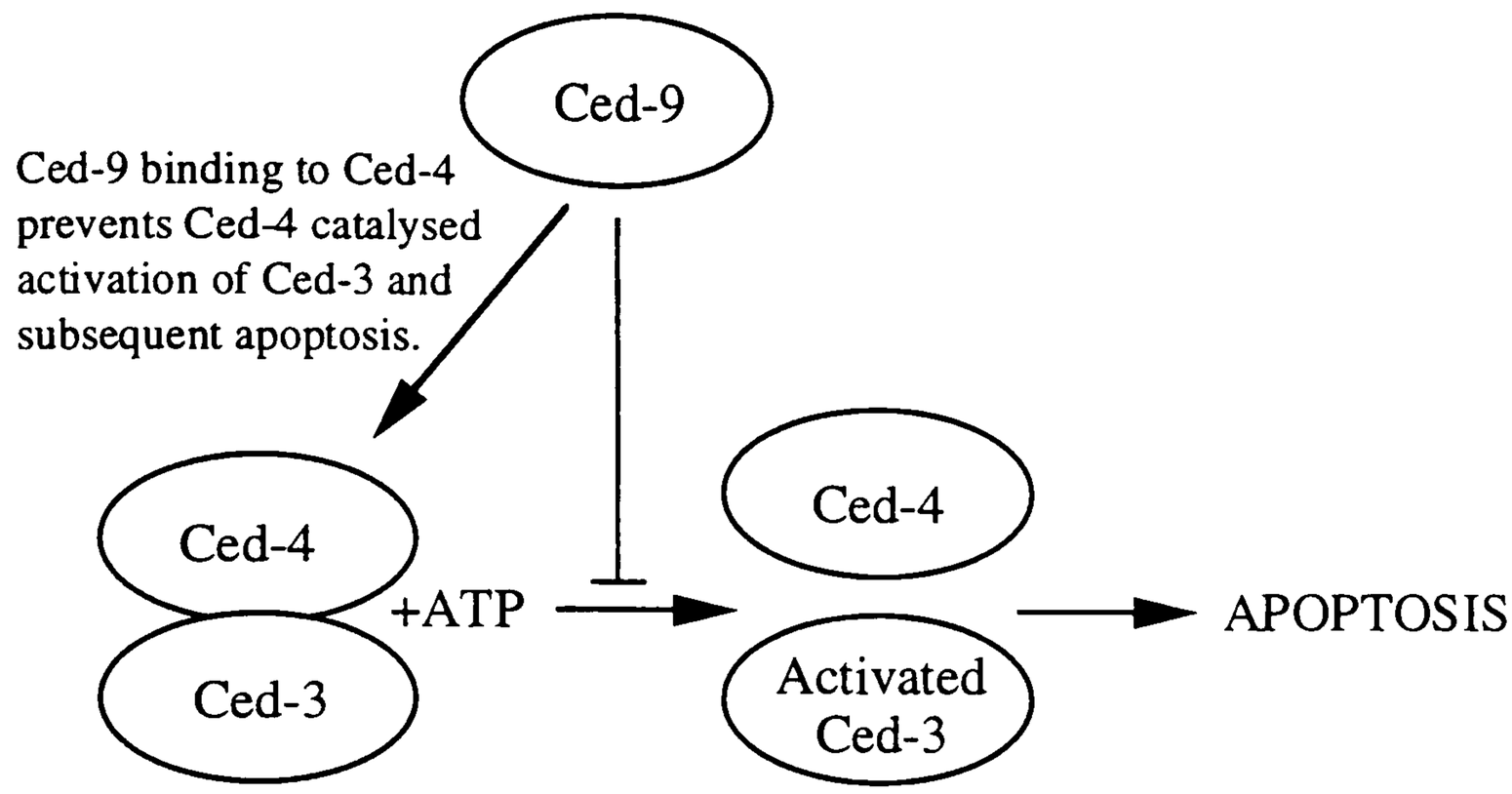
activation, by binding to Ced-4 (see Figure 1.3). The existence of homologues of each of these molecules in mammals suggests that this process is conserved, but is further complicated by the involvement of whole families of homologues for each of Ced-3, Ced-4 and Ced-9.

1.5.3.2 Mammalian Ced-9 homologues: The Bcl-2 family

As mentioned earlier, the first mammalian homologue of Ced-9 to be identified was Bcl-2, which can replace Ced-9 function in *C. elegans*. Many mammalian molecules with homology to Bcl-2 have now been identified, with some of the family acting similarly to Bcl-2 in that they act to prevent apoptosis (Bcl-X_L, Bcl-w, Brag-1, Mcl-1, A1/Bfl-1) and others acting to promote apoptosis (Bax, Bak, Bcl-X_S, Bad, Bid, Bik/Nbk, Bid, Hrk) (reviewed by McDonnel *et al*, 1996; Yang & Korsmeyer, 1996; Reed, 1997; Kroemer, 1997).

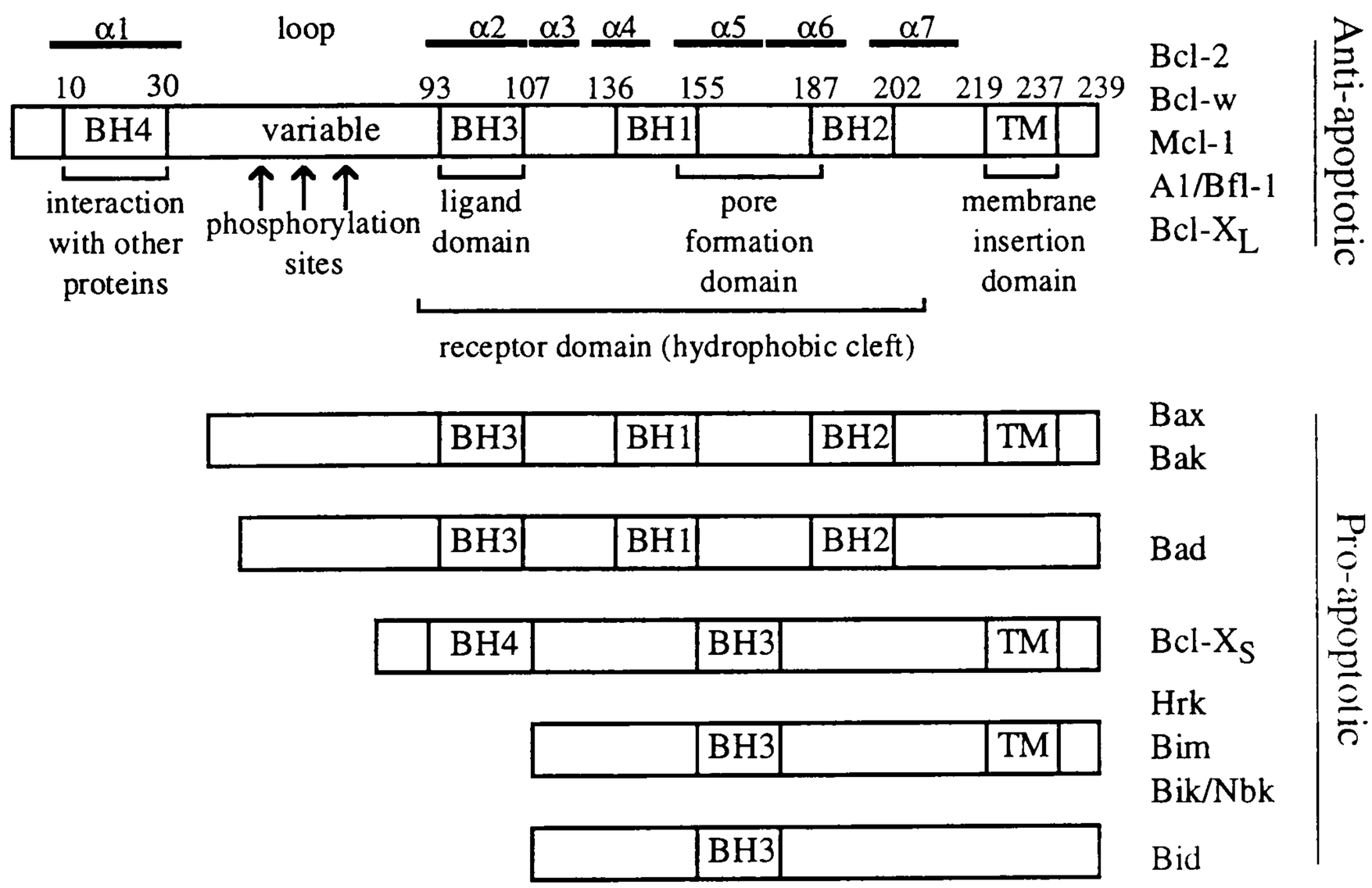
The Bcl-2 family members are able to form homodimers and heterodimers, and it is the relative amount of anti-apoptotic dimers and pro-apoptotic dimers which control entry into apoptosis, rather than the absolute levels of the individual proteins. If there is a majority of pro-apoptotic dimers, then apoptosis will commence, whereas a majority of anti-apoptotic dimers prevent entry into apoptosis. Figure 1.4 shows the protein domain structures of different members of the Bcl-2 family. The BH1 and BH2 domains are needed for homodimerization of anti-apoptotic Bcl-2 family members, and heterodimerization with pro-apoptotic Bcl-2 family members (such as Bax and Bak). The BH3 domain is also needed for heterodimerization between pro- and anti-apoptotic Bcl-2 family proteins, with the BH3 domain serving as a death 'ligand' that binds within the hydrophobic pocket that forms the receptor domain. The BH4 domain is restricted to anti-apoptotic Bcl-2 family members (with the exception of Bcl-X_S which is pro-apoptotic when overexpressed and results from alternate splicing of Bcl-X (Boise *et al*, 1993), but its physiological role is yet to be demonstrated) and is needed

Figure 1.3 Ced-9 inhibits Ced-3 activation by Ced-4



Model for apoptosis control in *C. elegans* by the Ced-3, -4 and -9 proteins. See main text for details and references.

Figure 1.4 Conserved motifs in Bcl-2 related proteins



Proteins not shown to scale. Positions of the domains in the Bcl-2 protein are shown (Bcl-2 = 239 a.a's). BH are regions of Bcl-2 homology. BH4 is needed for interaction with Ced-4, Bad, Raf-1, Bag-1, calcineurin, R-ras, H-ras, p53-BP2, Nip1, Nip2, Nip3. Adapted from Kroemer (1997) and Reed (1997).

for interaction with molecules that do not show homology with Bcl-2. The presence of the BH4 domain is essential for the anti-apoptotic activity of Bcl-2 family members (Kroemer, 1997). It is this BH4 domain that allows signalling events within the cell to alter the balance of anti- and pro-apoptotic effects of the Bcl-2 family proteins.

But how do the Bcl-2 family regulate entry into apoptosis? Studies to date show that these proteins have two distinct functions that can regulate entry into apoptosis: they can act as docking proteins, and as pore-forming proteins. Bcl-2 can also prevent formation of reactive oxygen species (ROS) thus inhibiting pro-oxidant induced death. However, the action of ROS is not necessary for apoptosis, rather ROS can initiate apoptosis in some circumstances (Kroemer, 1997).

The three dimensional structure of Bcl-X_L shows considerable similarity with the pore forming domain of certain bacterial toxins, especially the diphtheria toxin, and is able to form ion channels in synthetic lipid vesicles and lipid bilayers (Minn *et al*, 1997). In order to be able to form these ion channels, the two core alpha helices ($\alpha 5$ and $\alpha 6$, see Fig 1.4) are required (Reed, 1997). The pro-apoptotic Bcl-2 family member Bax can also form ion channels (Reed, 1997) suggesting either 1) the channels formed by anti-apoptotic Bcl-2 related proteins are protective against apoptosis whilst pro-apoptotic Bcl-2 related proteins form apoptosis inducing channels or 2) interaction between pro- and anti-apoptotic Bcl-2 family members control channel formation such that only when pro-apoptotic Bcl-2 family members are available can channels be formed. There is evidence that the channels formed by either Bcl-2 or Bax have distinct characteristics, which may allow these channels to perform different physiological roles (Schlesinger *et al*, 1997).

The formation of channels *in vivo* would be expected to occur in mitochondria, endoplasmic reticulum and nuclear membranes, where the Bcl-2 related proteins are located. The pores may then allow factors that are normally separated by these

membranes to translocate, interact and initiate apoptosis. This theory is strengthened by recent observations of the factors required for caspase activation.

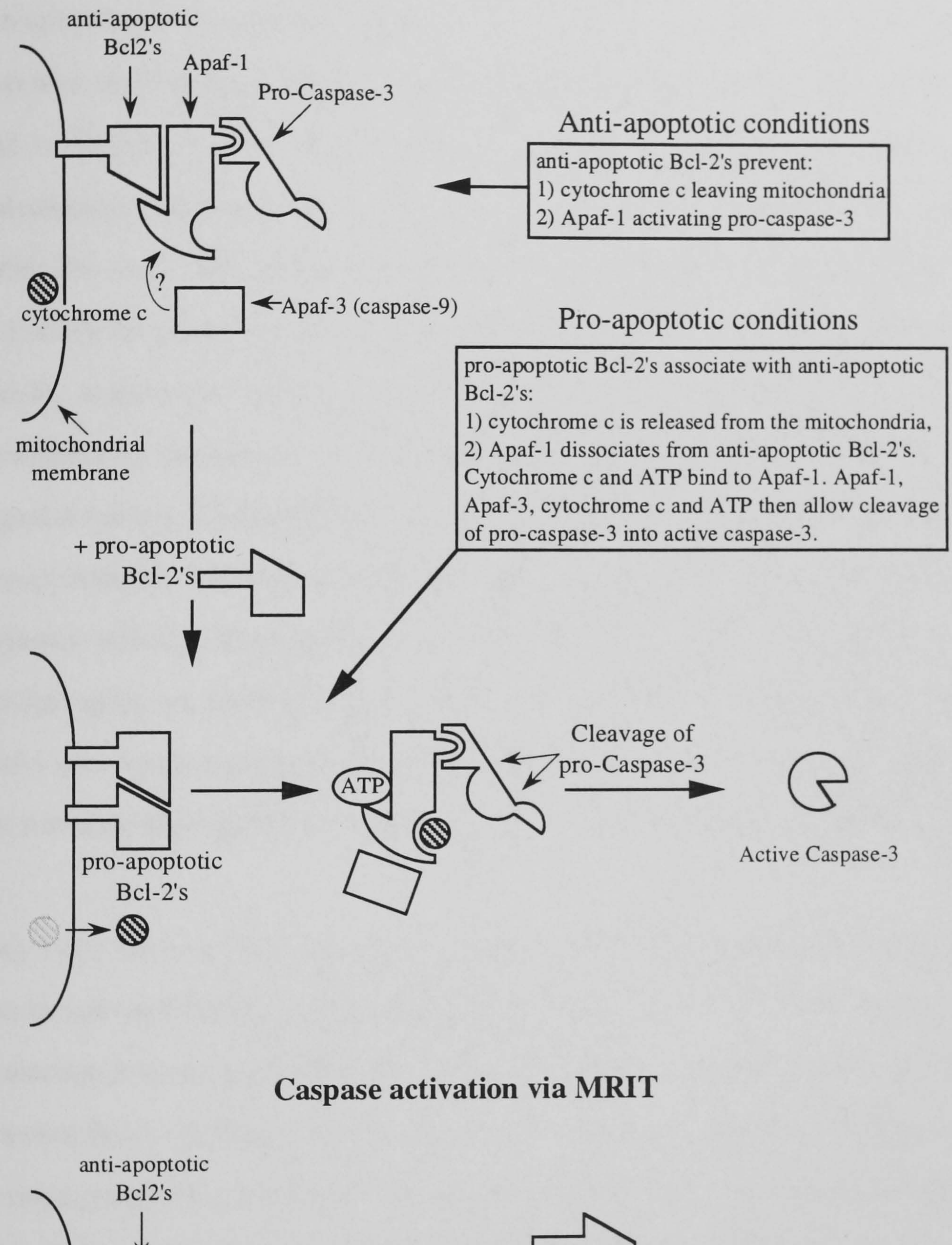
1.5.3.3 Bcl-2 family controlled caspase activation via mammalian Ced-4 homologues

Caspases can be activated by combining Apaf-1, cytochrome c (Apaf-2), Apaf-3 and dATP in cell free systems (Zou *et al*, 1997). Together, Apaf-1, -2, and -3 constitute a functional homologue of Ced-4 in mammalian cells. Apaf-1 and Apaf-3 are both cytosolic, whereas cytochrome c is contained within the mitochondria. Therefore, cytochrome c is required for activation of caspases by Apaf-1 (a Ced-4 homologue) and Apaf-3. If the Bcl-2 family is able to control the release of cytochrome c from mitochondria, then this would provide a mechanism for the regulation of apoptosis by the Bcl-2 family proteins. This was confirmed with the publication of two papers showing that Bcl-2 prevents the release of cytochrome c from mitochondria (Yang *et al,* 1997; Kluck *et al*, 1997). Yang *et al* (1997) showed that overexpression of Bcl-2 in HL-60 cells prevented the release of cytochrome c from mitochondria and inhibited apoptosis. Kluck *et al* (1997) showed that cytochrome c was spontaneously released from mitochondria in a cell free system, leading to caspase activation, and that Bcl-2 prevented this release. Bcl-2 can only prevent release of cytochrome c in this system; addition of exogenous cytochrome c will still activate the caspases, even in the presence of excess Bcl-2. Bcl-2 is also only able to prevent the release of cytochrome c from mitochondria on which Bcl-2 is located in this cell free system. The release of cytochrome c precedes the permeability transition in mitochondria, where a non-specific channel forms between the mitochondrial membranes. This demonstrates that cytochrome c translocation does not occur following a general breakdown of mitochondrial membranes, but is released by a specific mechanism.

Very recently, the previously unidentified molecule, Apaf-3, has been isolated and identified as Caspase-9 (Li *et al*, 1997). Apaf-1 binds to Apaf-3 (caspase-9) in a cytochrome c and dATP dependent manner, resulting in activation of caspase-9, which in turn activates caspase-3.

The second way in which the Bcl-2 family control apoptosis is through interaction with other proteins, via the BH4 domain found on anti-apoptotic Bcl-2 related proteins. These interactions remove these proteins from the cytosol, and by binding to them, bring them onto intracellular membranes, where they are either inactivated or used to interact with other membrane-associated proteins. In *C. elegans* Ced-9 removes Ced-4 from the cytosol, preventing it from activating Ced-3. In mammals, the anti-apoptotic Bcl-2 related proteins are predicted to bind the mammalian Ced-4 homologue(s), preventing activation of the Ced-3 homologues (the caspases), as Bcl-X_L is known to bind Ced-4 (Wu *et al*, 1997). The mammalian protein MRIT can cause caspase activation, and is a functional homologue of Ced-4 (Han *et al*, 1997). MRIT binds both Bcl-X_L and caspases, and the anti-apoptotic Bcl-2 related proteins can inhibit MRIT mediated apoptosis (Han *et al*, 1997). Whether cytochrome c is needed for this activation process (as has been shown for Apaf-1) has not been tested, but MRIT is significantly different from Apaf-1 in that it has a domain similar to the catalytic domain of the caspases, but lacks the key residues required for protease activity. Therefore, MRIT may allow activation of caspases, in a Bcl-2 family regulated manner, without the need for cytochrome c. The binding of Ced-4 by Ced-9 (or Bcl-X_L) in *C. elegans* is prevented by overexpression of the pro-apoptotic Bcl-2 related proteins Bax, Bak and Bik (Reed, 1997). It is then predicted that in mammals pro-apoptotic Bcl-2 family members will bind to anti-apoptotic Bcl-2 family members, thereby releasing the mammalian Ced-4 homologue(s), allowing activation of the caspases (see Fig 1.5).

Figure 1.5 Theoretical model for Bcl-2 family controlled caspase activation via mammalian Ced-4 homologues



1.5.3.4 Role of other molecules able to bind to Bcl-2 related proteins

Proteins with no homology to the Bcl-2 family are able to bind to or associate with anti-apoptotic Bcl-2 related proteins via the BH4 domain (see Fig 1.4). Such binding may alter the function of the Bcl-2 family members, as typified by the protein kinase Raf-1 (Wang *et al*, 1994). By associating with Raf-1, Bcl-2 targets Raf-1 to the mitochondria, where it is able to phosphorylate the pro-apoptotic Bad (Wang *et al*, 1996; Zha *et al*, 1996). Bad will associate with Bcl-2 or Bcl-X_L, displacing Bax and promoting apoptosis, but will not associate with A1 or Mcl-1 (anti-apoptotic), Bax, Bcl-X_S, or itself (pro-apoptotic) (Yang *et al*, 1995). The phosphorylation of Bad can be initiated by the addition of the survival factor IL-3 to FL5.12 cells, indicating a signal transduction pathway that regulates the activity of Bcl-2 family members. Bad phosphorylation causes its dissociation from Bcl-2 and Bcl-X_L and its binding to the cytosolic protein kinase regulator protein 14-3-3, which allows Bcl-2 and Bcl-X_L to prevent apoptosis. Other substrates for Raf-1 on the mitochondria should exist, as Raf-1 does not phosphorylate Bcl-2 or Bcl-X_L itself, yet artificially targeting Raf-1 to the mitochondria suppresses apoptosis in cells lacking the Bad protein (Reed, 1997).

Bag-1 also binds to Bcl-2 and acts to prevent apoptosis (Takayama *et al*, 1995). Raf-1 can be activated by Bag-1, and expression of both Bcl-2 and Bag-1 provides better protection from apoptosis than expression of either alone, suggesting a co-operation between Bcl-2 and Bag-1. In cells requiring hepatocyte growth factor or platelet derived growth factor, withdrawal of these growth factors causes Bag-1 binding to the growth factor receptors, preventing co-operation with Bcl-2 (Kroemer, 1997).

The protein phosphatase, calcineurin, associates with Bcl-2, with overexpression of calcineurin causing apoptosis (Reed, 1997). The mechanism of calcineurin induced apoptosis is not known.

The association of Bcl-2 with Ras (a key signal transducer in the regulation of proliferation and differentiation of eukaryotic cells, Chen & Faller, 1996) links the regulation of Bcl-2 related proteins with signal transduction, and also with the proliferation status of the cell. Another link with the cellular status is provided by the association of p53-BP2 (p53 binding protein 2) with Bcl-2. p53 is a tumour suppresser involved in controlling the cell cycle that can trigger apoptosis (see reviews by: Lane *et al*, 1994; Götz & Montenarch, 1995; Wyllie, 1997; Levine, 1997).

The phosphorylation of Bcl-2 has also been observed (Haldar *et al*, 1994), thus allowing apoptosis to proceed. This suggests that the phosphorylation state of the Bcl-2 related proteins is vital in determining their functional status. In this way the Bcl-2 family members can be deactivated rapidly by kinases or phosphatases, allowing rapid responses to external stimuli through signal transduction cascades. This allows apoptosis to be controlled in mammals not just by the absolute levels of pro- and anti-apoptotic Bcl-2 family proteins, but by activation and deactivation of these proteins in response to pro- and anti-apoptotic stimuli.

1.5.3.5 Mammalian Ced-3 homologues: The Caspases

The effector of apoptosis in *C. elegans* is the Ced-3 protein, which is a homologue of ICE (as mentioned in section 1.5.3.1). There is now a growing family of ICE homologues, termed caspases (cysteinyl aspartic-specific proteinases) identified in mammals (recent reviews by: Nicholson & Thornberry, 1997; Porter *et al*, 1997; Villa *et al*, 1997; Zhivotovsky *et al*, 1996). All the caspases cleave proteins after an aspartate residue, with the four amino acids before the cleavage site required for recognition and cleavage of a protein by caspases. These four amino acids are termed P_1 to P_4, with the aspartate immediately before the cleavage point being P_1. Mammalian caspases may be grouped into three groups by their P_4 to P_1 specificity and by the proposed role of each caspase (see Table 1.1). Group 1 caspases are involved primarily in the immune

Table 1.1 The proposed roles and specificities of the caspases

Caspase Grouping	P_4 -P_1 specificity	Consensus	Role (proposed)
Group 1			
Caspase-1 (ICE)	WEHD	WEHD	Immune response-maturation of multiple pro-inflammatory cytokines
Caspase-4(ICE_{rel}-II, TX, ICH2)	(WL)EHD		
Caspase-5(ICE_{rel}-III, TY)	(WL)EHD		
Group 2			
CED-3	DETD	DExD	Cleavage of DxxD apoptotic substrates
Caspase-2 (ICH1)	DEHD		
Caspase-3 (CPP32, Apopain, Yama)	DEVD		
Caspase-7 (Mch3, ICE-LAP3, CHH-1)	DEVD		
Group 3			
Caspase-6 (Mch2)	VEHD	(IVL)ExD	Activation of group 2 caspases and other group 3 caspases.
Caspase-8 (MACH, FLICE, Mch5)	LETD		
Caspase-9 (ICE-LAP6, Mch6)	LETD		Cleavage of non-DxxD apoptotic substrates
Caspase-10 (Mch4)	xExD		
Granzyme B	IEPD		

Caspases known to date (10) are shown with granzyme B included as this protein is also able to activate the caspases. Adapted from Nicholson & Thornberry, 1997.

response, but can induce apoptosis if overexpressed in certain cells types, such as fibroblasts (Miura *et al*, 1993). The group 2 caspases dominate the cleavage of specific substrates during the death of a cell by apoptosis, which have the P_4 to P_1 sequence DxxD. The group 3 caspases activate other group 3 caspases and group 2 caspases, and also cleave apoptosis specific-substrates, which do not have the P_4 to P_1 DxxD consensus. A large hydrophobic amino acid such as tryptophan is usually the P_4 residue for group 1 caspases, an aspartate is essential at P_4 for group 2 caspases, while group 3 prefer branched chain aliphatic residues at P_4 (Nicholson & Thornberry, 1997).

The activation of mammalian caspases by mammalian Ced-4 homologues and other mechanisms (see section 1.5.3.6) results in the cleavage of specific proteins within the cell, rather than the wholescale destruction of all cellular proteins. The result of the cleavage of specific substrates by caspases are i) to halt cell cycle progression, ii) disablement of homeostatic repair mechanisms, iii) initiation of detachment from surrounding tissue, iv) disassembly of structural components and v) marking of the apoptotic cell for phagocytosis (Nicholson & Thornberry, 1997). Examples of cleaved proteins include protein kinase δ and the retinoblastoma protein which are both involved in controlling progression through the cell cycle. Poly (ADP-ribose) polymerase (PARP) is a nuclear protein which is needed to maintain genome integrity and this is cleaved by caspase-3 (Tewari *et al*, 1995). The production of mRNA is disrupted by cleavage of hnRNP C1 and C2 (heteronuclear proteins) which are needed for pre-mRNA splicing (Waterhouse *et al*, 1996). One of the proteins involved in cell adhesion (focal adhesion kinase) is also cleaved by effector caspases (Nicholson & Thornberry, 1997). Structural proteins cleaved include actin, but this cleavage may be mediated by proteases other than caspases in certain cell types (Brown *et al*, 1997). Recognition of an apoptotic cell can be mediated by phosphatidylserine exposure. This exposure occurs after caspase activation (Naito *et al*, 1997) but has not been shown to be a direct result of cleavage of a protein by a caspase. The fragmentation of DNA is

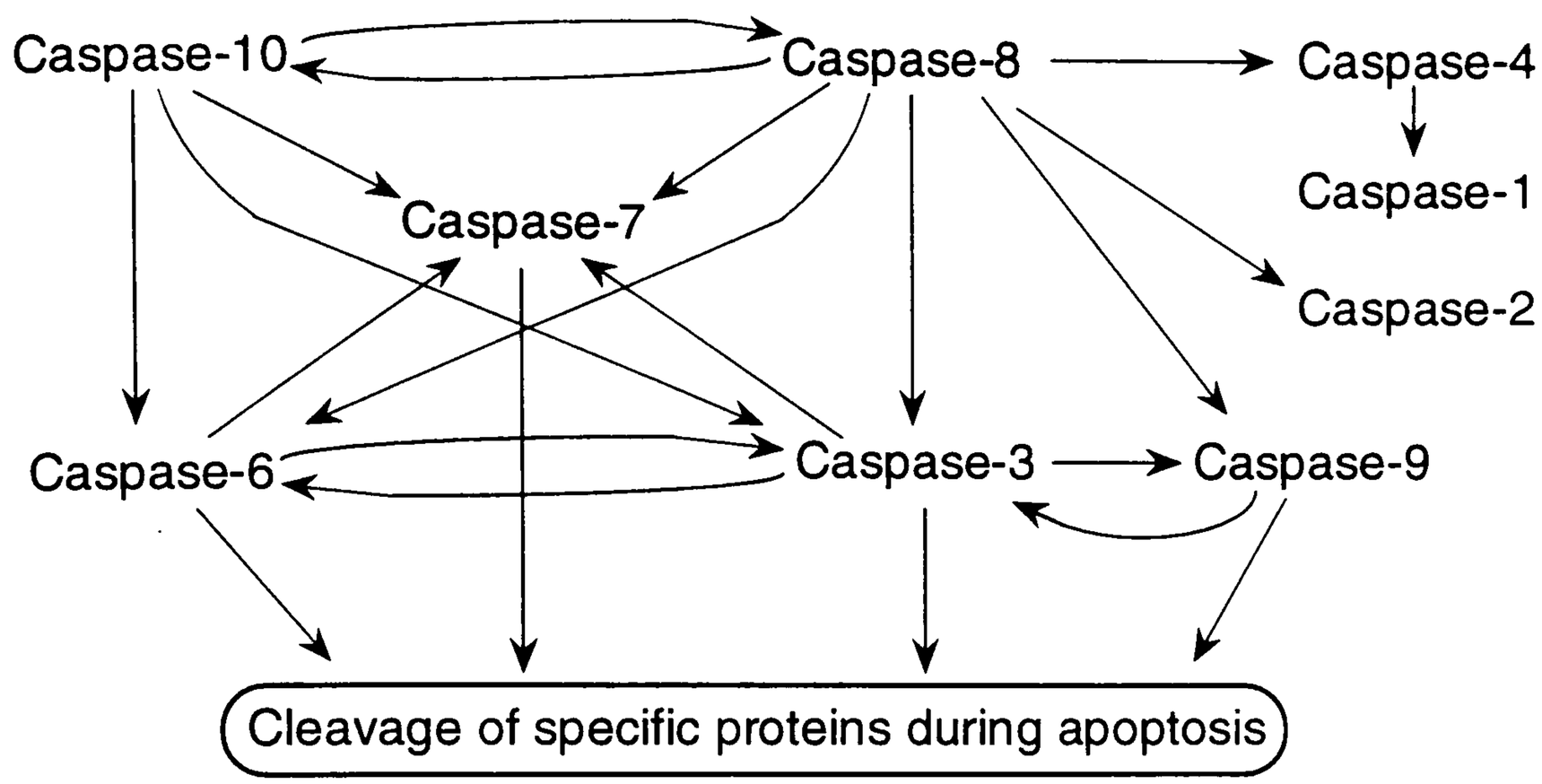
also triggered by caspase activation. Caspase-3 will cleave DNA fragmentation factor (DFF), resulting in its activation. This activated protein then initiates DNA fragmentation without further requirement of caspase-3 (Liu *et al*, 1997).

1.5.3.6 Caspase activation

Caspases are synthesized as inactive proenzymes, which consist of 3 domains; a prodomain and two catalytic domains. The dormant proenzyme becomes active following cleavage of the proenzyme to remove the prodomain and separate the two catalytic domains, which then form an active caspase tetramer consisting of two large and two small catalytic subunits. These points of cleavage occur after an aspartate residue, and are in this way similar to all sites where caspases cleave, allowing an active caspase to cleave other procaspases. The caspase prodomains are larger in some caspases than others. Caspases-1, -2, -4, -5, -8 and -10 have long prodomains, while caspases-3, -6, -7 and -9 have short prodomains (Villa *et al*, 1997). It is notable that the caspases known to cleave the specific cellular proteins which lead to the characteristic signs of apoptosis have short prodomains, whilst the long-prodomain caspases cleave other caspases (with the exception of the group 1 caspases which are involved primarily with the immune response). This suggests that the short prodomain caspases are 'effector' caspases which actually execute the cell. The longer prodomain caspases are 'regulatory' caspases whose activation leads to activation of the 'effector' caspases.

This activation of caspases requires the cleavage of the procaspases at a site recognized by other caspases, but what activates the first caspase? The mammalian Ced-4 homologues Apaf-1 (with cytochrome c) and MRIT are both able to activate caspases (see section 1.5.3.3), by an unknown mechanism. Following the activation of one or more key caspases a caspase activation cascade may occur as shown in Figure 1.6.

Figure 1.6 Theoretical proteolytic cleavage/activation cascade of caspases



The caspase activation cascade shown is based on *in vitro* observations and may not be a true representation of events *in vivo*. Caspase-5 is not included as this has only been shown to be a substrate of itself. Adapted from Zhivotovsky *et al*, 1997; Villa *et al*, 1997; Porter *et al*, 1997; Lee *et al*, 1997.

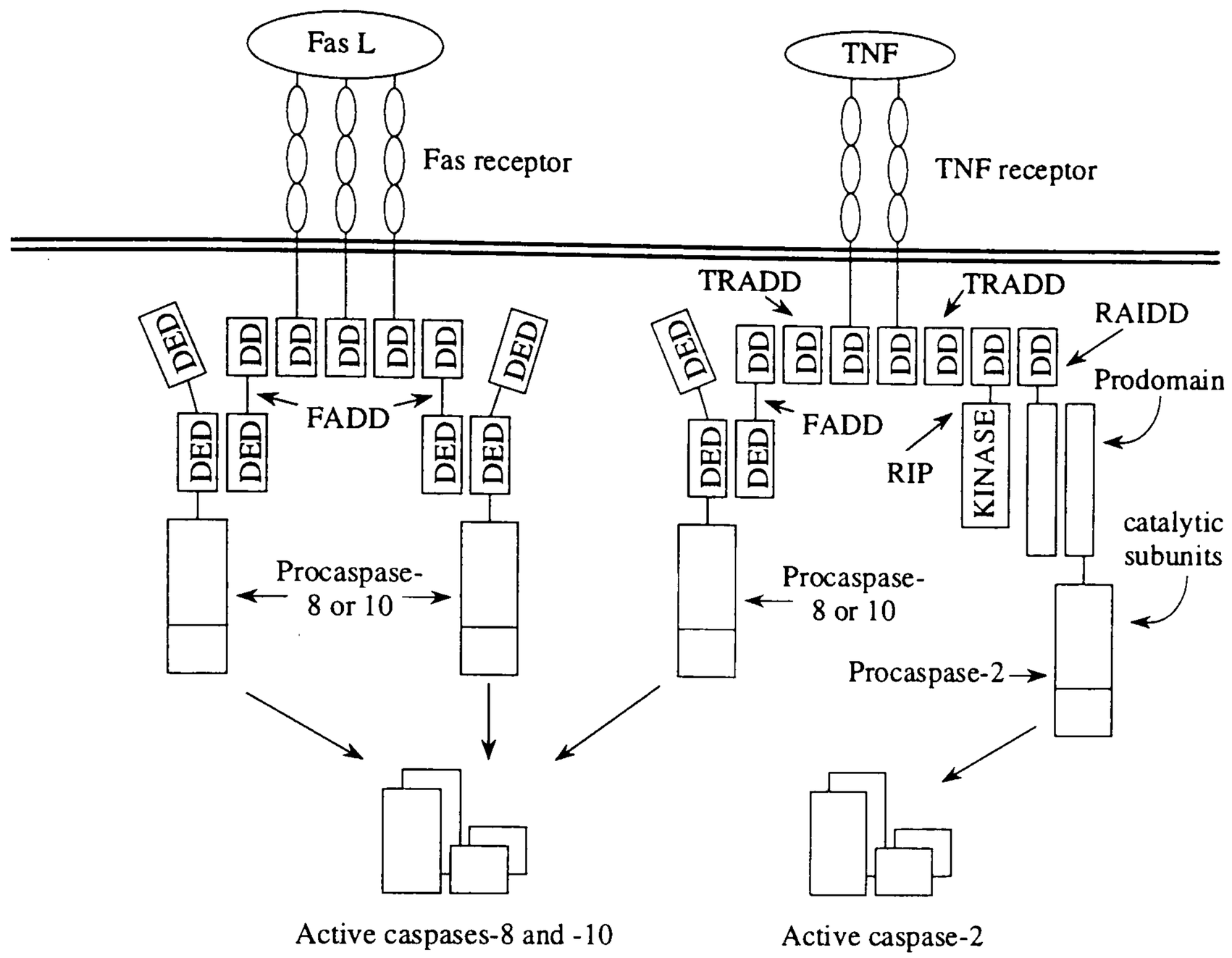
This cascade could then activate all caspases needed to complete the apoptotic program.

The only other known protein that cleaves caspases is granzyme B, which is used by cytotoxic T lymphocytes in the destruction of target cells (Shi *et al,* 1997). Artificial synthesis of very high levels of different procaspases results in autolytic processing to active caspases, and concentration of isolated procaspases by ultrafiltration also activates the procaspases. This suggests that procaspases are able to activate one another if brought together in the correct manner. This initial activation of caspases can be mediated by the activation of either the Fas-receptor or the TNF-receptor-1 (p55) (recent reviews by: Wallach *et al*, 1997; Nagata, 1997; Wallach, 1997; Darnay & Aggarwal, 1997), as shown in Figure 1.7.

The activation of procaspases 8 or 10 following binding of the Fas ligand to its receptor, involves adaptor molecules which link the procaspases to the Fas receptor intracellular domains. This docking is mediated by a domain termed the death domain (DD) of about 90 amino acids, which is found on the intracellular region of both the Fas receptor and the TNF-receptor-1. Similar DD's are found on the adaptor molecule FADD, which associates with the DD on the Fas receptor. FADD has another domain termed the death effector domain (DED), which is also found in the prodomain of caspases 8 and 10. This DED mediates the binding of these procaspases to FADD, thereby recruiting the procaspases to the Fas receptor. The procaspases are then activated, either by autocatalysis, or by virtue of their close proximity to one another thereby allowing each to activate the other.

The activation of caspases following TNF-receptor-1 ligation involves similar death domains found on the intracellular region of the receptor. These DD's are bound to firstly by the DD containing protein TRADD, which then recruits FADD, in turn recruiting the procaspases 8 or 10. Procaspase 2 can also be activated following TNF-

Figure 1.7 Activation of caspases through Fas receptor and TNF receptor-1



DD represents the Death Domain through which the receptors recruit other death domain containing molecules (FADD,TRADD, RIP, RAIDD). FADD also has a Death Effector Domain (DED) which recruits procaspases-8 and -10. Procaspase-2 is recruited by RAIDD, which has a death domain and a domain similar to the prodomain of procaspase-2. See main text for details and references.

receptor-1 activation, with the DD-containing protein RIP binding to TRADD, which then allows another DD containing protein RAIDD to bind to TRADD. RAIDD has a domain similar to the prodomain of procaspase-2, through which procaspase-2 is recruited before being activated by proteolytic cleavage.

1.5.3.7 Regulation of Caspase activation

Activation of the Fas receptor or TNF-receptor-1 does not lead to apoptosis in all circumstances, as cell death can be prevented by the activity of other molecules. For example, endothelial cells which express the Fas receptor do not become apoptotic following activation of the receptor (Richardson *et al*, 1994), and B cells may proliferate in response to Fas depending upon the signal from the B cell antigen receptor (Rathmell *et al*, 1996). Neutrophils, which are normally extremely sensitive to Fas- and TNF-induced apoptosis, become resistant to TNF- and Fas-induced apoptosis following migration through the endothelium, LPS or cytokine treatment (Tsuchida *et al*, 1995; Marshall *et al*, 1996).

A number of mechanisms have now been identified which allow TNF-treated cells to avoid commitment to apoptosis. TNF induced apoptosis can be prevented by activation of NFκB. This activation leads to transcription of genes, one or more of which are presumed to inhibit apoptosis (for example, see Nagata, 1997). This activation of transcription is mediated by a protein termed TRAF-2, which activates NIK(MAPKKK), itself leading to activation of NFκB (for example, see Wallach, 1997). The activation of TRAF-2 can be mediated by the TNF-receptor-2, which cannot signal for apoptosis, but can signal for survival via a domain termed TRAF, which is also found in TRAF-2. The TNF-receptor-1 can also recruit and activate TRAF-2, via the adaptor proteins RIP and TRADD, both of which also contain the TRAF domain (Wallach, 1997). Two other proteins (cIAP-1 and -2) which bind to TRAF-2 can inhibit apoptosis, when not associated with TRAF-2, by an unknown

mechanism (Wallach, 1997). These proteins do not effect Fas induced apoptosis as they are not recruited to the Fas signalling complex. The protein Sentrin is also able to block apoptosis following TNF treatment, by binding to the DD of the TNF-receptor-1 (Darnay & Aggarwal, 1997). Sentrin has no DD and the mechanism by which this is achieved is unknown.

Fas induced apoptosis can be inhibited by the protein tyrosine phosphatase FAP-1 (Sato *et al*, 1995), again by an unknown mechanism. The FAP-1 protein associates with the carboxy terminus 15 amino acids of the Fas receptor and is found in tissues that show resistance to Fas-mediated cell death. Its role as an inhibitor of Fas-mediated apoptosis was shown by overexpression of the molecule in various cell lines, with the level of expression correlating with the degree of protection from apoptosis.

A molecule called FLIP, identified by five independent groups, was shown to either block Fas or TNF induced apoptosis (by 3 groups) or to induce apoptosis (by 1 group), with the fifth group remaining undecided as to its function (reviewed by Wallach, 1997b). The FLIP protein contains DED's and a domain similar to the carboxy-terminal protease precursor of caspases 8 and 10 which, however, lacks certain sequence features required for protease activity. It is thought to inhibit apoptosis by preventing binding of the caspases to the DED of FADD, and also by binding to the protease precursor regions of caspases, thereby preventing their activation. FLIP can also bind to TRAF-2, displacing cIAP-1 and -2 which are then thought to be able to prevent cell death. The differences in the results obtained, and subsequent interpretation by these groups, may be explained by the different methodologies used. The artificial over-expression of a protein in a cell line is also far from physiologically normal, so the results obtained must be interpreted with care. Whatever the physiological role of FLIP may be, it is certainly involved in modulating the cellular response to Fas and TNF receptor activation.

1.5.3.8 Modulation of Caspase activity by Bcl-2 family members

The Bcl-2 family proteins are generally thought to act upstream of the caspases (Salvesen & Dixit, 1997). However, there have been a number of reports of the anti-apoptotic Bcl-2 family members preventing apoptosis after exposure of cells to Fas, which would normally result in apoptosis. This has been shown for both Bcl-2 and Bcl-X_L but the mechanism of action is not known (Nagata, 1997).

Two groups demonstrated the ability of Bcl-2 to inhibit cell death by generating transgenic mice which express Bcl-2 in the liver (Rodriguez *et al,* 1996; Lacronique *et al*, 1996). Administration of Fas to transgenic mice still caused some signs of apoptosis in the liver, but this was much less severe than in control mice. Bcl-X_L overexpression in Jurkat T cells substantially inhibits cell death following Fas treatment (Boise & Thompson, 1997). This work shows that Fas treatment still caused activation of caspases, as demonstrated by complete cleavage of PARP 6 h after Fas treatment. In the continuous presence of Fas, the Bcl-X_L expressing cells showed a slight loss of viability after 48 h, indicating that protection was not complete. This paper also demonstrated that if Fas was removed after 6 h (when PARP cleavage is complete), the Bcl-X_L expressing cells are able to recover and continue to divide. This suggests that caspase activation is not the point of commitment to apoptosis as is widely thought. The mechanism by which Bcl-X_L does protect from apoptosis following caspase activation remains unknown, but protected cells did not show a loss of mitochondrial membrane potential. This could indicate that caspase activation via Fas needs to be further amplified by release of factors (such as cytochrome c) from the mitochondria, in these cells.

Although the precise mechanisms by which Bcl-2 family members modulate caspase activation are not known, some molecules which may be involved in this process have been identified. One such molecule is called Bag-1, which is a Bcl-2 binding protein

with no homology to any other Bcl-2 family members (Takayama *et al*, 1995). Induction of apoptosis by activation of caspases by Fas was not inhibited by Bag-1 alone, and slightly inhibited by Bcl-2 in Jurkat T cells. Combination of Bag-1 and Bcl-2 prevented apoptosis in around 75% of the cells, indicating that these molecules can act together to prevent apoptosis following Fas receptor activation.

There has been one demonstration of an anti-apoptotic Bcl-2 family member, namely Mcl-1, binding to FADD, and blocking Fas induced apoptosis (Wei *et al*, 1996). This interaction involves the N-terminal region of the Mcl-1 molecule, which is not present in any other known Bcl-2 family members, so is likely to be a unique property of Mcl-1.

Another possible mechanism for prevention of apoptosis following Fas treatment is provided by the identification of a novel Fas-binding molecule, called Daxx (Yang *et al*, 1997b). This work indicates that, in some cell types, endogenous Daxx is required for Fas induced apoptosis, which is regulated by Bcl-2. These events occur downstream of Fas, and therefore allow the Fas death signal to be regulated by Bcl-2.

The mammalian Ced-4 homologue Apaf-1 (see section 1.5.3.3), together with cytochrome c are able to activate the caspases. The Apaf-1 protein is predicted to be prevented from activating caspases by binding to anti-apoptotic Bcl-2 family members (Reed, 1997). A second Ced-4 homologue, MRIT, can also activate caspases, and this activation is inhibited by anti-apoptotic Bcl-2 family members (Han *et al,* 1997). Activation of the caspases by these two proteins appears to represent a separate mechanism to that employed following Fas or TNF treatment, in that their function may be directly controlled by the Bcl-2 family of proteins: this has yet to be conclusively shown for Apaf-1, but has been shown for MRIT (Han *et al*, 1997).

1.5.3.9 Problems with the current understanding of the control of apoptosis

Although the progress made over the past few years into the mechanisms underlying the control of apoptosis have been immense, there are still many aspects of these processes which are not understood. This is exemplified by the role of the Bcl-2 family of proteins in controlling entry into apoptosis. It is known that the Bcl-2 family can prevent or trigger apoptosis, but the details of how these events are achieved are far from clear.

Some difficulties in understanding the roles that these proteins play have arisen from experiments where conflicting and even opposing results have been described. For example, the Bcl-2 protein is well known for its anti-apoptotic activity, but when artificially expressed at very high levels, Bcl-2 actually increases cell death (Chen *et al*, 1996b). Conversely, the pro-apoptotic protein Bax can prevent apoptosis when overexpressed in neuronal cells (Middleton *et al,* 1996). The pro-apoptotic protein Bak can also inhibit cell death when expressed in an Epstein-Barr virus transformed cell line (Kiefer et al, 1995). These unusual results were all obtained in cells where the molecules were artificially expressed, so the systems used were far from physiological. However, the fact that these molecules can behave in such unexpected ways demonstrates that our understanding of their actions are limited.

The Bcl-2 molecules are thought to act primarily on mitochondrial membranes, to prevent apoptosis, but one report shows that when Bcl-2 is restricted to just the endoplasmic reticulum the protection from apoptosis can be greater than when Bcl-2 is located on the mitochondria (Zhu *et al*, 1996).This report also showed that the differently located Bcl-2 proteins bound Bax equally well, even in circumstances where apoptosis was not prevented. Thus Bax binding by Bcl-2 is not necessarily sufficient to prevent apoptosis.

The expression of Bax can induce apoptosis by mechanisms involving the activation of caspases, but inhibition of caspases does not always prevent cell death (Xiang *et al*, 1996). The death that occurs in such cases is distinct from apoptosis, in that caspase substrates were not cleaved. However, the mitochondrial function ceased and cell death occurred after this cessation. Another report shows that caspase inhibition does not stop cell death induced by oncogene expression, DNA damage, or Bak (McCarthy *et al*, 1997). Again, the death that results is distinct from apoptosis, but death does occur by a process involving cytoplasmic blebbing. The apoptosis induced by Fas was however completely prevented by inhibition of caspases (Tewari *et al*, 1995). This suggests that apoptosis initiated by agents whose signal for death is not initially through the activation of a caspase activate two distinct death programs. These two programs would normally run in parallel, giving the conventional apoptotic morphology. If caspases are inhibited then the death process is still initiated but the death is not typically apoptotic. This indicates the absolute requirement of caspases for the correct completion of the apoptotic program (Salvesen & Dixit, 1997). This is further demonstrated by work showing that a caspase-3 cleavage product of gelsolin is able to cleave actin filaments *in vitro*, and that this cleaved gelsolin is required for the normal morphological changes associated with apoptosis (Kothakota *et al*, 1997).

Another problem with the current understanding of the control of apoptosis is demonstrated in a report stating that Bcl-X_L but not Bcl-2 is able to protect a B cell line from apoptosis (Gottschalk *et al*, 1994). This observation shows that not all anti-apoptotic Bcl-2 family members act in the same way in a particular cell type. This is further demonstrated by the artificial expression of Bcl-2 in neutrophils. Although Bcl-2 expression does delay the onset of apoptosis in these cells, it does not prevent the expression of apoptotic surface epitopes that signal engulfment by phagocytic macrophages (Lagasse & Weissman, 1994).

1.6 Neutrophil Apoptosis

The mature neutrophil is a terminally differentiated cell, which spontaneously undergoes apoptosis both *in vivo* and *in vitro*, before phagocytosis by neighbouring phagocytes (Savill *et al*, 1989). This process of apoptosis, followed by removal of the apoptotic neutrophil, is important in the resolution of inflammation (section 1.4.3). The lifespan of the circulating neutrophil *in vivo* is difficult to determine, but it is proposed that the half-life is between 5 and 6 h (Savill *et al*, 1989), with the entire circulating pool removed within 72 h (Haslett, 1992).

The functional capacity of neutrophils is lost during apoptosis, with neutrophils aged *in vitro* losing functions such as chemotaxis, phagocytosis, granule release and oxidative burst (Whyte *et al*, 1993). The morphology of the neutrophil also undergoes major changes associated with apoptosis, such as decreased cell volume, compaction of chromatin (which is observed as loss of the characteristic multilobed appearance of the neutrophil nucleus) and fragmentation into internucleosomal fragments, similar to that seen in most cells during apoptosis (section 1.5.2). There is also a loss of expression and/or function of certain cell surface proteins, resulting in a loss of the ability to adhere to many surfaces (Dransfield *et al*, 1995). The low affinity receptor for IgG immune complexes FcγRIIIb (CD16), which is normally abundant on the neutrophil cell surface, is lost on apoptotic neutrophils (Dransfield *et al*, 1994), in parallel with an increase in phosphatidylserine exposed on the neutrophil outer membrane leaflet, as measured by binding of Annexin V (Homberg *et al*, 1995).

1.6.1 Agents which modulate entry into apoptosis

The lifespan of the mature neutrophil can be prolonged or shortened by various treatments, by delaying or accelerating their entry into apoptosis. There have been

numerous studies of agents suspected to be able to modulate neutrophil apoptosis, which are summarised in Table 1.2.

Many of the agents that are able to modulate neutrophil apoptosis are involved in inflammation. The interleukins-1β, -2, -4, -6, -12, and -15, are all able to delay neutrophil apoptosis. The ability of IL-2 to delay apoptosis is not confirmed in all studies, but this is likely due to the concentrations used. Many agents that delay neutrophil apoptosis also increase gene expression (Table 1.2), and IL-2 is able to delay apoptosis only at concentrations that are high enough to increase gene expression. IL-6 has been reported to accelerate, delay, or have no effect on neutrophil apoptosis, but the literature clearly favours the ability of IL-6 to delay apoptosis. IL-6 is able to increase gene expression in neutrophils (Colotta *et al*, 1992), and the work of Biffl *et al* (1995, 1996) shows that IL-6 is able to delay neutrophil apoptosis in a neutrophil, and IL-6, concentration dependant manner, explaining the lack of action found by other investigators. Of the other interleukins investigated, only IL-10 has any effect on neutrophil apoptosis. IL-10 alone has no effect on neutrophil apoptosis, but it is able to prevent the ability of other agents to delay apoptosis (Keel *et al*, 1997).

The colony stimulating factors, granulocyte macrophage-colony stimulating factor (GM-CSF), and granulocyte-colony stimulating factor (G-CSF) are both effective in delaying neutrophil apoptosis, while macrophage-colony stimulating factor (M-CSF) has no effect. Interferon-γ (IFN-γ) also delays neutrophil apoptosis, whilst also increasing gene expression (Klebanoff *et al*, 1992).

Most of these agents that can delay neutrophil apoptosis also prime the neutrophil (Edwards, 1994), but the pro-inflammatory, neutrophil priming molecule, tumour necrosis factor-α (TNF-α) has been reported to have varying effects on neutrophil apoptosis. In common with many other agents that activate neutrophil gene

Table 1.2 Agents investigated which may modulate neutrophil entry into apoptosis

Agent	Accelerate (↑), Delay (↓), No Effect (-), (References) and Notes
IL-1β	↓ (1, 2) Increases mRNA and protein synthesis (1).
IL-2	↓ (3, 4), - (5) Increases mRNA and protein synthesis (3): low concentration used in (5) had no effect on protein synthesis.
IL-3	- (6, 7) Delays eosinophil apoptosis (7).
IL-4	↓ (8), - (6) Increases gene expression (8).
IL-5	- (7) Delays eosinophil apoptosis.
IL-6	↓ (1, 2, 9, 10), - (6, 12), ↑ (11) Increases gene expression (1); only effective at higher neutrophil concentrations (10).
IL-7	- (13) No CDw127 component of receptor.
IL-8	- (1, 6, 12)
IL-10	- (12, 14, 15) Prevents action of other agents able to delay apoptosis (12).
IL-12	↓ (12)
IL-13	- (5) Only tested at low concentration: Higher concs increase gene expression.
IL-15	↓ (4) Increases gene expression: no priming of respiratory burst.
GM-CSF	↓ (1, 2, 12, 16, 17, 18, 19, 20, 21, 22, 23, 24) Increases gene expression
G-CSF	↓ (1, 6, 12, 20, 25, 26, 27) Increases gene expression, but less effectively than agents such as GM-CSF.
M-CSF	- (1)
IFN-γ	↓ (1, 12, 28) Increases protein synthesis (1).
TGF-β	↓ (29), - (6)
TNF-α	↑ (30, 31, 32, 33, 34, 35, 36, 37, 38, 39), - (26), ↓ (1, 12), ↓↑ (23) See main text for details, and explanation of conflicting results.
Fas	↑ (2, 37, 40, 41, 42, 43, 44, 45, 46, 47)
LPS	↓ (1, 12, 16, 17, 23, 30, 47) Increases gene expression
fMLP	- (1, 6, 23, 48), ↓ (16), ↑ (19, 36) See main text for details.
C5a	- (1, 6), ↓ (16), ↑ (19) See main text for details
Glucocorticoids	↓ (7, 17, 29, 40, 49)
LTB_4	↓ (19, 23)
PAF	- (23) PAF primes neutrophils, see main text for details.
Act D	↑ (50) Blocks transcription, see main text for details.
CHX	↑ (26, 38, 50) Blocks translation, see main text for details.
Saporin	↑ (24) Inactivates ribosomes, preventing protein synthesis.
S.F.	↑ (51) Synovial fluid from inflammed joints.
NO	↑ (52)
Phagocytosis	↑ (33, 53) *E. coli* ingestion induced apoptosis, see main text for details.
Heat	↑ (33) Heat shock induced apoptosis.
Nicotine	↓ (54) Thought to involve new protein synthesis.
Platelets	↓ (55) Mechanism unknown, but thought to involve platelet surface proteins.
Adenosine	↓ (57) ATP, Ap_3A, Ap_4A, Ap_5A, Ap_6A also effective (21, 56).
Proteases	↑ (58) Pronase, trypsin, chymotrypsin and elastase were used
Hypoxia	↓ (59, 60) Oxidative stress and antioxidants discussed in main text.
↑ cAMP	↓ (61, 45), ↑ (62) See main text for discussion.
↑ $[Ca^{2+}]_i$	↓ (63, 64, 65) Transient increases delay apoptosis, see main text.
CD11a/b	↓ (66, 32, 47, 48, 67) Cross-linking β_2 integrins delays apoptosis.
L-selectin	↑ (48, 67) Cross-linking L-selectin accelerates apoptosis.

Table 1.2 continued, references.

1) Collota *et al*, 1992; **2**) Iwai *et al,* 1994; **3**) Pericle *et al*, 1994;
4) Girard *et al*, 1996; **5**) Girard *et al*, 1996a; **6**) Brach *et al*, 1992;
7) Meagher *et al*, 1996; **8**) Girard *et al*, 1997; **9**) Biffl *et al*, 1995;
10) Biffl *et al*, 1996; **11**) Afford *et al*, 1992; **12**) Keel *et al*, 1997;
13) Girard *et al*, 1997a; **14**) Cox, 1996; **15**) Ward *et al*, 1996;
16) Lee *et al*, 1993; **17**) Cox, 1995; **18**) Yousefi *et al*, 1994;
19) Hébert *et al*, 1996; **20**) Cox*et al*, 1994; **21**) Gasmi *et al*, 1996;
22) Wei *et al*, 1996a; **23**) Murray *et al*, 1997; **24**) Bergamaschi *et al*, 1996;
25) Adachi *et al*, 1994; **26**) Sullivan *et al*, 1996; **27**) Niessen *et al*, 1997;
28) Klebanoff *et al*, 1992; **29**) Ward *et al*, 1996a; **30**) Hachiya *et al*, 1995;
31) Takeda *et al*, 1993; **32**) Ginis & Faller, 1997; **33**) Watson *et al*, 1996;
34) Gon *et al*, 1996; **35**) Murray *et al*, 1996; **36**) Kettritz *et al*, 1997;
37) Watson *et al*, 1997; **38**) Tsuchida *et al*, 1995; **39**) Chilvers *et al*, 1995;
40) Liles & Klebanoff, 1995; **41**) Liles *et al*, 1996; **42**) Hsieh *et al*, 1997;
43) Kasahra *et al*, 1997; **44**) Hu *et al*, 1997; **45**) Yasui *et al*, 1997;
46) Jimenez *et al*, 1997; **47**) Watson*et al*, 1997a; **48**) Watson *et al*, 1997b;
49) Kato *et al*, 1995; **50**) Whyte *et al*, 1997; **51**) Bell *et al*, 1995
52) Brennan *et al*, 1996; **53**) Watson *et al*, 1996a; **54**) Aoshiba *et al*, 1996;
55) Andogegui *et al*, 1997; **56**) Gasmi *et al*, 1996; **57**) Walker *et al*, 1997;
58) Trevani *et al*, 1996; **59**) Hannah *et al*, 1995; **60**) Mecklenburgh *et al*, 1996;
61) Rossi *et al*, 1995; **62**) Aoshiba *et al*, 1995; **63**) Whyte *et al*, 1993a;
64) Cousin *et al*, 1996; **65**) Cousin *et al*, 1996a; **66**) Watson *et al*, 1996b;
67) Watson *et al*, 1996c.

expression, Colotta *et al* (1992) showed TNF-α delayed neutrophil apoptosis after 24 h culture *in vitro*. This is somewhat surprising as TNF-α is able to induce caspase activation (section 1.5.3.6). Many reports have since shown that TNF-α rapidly induces neutrophil apoptosis, with a recent report showing that TNF-α firstly induces neutrophil apoptosis in susceptible cells, but then is able to delay apoptosis (Murray *et al*, 1997). The reaction of neutrophils to TNF-α is modulated by other factors (see section 1.6.3) with the balance between the pro-apoptotic effect and the anti-apoptotic effect being reflected in the differing responses of individual neutrophils within a population. Similarly, activation of the Fas receptor (CD95) activates the caspases, leading to apoptosis, but this can also be modulated by other factors (see 1.6.3).

Other agents associated with inflammation are also able to modulate neutrophil apoptosis, such as lipopolysaccharide (LPS) which is a potent inhibitor of neutrophil apoptosis. This molecule also activates gene expression and primes the neutrophil. The chemotactic peptide fMLP, and complement fragment C5a are reported to have varying effects on apoptosis. C5a has been reported by two groups to have no effect on neutrophil apoptosis, but was used at concentrations in the nanomolar range (which trigger chemotaxis). In the micromolar range C5a has been shown to either delay apoptosis, or to accelerate apoptosis (see Table 1.2), so the action of this factor remains to be confirmed. fMLP (nanomolar to micromolar concentrations) was shown to act in the same manner as C5a by the groups who studied C5a (shown in Table 1.2), but later work by Haslett's group (Murray *et al*, 1997) show that fMLP has no effect on neutrophil apoptosis. These results may reflect the ability of fMLP to activate the neutrophil respiratory burst, producing reactive oxygen species, as indicated by Kettritz *et al* (1997). This work shows that addition of superoxide dismutase (which is involved with the neutralisation of ROS) is sufficient to prevent the slight increase in apoptosis observed in fMLP stimulated neutrophils (section 1.6.2). Leukotriene B_4 (LTB_4), another chemotactic molecule, will delay neutrophil

apoptosis, but another chemotactic agent, platelet activating factor (PAF) has no direct effect on neutrophil apoptosis. Both these agents prime neutrophils, indicating that priming alone is not sufficient to delay neutrophil apoptosis.

The heat that may be experienced by inflammatory neutrophils can induce apoptosis. as does ingestion of *Escherichia coli* (Watson *et al*, 1996). The proteolytic enzymes released by neutrophils in inflammation can also induce neutrophil apoptosis (Trevani *et al*, 1996). This acceleration of apoptosis may aid in the resolution of acute inflammation (section 1.4.3).

Migration of neutrophils across the endothelium delays neutrophil apoptosis, mediated through signalling following β_2 integrin occupation (Table 1.2). Apoptosis is accelerated by activation of L-selectin (Table 1.2), which is also involved in adhesion to the endothelium. Endothelial transmigration will accelerate apoptosis in unprimed neutrophils (Watson *et al*, 1997b). Primed neutrophils lose surface L-selectin, and upregulate their β_2 integrins (Watson *et al*, 1997b). These findings indicate that neutrophil apoptosis is modulated by the combined effects of many different factors *in vivo*.

Glucocorticoids (but not non-glucocorticoid steroids (Cox, 1995)) delay neutrophil apoptosis. Glucocorticoids exert their effects on target cells by increasing transcription of target genes, but do not prime neutrophils (Cox, 1995). The role of active gene expression in delaying neutrophil apoptosis is further demonstrated by reports showing that Actinomycin D (Act D), which inhibits transcription, cycloheximide (CHX) and saporin, which both inhibit translation, all accelerate entry of neutrophils into apoptosis (Table 1.2). These agents are also able to prevent GM-CSF delayed neutrophil apoptosis (Brach *et al*, 1992; Bergamaschi *et al*, 1996), indicating the activation of gene expression by GM-CSF is essential for the subsequent delay in apoptosis. This is in contrast to many other cell types, where

inhibition of protein synthesis protects from apoptosis. There is no acceleration of apoptosis in inflammatory or migrated neutrophils upon addition of CHX (Tsuchida *et al*, 1995), suggesting that the protective protein(s) have already been synthesised. This has led to the suggestion that the neutrophil is pre-programmed to undergo apoptosis, unless a protective factor is constantly produced (Stringer *et al*, 1996), whereas many other cell types are pre-programmed to survive, and require the synthesis of new protein(s) in order for apoptosis to be induced.

1.6.2 Oxidative stress

Oxidative stress can induce apoptosis in many cell types, although this process is not an essential feature of apoptosis (Jacobson, 1996). The neutrophil is able to produce reactive oxygen species (ROS) via the NADPH oxidase (Edwards, 1994), and it is possible that these ROS may initiate apoptosis in the neutrophil. Hypoxia potently delays neutrophil apoptosis (Table 1.2) but accelerates apoptosis in many other cell types (Hannah *et al*, 1995). Addition of H_2O_2 to the supraphysiological concentration of 1.5 mM induces neutrophil apoptosis (Hannah *et al*, 1995). These results suggest a role for oxidative stress in inducing neutrophil apoptosis, but the addition of antioxidants to the medium has no effect on the rate of apoptosis, except catalase, which can inhibit apoptosis, but by a mechanism that may be independent of ROS (Hannah *et al*, 1995). Many groups have examined the effect of antioxidants on neutrophil apoptosis, with the majority of publications indicating that antioxidants have no effect on spontaneous neutrophil apoptosis (Hannah *et al,* 1995; Watson *et al*, 1996a; Watson *et al*, 1996d; Mecklenburgh *et al*, 1996; Kasahara *et al*, 1997). However, these reports must be interpreted with care, as many of the antioxidants used are unlikely to gain entry into neutrophils, so detoxifying only extracellular oxidants. The ability of catalase to inhibit neutrophil apoptosis may indeed be by a mechanism independent of its antioxidative property, but could also be due to removal of extracellular H_2O_2, which is relatively long lived (compared to other ROS

produced by neutrophils, Edwards, 1994) so may be able to re-enter the cell, thereby leading to oxidative stress. The intracellular levels of H_2O_2 in neutrophils are diminished by addition of catalase into the culture media (Kasahara *et al*, 1997), suggesting H_2O_2 is indeed able to re-enter the cell.

Two reports indicate that SOD can delay spontaneous neutrophil apoptosis (Kettritz *et al*, 1997; Oishi *et al*, 1997), but this may be explained by the lack of serum used in the culture. Neutrophils cultured without serum enter apoptosis more quickly, and serum free media is far from physiological. Also, serum contains numerous antioxidants (Payne *et al*, 1995), without which the cultured neutrophils could be extra sensitive to oxidative damage, so added antioxidants would overcome this stress.

Glutathione (GSH) is a tripeptide found in many cells, which helps prevent oxidative stress, by converting H_2O_2 into water, and maintains protein disulphide bonds (Payne *et al*, 1995). Artificially increasing the intracellular concentration of GSH in neutrophils (by various means) has no effect on the rate of spontaneous apoptosis (Watson *et al*, 1996b; 1997a). Depletion of GSH does, however, increase the rate of spontaneous apoptosis, and this is not prevented by the addition of the cell permeable antioxidant, pyrolidine dithiocarbamate (Watson *et al*, 1996d). These observations led the authors to suggest that ROS are not mediators of neutrophil apoptosis, rather it is the redox state of the cell that can initiate apoptosis. The depletion of GSH resulted in increased protein tyrosine phosphorylation, and inhibition of this phosphorylation prevented the accelerated apoptosis following GSH depletion. Therefore, there is a signalling pathway which in response to GSH depletion triggers neutrophil apoptosis. The inability of pyrolidine dithiocarbamate to prevent apoptosis following GSH depletion implies it is the redox state of the cell, not ROS, that induce tyrosine phosphorylation and apoptosis. However, ROS production would be predicted to deplete GSH, as GSH would be utilised to remove H_2O_2

produced by superoxide dismutase during the oxidative burst. Therefore, production of ROS could also induce neutrophil apoptosis.

The rate of neutrophil apoptosis in chronic granulomatous disease (CGD) in which the neutrophil has a diminished or absent ability to produce ROS (Edwards, 1994), is significantly slower than for normal neutrophils *in vitro* (Kasahara *et al*, 1997). This would also appear to implicate ROS in the apoptosis of neutrophils, with the authors concluding ROS are major mediators of apoptosis in neutrophils (Kasahara *et al*, 1997). Addition of H_2O_2 restores CGD neutrophils to a normal rate of apoptosis (Kasahara *et al*, 1997). Taken together these reports indicate that ROS are able to induce neutrophil apoptosis, and that the depletion of GSH is a mechanism by which apoptosis can be triggered following ROS production.

1.6.3 Fas and TNF-α induced neutrophil apoptosis

Both Fas and TNF-α rapidly induce apoptosis in mature neutrophils (Table 1.2). Curiously, induction of apoptosis by TNF-α cannot induce apoptosis in bone marrow neutrophils or in migrated neutrophils (Tsuchida *et al*, 1995). The addition of CHX at a concentration too low to affect spontaneous apoptosis, increases the susceptibility of mature neutrophils to TNF-α, and also restores TNF-α induced apoptosis to bone marrow neutrophils, without affecting the susceptibility of migrated neutrophils (Tsuchida *et al*, 1995). Transmigration results in much fewer TNF-α binding sites per cell, and also a 20-fold increase in the dissociation constant, which may explain the resistance of inflammatory neutrophils to TNF-α mediated apoptosis (Tsuchida *et al*, 1995). This indicates that *de novo* protein synthesis is involved in neutrophil resistance to TNF-α induced apoptosis. This is in agreement with the role of NF-κB in resistance to TNF-α induced apoptosis (section 1.5.3.7). The glucocorticoid dexamethasone also inhibits TNF-α induced apoptosis (Kato *et al*, 1995) as does LPS if added before TNF-α (Hachiya *et al*, 1995), and both

treatments may induce *de novo* protein synthesis to be effective. Surprisingly, neutrophils cultured for 6 h are resistant to TNF-α induced apoptosis, as are PAF primed neutrophils (Murray *et al*, 1997). The expression of both TNF receptors (p55 and p75) decreased to around 30% in 6 h aged neutrophils, which may explain the resistance of these cells. This work also demonstrates the need for both the TNF (p55 and p75) receptors in neutrophil apoptosis. TNFR55 mediates the priming signal and will result in delayed apoptosis if TNFR75 is blocked, whereas both receptors together are needed to signal for cell death.

Neutrophils express both Fas and the Fas ligand (FasL) (Iwai *et al*, 1994), and also release soluble Fas when cultured *in vitro* (Liles *et al*, 1996). It has been suggested that the co-expression of both Fas and FasL by neutrophils will commit neutrophils to apoptosis via engagement of Fas by the FasL (Liles & Klebanoff, 1995). Addition of a FasL neutralising agent (Ab or soluble Fas) has been shown to retard spontaneous neutrophil apoptosis (Liles *et al*, 1996; Hsieh *et al*, 1997). Liles *et al* (1996) conclude that the Fas/FasL pathway is an important, but not exclusive, mechanism involved in the control of spontaneous neutrophil apoptosis.

Apoptosis induced by Fas can be modulated by a number of agents. Liles *et al* (1996) showed that G-CSF reduces Fas induced apoptosis to about 50 % of those cells killed by treatment with FasL alone. GM-CSF, IFN-γ, TNF-α and dexamethasone also reduce Fas induced apoptosis, but less effectively than G-CSF, whilst inhibition of tyrosine kinases with genistein also reduces Fas induced apoptosis with a similar potency to G-CSF (Liles *et al*, 1996). Similar results with G-CSF and GM-CSF were obtained by Hu *et al* (1997), although they found that GM-CSF was more effective than G-CSF. Similar to TNF-α, Fas induced apoptosis is diminished by migration, CD11b cross-linking, and pre-treatment with LPS (Watson *et al*, 1997a). The protection conferred by LPS and CD11b cross-linking is only transient, and by 4 h the effect is lost (Watson *et al*, 1997a).

Superoxide anion will inhibit Fas-mediated death in Jurkat cells without the need for protein synthesis or phosphorylation (Clément & Stamenkovic, 1996), but the mechanism for this is unknown. Watson *et al* (1997a) showed that in the neutrophil LPS treatment, CD11b cross-linking or migration can all increase intracellular levels of GSH. These treatments slightly increase (double) the formation of ROS, whereas full activation of the respiratory burst with PMA results in a 14-fold increase in ROS. This slightly increased production of ROS may account for the increase in GSH levels. Alternatively, the increase in GSH levels may be a physiological response to recruitment of neutrophils to a site of inflammation, conferring enhanced protection from ROS. This work clearly shows that increasing GSH within the neutrophil (by addition of GSH, or N-acetylcysteine), to similar levels found after activation, reduces the potency of Fas in inducing apoptosis, though not to the same level as LPS or CD11b cross-linking. The use of buthionine sulfoximine (BSO), which prevents synthesis of new GSH, allowed further investigation of the role of GSH. LPS was still effective in delaying spontaneous apoptosis when BSO was added, but BSO almost completely prevented LPS mediated protection from Fas induced apoptosis. The effect of LPS on Fas-induced apoptosis was almost completely restored by addition of GSH to the levels found after LPS treatment. These results suggest that increasing GSH alone confers some resistance to Fas induced apoptosis, but that the full effect of LPS is mediated by factors other than the level of GSH.

It is clear that induction of apoptosis by either TNF-α, or Fas activation is controlled by the status of the neutrophil, and that many factors can effect the response to either of these stimuli.

1.6.4 Involvement of intracellular signalling in neutrophil apoptosis

The complexity of the interaction between different factors in controlling entry into neutrophil apoptosis is demonstrated by the effects of different signalling molecules and pathways on this process. Agents that give a transient rise in intracellular free calcium ($[Ca^{2+}]_i$) are able to delay spontaneous neutrophil apoptosis (Table 1.2), in contrast to many other cell types where apoptosis is accelerated (Whyte *et al*. 1993a). It was also shown by Whyte *et al* (1993a) that removal of free calcium accelerates apoptosis in the neutrophil. This action may involve protein kinase C (PKC), which is activated by $[Ca^{2+}]_i$, as inhibition of PKC with staurosporine (10 μmol/L) accelerates neutrophil apoptosis (Cousin *et al*, 1996b), although care must be taken with the interpretation of these results, as at 10μmol/L staurosporine will inhibit other protein kinases (Badwey *et al*, 1991). However, cross-linking of L-selectin (which accelerates neutrophil apoptosis) or addition of fMLP or C5a (which do not affect neutrophil apoptosis) to neutrophils also increases $[Ca^{2+}]_i$. Therefore, not all agents that give an increase in $[Ca^{2+}]_i$ delay neutrophil apoptosis, indicating that this signal can be modulated by other signals. Alternatively, in some circumstances the rise in $[Ca^{2+}]_i$ may have no role in signalling to apoptosis, as rises in $[Ca^{2+}]_i$ can result in activation of different signal transduction pathways depending on the nature of the calcium signal (Berridge, 1997).

Cyclic AMP (cAMP) has also been implicated in controlling entry of neutrophils into apoptosis, with agents that increase cAMP (such as PDE_2, PGE_2 mimics. forskolin, and cAMP analogues) delaying spontaneous apoptosis (Table 2.1). and also protecting against Fas induced apoptosis (Yasui *et al*. 1997). One report (Aoshiba *et al*, 1995) showed an increase in apoptosis following a rise in cAMP. but these results may be misleading because in this report neutrophils were cultured in serum free media, which accelerates apoptosis. The activation of protein kinase A (PKA) is necessary for the cAMP mediated delay in apoptosis. as inhibition of PKA

negates this action (Rossi *et al*, 1995). This delay in apoptosis following a rise in cAMP is thought to be due to regulation of genes, transcription factors and proteins involved in apoptosis (Rossi *et al*, 1995).

Not all agents that cause an increase in cAMP levels delay neutrophil apoptosis. The phosphodiesterase inhibitor theophylline, which mediates an increase in cAMP, causes an acceleration of neutrophil apoptosis, despite the rise in cAMP (Yasui *et al*, 1997). This again demonstrates that a signal which would be predicted to delay apoptosis may be modulated by other factors.

Inositol phosphates, and phosphatidylinositol phosphates are also involved in the modulation of neutrophil apoptosis, as inositol hexakisphosphate addition both primes the neutrophil and delays apoptosis (Murray *et al*, 1997). On the other hand inhibition of phosphatidylinositol 3-OH kinase by wortmannin increases the potency of TNF-α in inducing neutrophil apoptosis (Murray *et al*, 1996).

The inhibition of apoptosis by GM-CSF is prevented by inhibition of protein tyrosine kinases, with either genistein or herbimycin A (Yousefi *et al*, 1994). Conversely, genistein or herbimycin A are quite potent inhibitors of Fas-induced apoptosis in neutrophils (Liles *et al*, 1996). The tyrosine kinase Lyn is essential for transducing anti-apoptotic signals from GM-CSF (Wei *et al*, 1996). Lyn kinase is activated by a number of neutrophil stimulants (Gaudry *et al*, 1995), but many of these have no effect on apoptosis (e.g. IL-8). Thus, Lyn kinase activity is not always associated with delayed neutrophil apoptosis. Inhibition of tyrosine phosphatases with phenylarsine oxide (PAO) will accelerate apoptosis at low concentrations (0.1-0.5 μmol/L), which only result in a slight increase in phosphorylation on tyrosine residues (Simon *et al*, 1995). Apoptosis is, however, delayed at higher concentrations of PAO (1.5 μmol/L) where tyrosine phosphorylation is further

enhanced (Simon *et al*, 1995). Inhibitors of protein phosphatases 1 and 2A (okadaic acid and calyculin A), while having no effect on spontaneous neutrophil apoptosis, do prevent dexamethasone in delaying apoptosis (Cousin *et al*, 1996a). A further example of the importance of tyrosine phosphorylation in controlling neutrophil apoptosis is the IL-10 mediated inhibition of apoptosis delayed by LPS, TNF-α, IFN-γ, G-CSF, GM-CSF and IL-12 (Keel *et al*, 1997). This report showed that this inhibition is likely mediated by decreasing the level of tyrosine phosphorylation following treatment with these apoptosis delaying agents.

The sphingolipid ceramide has been shown to induce apoptosis in many cell types and is involved in signalling following treatment with TNF-α (Hannun & Obeid, 1995). Ceramide is involved in regulating the response to TNF-α in neutrophils, by delaying the cell spreading and respiratory burst triggered by TNF-α (Fuortes *et al*, 1996). However, ceramide does not induce apoptosis in neutrophils (Murray *et al*, 1996), giving a further example of the different responsiveness of neutrophils to a treatment which modulates apoptosis in other cell types.

The effects of some agents can be enhanced when used in combination. For example, dexamethasone-delayed apoptosis can be enhanced (when used at the optimal concentration) by GM-CSF, but not by LPS (Cox, 1995). This implies that there is more than one pathway that leads to delayed apoptosis in neutrophils. Whether these pathways act differently, or converge at a common point to control apoptosis, is unknown at present.

1.6.5 Bcl-2 family and Caspases

As discussed earlier (section 1.5.3.1-9) the Bcl-2 family and caspases are intimately associated with the control of entry into, and execution of the apoptotic program.

These proteins are the subject of intense research by many groups, but there has been relatively little research into their involvement in neutrophil apoptosis.

The caspase-3 (CPP32) protein is expressed in neutrophils at a relatively high level in comparison to other cells and tissues, which may reflect the high rate of apoptosis found in neutrophils (Krajewska *et al,* 1997). The expression of ICE (caspase-1) mRNA is increased in HL-60 cells differentiating into granulocytes, but there is no increase in caspase-3 mRNA (Watson *et al*, 1997c). There is also an increase in the levels of active caspases-1 and -3 in these differentiated cells, but this may merely reflect the increased rate of apoptosis after differentiation, rather than being the cause of this increase. Similarly, the levels of active caspases-1 and -3 are decreased in LPS-stimulated or inflammatory neutrophils (Watson *et al*, 1997), but again this may merely reflect the lower rate of apoptosis.

The role of Bcl-2 family members in neutrophil apoptosis is far from clear. As discussed in sections 1.5.2-3 the Bcl-2 family is located on the mitochondrial membrane, endoplasmic reticulum and nuclear envelope. The anti-apoptotic proteins Bcl-2 and Bcl-X_L are able to prevent caspase activation by preventing release of cytochrome c from the mitochondria, which requires the presence of these proteins on the mitochondrial membrane (Yang *et al*, 1997; Kluck *et al*, 1997). The localisation of Bax dimers to mitochondria is reported to be essential for Bax mediated cytotoxicity (Zha *et al*, 1996a). The neutrophil has very few, very small mitochondria (Bainton, 1980; Edwards, 1994), which would suggest little role of the Bcl-2 family in neutrophil apoptosis. However, Bcl-2 has been reported to be effective in inhibiting apoptosis when targeted to only the endoplasmic reticulum (Zhu *et al*, 1996), which indicates that the Bcl-2 family can exert an effect without mitochondrial localisation.

The Bax protein is expressed at relatively high levels in mature neutrophils (Ohta *et al*, 1995), which in view of the sparse mitochondria suggests Bax need not be located on mitochondria to exert its apoptotic action. There are a few reports of Bcl-2 being expressed in mature neutrophils (Kiehntopf *et al*, 1995; 1996), with one report even indicating that LPS treatment up-regulates Bcl-2 expression (Hsieh *et al*, 1997). However, the majority of reports state that mature neutrophils are essentially devoid of Bcl-2 (Afford *et al*, 1992; Hannah *et al*, 1994; Iwai *et al*, 1994; Lagasse & Weissmann, 1994; Hannah *et al*, 1995; Krajewski *et al*, 1995; Liles & Klebanoff, 1995; Wei *et al*, 1996; Watson *et al*, 1997c). Bcl-X_L is also absent from mature neutrophils (Krajewski *et al*, 1994; Ohta *et al*, 1995; Sanz *et al*, 1997), although Krajewski *et al* (1994) did find a small minority of granulocytes having slight Bcl-X_L immunoreactivity. Liles & Klebanoff (1995) also report the absence of Bcl-X in mature neutrophils, but have not fully published these observations. The paper of Ohta *et al* (1995) must be interpreted with care as they used post-nuclear extracts (nuclei removed, after lysis, by centrifugation) for their western blots. The work of Sanz *et al* (1997) does not investigate neutrophils, but examines HL-60 cells induced to differentiate towards neutrophil-like cells. The anti-apoptotic Mcl-1 protein has also been reported to be absent in mature neutrophils (Ohta *et al*, 1995; Krajewski *et al*, 1995; Yang *et al*, 1996a), but again these results must be interpreted with care: the Ohta paper used neutrophil extracts devoid of nuclei, the Krajewski paper looked only at bone marrow neutrophils, and the Yang paper used differentiating ML-1 cells which did show a slight induction of Mcl-1 after 1h but the authors concluded Mcl-1 was not induced.

The only other report to my knowledge that investigates Bcl-2 family members found in neutrophils shows that the anti-apoptotic protein A1 (Bfl-1/GRS) is up-regulated when 32D cl3 cells (murine) are differentiated into neutrophils with G-CSF (Lin *et al*, 1993). The authors showed that this protein was found in murine T cells, and myeloid cells, but not B cells. There have been no reports of this protein in

human neutrophils to my knowledge, though the mRNA is abundant in human leukocytes (Karson *et al*, 1996; Kenny *et al*, 1997), bone marrow and HL-60 cells (Choi *et al*, 1995; Moreb & Schweder, 1997). The relative abundance of A1 in different leukocyte species has not been tested.

The artificial expression of Bcl-2 protein in neutrophils does delay the morphological features of apoptosis and DNA fragmentation, but does not prevent the recognition of these cells by macrophages (Lagasse & Weissmann, 1994). This suggests that Bcl-2 is not normally involved in the control of neutrophil apoptosis, but does indicate that anti-apoptotic Bcl-2 family members can control some of the processes involved in neutrophil apoptosis. The morphological features of apoptosis in the neutrophil (including phosphatidylserine externalisation) can also be prevented by inhibition of the protease calpain, but cells treated in this way are still recognized by phagocytes (Knepper-Nicolai *et al*, 1996; 1996a). Together, these results suggest separate pathways exist within the neutrophil controlling flagging of the cell for phagocytosis, and other features of apoptosis.

Despite the numerous reports investigating neutrophil apoptosis, the molecular mechanisms by which entry into apoptosis is triggered remain undefined. The spontaneous apoptosis of the neutrophil is regulated in part by the Fas/FasL pathway (Liles *et al*, 1996). The induction of apoptosis by Fas is prevented by various treatments in the neutrophil, with the level of GSH implicated in this protection (Watson *et al*, 1997a). However, this mechanism has only been investigated for a few treatments that prevent Fas mediated apoptosis, so the possibility remains that this mechanism is not exclusively responsible.

The fact that some agents that delay neutrophil apoptosis are more potent when used in combination implies that more than one mechanism is involved in delaying neutrophil apoptosis. This is also indicated by the different consequences of altering

the levels of signalling molecules within the neutrophil. Many priming agents delay neutrophil apoptosis, but this does not hold for all priming agents. Indeed, there are examples of agents which delay neutrophil apoptosis without priming (e.g. glucocorticoids). Many of the agents that delay neutrophil apoptosis also enhance gene expression, suggesting that continued expression of key genes is required to prevent entry into apoptosis.

The presence of Bax would indicate that this molecule is involved in triggering apoptosis in the neutrophil, but it is not known how this may be controlled, as no anti-apoptotic Bcl-2 family members have been conclusively identified as yet in mature human neutrophils. The expression of Bcl-2 in the neutrophil does not prevent recognition of aged neutrophils as apoptotic (Lagasse & Weissmann, 1994), but not all members of the Bcl-2 family act in the same manner. Bcl-X_L, but not Bcl-2 rescues a B cell line from apoptosis (Gottschalk *et al*, 1994). Bcl-2 and Mcl-1 show different temporal and spatial patterns of expression (Yang *et al*, 1995a). The Mcl-1 protein is reported to interact with FADD via a region unique to Mcl-1 amongst the Bcl-2 related proteins. It is possible therefore that an anti-apoptotic Bcl-2 family member (other than Bcl-2) can control entry into neutrophil apoptosis, via interaction with Bax and other proteins.

1.7 Aims of this Thesis

There are many diseases in which the neutrophil is implicated (Malech & Gallin, 1987; Edwards & Hallet, 1997). Manipulation of neutrophil apoptosis could be of benefit to many of these problems. Acceleration of apoptosis may benefit inflammatory conditions, whereas delaying apoptosis may benefit neutropenic patients. The aims of this thesis are therefore to investigate the mechanisms controlling neutrophil apoptosis, in order to provide a better understanding of the

molecular processes underlying modulation of apoptosis in these clinically important cells.

The first aim of this thesis is to develop apoptosis assays in this laboratory, to enable accurate determination of the effects of various agents on the rate of apoptosis. This will involve the use of a multiple assays for the determination of apoptosis, to ensure the applicability of individual assays in measuring neutrophil apoptosis.

In particular the relationship between protein synthesis and neutrophil apoptosis will be investigated, using a variety of agents known to enhance gene expression in neutrophils and other cell types, by different mechanisms. The role of histone acetylation in controlling entry into apoptosis in neutrophils will also be investigated.

The maintenance of CD16 surface expression in neutrophils protected from apoptosis occurs by an undefined mechanism. I aim to identify the mechanisms responsible for this maintenance of expression.

Bacterial superantigens are reported to have direct effects on neutrophils *in vitro* (Hensler *et al*, 1991; 1993), but their effect on neutrophil apoptosis has not been addressed. I shall therefore investigate the role of these superantigens in delayed neutrophil apoptosis.

Finally, I aim to determine the role of the Bcl-2 family proteins in controlling neutrophil apoptosis, as there is as yet no clear role for this family of proteins in controlling apoptosis in the neutrophil.

CHAPTER 2 MODULATION OF NEUTROPHIL APOPTOSIS BY TRANSCRIPTIONAL ACTIVATORS

2.1 Introduction

The spontaneous apoptosis of the neutrophil can be delayed by various treatments (section 1.6.1), such as pro-inflammatory cytokines, colony stimulating factors, and glucocorticoids (see Table1.2 for detailed list of references). Many of the agents that delay neutrophil apoptosis also increase gene expression, whilst inhibition of RNA or protein synthesis accelerates neutrophil apoptosis. These observations imply a role for active protein synthesis in neutrophil survival. There also appears to be a link between the priming process and delayed apoptosis, with many of the agents that are able to delay apoptosis also priming the neutrophil (i.e. Lee *et al*, 1993; and see table 1.2). Priming of the neutrophil can lead to increased gene expression (Edwards, 1994), again implicating active gene expression in rescue from apoptosis. However, not all priming agents delay apoptosis, for example fMLP and PAF (section 1.6.1). There are also agents which can delay neutrophil apoptosis without priming, such as glucocorticoids and IL-15 (i.e. Cox, 1995; Girard *et al*, 1996).

The drugs sodium butyrate, hexamethylene-bis-acetamide (HMBA) and 5-azacytidine (5-Azac) are all able to increase gene expression in other cell types by the mechanisms outlined below. Sodium butyrate causes histone hyperacetylation, by inhibiting histone deacetylase, which alters chromatin structure and results in a general increase in total RNA synthesis in many cell types (Kruh, 1982). Butyrate can also increase DNA excision repair, probably as a result of histone hyperacetylation increasing accessibility to chromatin (Smerdon *et al*, 1981; Dresler, 1985). There are also changes in the phosphorylation patterns of many nuclear proteins as a result of butyrate treatment (Boffa *et al*, 1981). The general increase in gene expression observed following butyrate treatment is not associated with increased levels of all gene products, for

example the oncogene p53 is down-regulated in 3T6 mouse fibroblasts by butyrate (Wintersberger & Mudrak, 1984).

The drug 5-azacytidine is also able to activate gene expression in many systems (Doerfler, 1983). This cytidine analogue is able to bind to and inhibit DNA methyltransferases, which would normally methylate cytosine residues in chromatin. The degree of CG methylation in chromatin is related to the transcriptional activity: a high degree of DNA methylation correlates with gene inactivation, whereas low levels of DNA methylation are associated with transcriptionally active parts of the genome (i.e. house keeping genes lack methyl groups) (Kolata, 1985).

Hexamethylene-bis-acetamide (HMBA) causes DNA demethylation through the replacement of 5-methylcytosine with cytosine (Bernstein & Kappes, 1988; Razin *et al*, 1986). The ability of HMBA to cause DNA hypomethylation suggests that this agent will have a similar effect to 5-azacytidine on gene expression.

DNA methylation is also modulated by butyrate treatment, but rather than hypomethylation of active genes there is increased methylation of active chromatin following butyrate treatment (Boffa *et al*, 1994). These authors suggest the hypermethylation of active genes following butyrate treatment is a result of increased accessibility of DNA methyltransferases to these regions of chromatin, though other factors are also thought to be involved. This hypermethylation does not prevent increased gene expression of the active genes, and the significance of this hypermethylation is not known.

The agents butyrate, 5-azacytidine and HMBA all increase gene expression in human neutrophils (Stringer, 1994; Humphreys *et al*, 1988), with a similar pattern of proteins upregulated by all treatments, as analysed by 2D-SDS-PAGE (Stringer, 1994). The pattern of gene expression is also similar to that seen with GM-CSF treated

neutrophils, with GM-CSF, butyrate, HMBA, and 5-azacytidine all giving an approximately two-fold increase in protein biosynthesis (Stringer, 1994).

Butyrate is known to delay neutrophil apoptosis (Stringer, 1994; Stringer *et al*, 1996) but the effects of 5-azacytidine and HMBA are less clear. Neither of butyrate, 5-azacytidine nor HMBA prime the neutrophil (Stringer, 1994). These agents may thus be used to delineate the mechanisms that control the priming process and rescue from apoptosis. Such agents could be of benefit to patients of cytotoxic therapy who become neutropenic. By extending the lifespan of the mature neutrophil the severity of neutropenia may be decreased, thereby lowering the risk of morbidity and mortality from infections. The priming of neutrophils by apoptosis delaying agents such as cytokines can cause problems, such as pulmonary accumulation and increased adhesion to endothelia, which impairs the normal function of neutrophils. Diminishing the severity of neutropenia without priming the neutrophil is greatly preferable to such treatments which also stimulate adhesion.

The ability of these transcriptional activators to delay neutrophil apoptosis was therefore investigated and compared to GM-CSF, by measurement of neutrophil apoptosis assessed by DNA fragmentation, cellular morphology, preservation of function, chromatin condensation and AnnexinV binding to phosphatidylserine residues exposed on the cell surface.

2.2 Materials and Methods

2.2.1 Materials

Neutrophil isolation medium was from Cardinal Associates (Sante Fe, NM), RPMI 1640 was from ICN Biomedicals Ltd. (Thame, Oxfordshire, UK), fetal calf serum and

L-Glutamine were from Gibco BRL (Paisley, UK). Recombinant human GM-CSF (non-glycosylated) was a gift from Glaxo (Greenford, UK) with an activity of > 1.5 MU/mg. Cycloheximide was from Calbiochem-Novabiochem Ltd. (Nottingham, UK). 30% solution of acrylamide/bis-acrylamide was from Severn Biotech Ltd. (Kidderminster, UK). All other specialist reagents were from Sigma (Poole, UK), with all other reagents used being of the highest quality available.

2.2.2 Neutrophil isolation

Neutrophils were isolated from heparinized venous blood from healthy volunteers by one step centrifugation through Neutrophil Isolation Medium (NIM) as described in the manufacturer's instructions. Briefly, ~13 mL blood was layered over ~7 mL NIM, centrifuged at 600g for 15-20 min, then the neutrophil layer collected. After hypotonic lysis to remove contaminating erythrocytes (Edwards *et al*, 1987), neutrophils were resuspended in RPMI 1640 medium supplemented with 2.5% fetal calf serum and 2 mmol/L L-glutamine, and counted using a Fuchs-Rosenthal haemocytometer slide. In all cases purity was > 97% as assessed by Wrights staining, with viability of neutrophils (as assessed by trypan blue exclusion) > 95% after purification.

2.2.3 Neutrophil culture

Neutrophils were resuspended at 5 x 10^6 cells/mL in RPMI 1640 medium supplemented with 2.5% fetal calf serum and 2mmol/L L-Glutamine, and incubated in polypropylene conical tubes at 37^0C with gentle agitation in the absence (control) or presence of: rhGM-CSF (50 U/mL); sodium butyrate (0.4 mmol/L); 5-azacytidine(5-azac) (50 μmol/L); hexamethylene-bis-acetamide (HMBA) (1 mmol/L); cycloheximide (CHX) (10 μg/mL). At various times (as indicated) cells were removed and processed as described below.

2.2.4 DNA gel electrophoresis

The extraction and electrophoresis of fragmented DNA was assessed using a previously described method (Collota *et al*, 1992) with some modifications. Briefly, 5 x 10^6 neutrophils were washed with cold phosphate-buffered saline (PBS) (10 mmol/L potassium phosphate; 0.9%(w/v) NaCl, pH 7.4), lysed with 10 mmol/L Tris, pH 7.5; 1 mmol/L EDTA; 0.2%(v/v) Triton X-100, then centrifuged at 13,000g at 4°C for 20 min exactly as described above. Centrifugation resistant low molecular weight DNA in the supernatants was transferred to separate microfuge tubes, mixed with 20 μg/mL RNase, and incubated for 1 h at 37°C. Low molecular weight DNA was then extracted twice with 1 volume phenol/chloroform/iso-amyl alcohol (25:24:1, respectively) and once with chloroform/iso-amyl alcohol (24:1). DNA in the extracts was then precipitated with 0.5 mol/L NaCl and 1 volume iso-propanol for 18 h at -20°C. The samples were then centrifuged for 10 min at 13,000g, and 200 μL of 70% ethanol was gently added to the precipitate; the samples were re-centrifuged for 2 minutes at 13,000g, air dried, and resuspended in water. Loading buffer (2.5%(w/v) ficoll; 0.025%(w/v) bromophenol blue; and 0.025%(w/v) xylene cyanol) was added to each sample; these were heated at 75°C for 5 min, snap cooled, and then electrophoresed along with DNA markers on a 1%(w/v) agarose gel containing 1 μg/mL ethidium bromide at 30 V in Tris-acetate buffer. DNA was visualised under UV and then photographed.

2.2.5 Quantitation of fragmented DNA

Quantitation of low molecular weight DNA was carried out as described previously (Collota *et al*, 1992). Briefly, 2.5 x 10^6 neutrophils were centrifuged in microfuge tubes at 6,000g for 2 min, washed with cold PBS and then lysed as above. After 15 min of incubation on ice, low and high molecular weight DNA were separated by centrifugation at 13,000g at 4°C for 20 min. Centrifugation resistant low molecular

weight DNA in the supernatant was transferred to separate tubes and precipitated overnight at 4°C with 12.5%(w/v) trichloroacetic acid (TCA). Cold TCA was also added to the pellets to a concentration of 12.5%(w/v), which were left overnight at 4°C. Samples were then centrifuged at 13,000g at 4°C for 7 min, and DNA in the precipitates was extracted with 30 µL of 5 mmol/L NaOH and 30 µL of 1 mol/L perchloric acid at 70°C for 20 min. Then, 120 µL diphenylamine reagent (Burton, 1955) was added to each sample and incubated overnight at 37°C. 120 µL from each sample was then transferred to flat-bottomed 96-well plate and the absorbance at 600 nm was measured using a Bio-Rad 3550 plate reader. The percentage fragmented DNA was then calculated from values obtained for low and high molecular weight DNA from each sample, in duplicate.

2.2.6 Morphological assessment of apoptosis

Cytocentrifuge preparations of 10^5 cells, made up to a volume of 200 µl with sterile PBS, were prepared at timed intervals during incubation, using a Shandon cytospin centrifuge. Cells were then stained using May-Grünwald-Giemsa. Cells were assessed for morphological changes characteristic of apoptosis (nuclear condensation, vacuolation), with the use of a x40 objective. At least 500 cells per slide were counted. This method has been previously demonstrated to correlate closely with other parameters of apoptosis (Savill *et al*, 1989).

2.2.7 NADPH oxidase activity

Chemiluminescence was assayed in a reaction mixture containing an equal volume of neutrophils from culture and 10 µmol/L luminol. After the addition of stimuli (fMLP at 1 µmol/L or PMA at 0.1 µg/ml), photon emission was measured using a LKB Wallac 1251 luminometer in a final volume of 1 ml (Edwards *et al*, 1987). Chemiluminescence relative to control cultures were then calculated.

2.2.8 Propidium iodide staining of chromatin

Neutrophils were processed for propidium iodide staining similarly to previously published methods (Dransfield *et al,* 1994; Nicoletti *et al*, 1991) with some modifications. Neutrophils were removed from culture and washed 3 times in cold PBS, before fixing in cold 70% ethanol for 15 min. Cells were washed once more, before resuspending in cold PBS with 2 μg/mL propidium iodide and leaving in the dark for 1 h before analysis using a Becton Dickinson Ortho Diagnostics Cytron analyser. Fluorescence distributions represent a total of 5,000 gated events.

2.2.9 Annexin V isolation and FITC-labelling

Isolation of Annexin V

Annexin V was purified from chicken liver following the method of Bousted *et al* (1993; 1988). Briefly, tissue (50g) was homogenised in 250 mL of: 0.15 mol/L NaCl; 5 mmol/L EGTA; 0.25 mmol/L phenylmethanesulphonyl fluoride (PMSF); 10 mmol/L Hepes pH 7.4 and centrifuged in a Sorvall SS-34 rotor at 25,000g for 30 min. $CaCl_2$ was added to the supernatant to a final concentration of 6 mmol/L. After 15 min on ice, the fraction was centrifuged for 30 min at 25,000g. The pellet was washed twice with 100 mL of: 0.15 mol/L NaCl; 1 mmol/L $CaCl_2$; 10 mmol/L Hepes pH 7.4, by resuspension and centrifugation (Sorvall SS-34 rotor, 25000g for 30 min) and then twice with 100 mL of: 1mmol/L $CaCl_2$; 10 mmol/L Hepes pH 7.4. The pellet was then resuspended in 15 mL of: 10 mmol/L EGTA; 10 mmol/L Hepes pH 7.4 and centrifuged at 100,000g for 30 min in a Sorvall AH-650 rotor. All steps were performed at 4°C. The final supernatant was dialysed against 20 mmol/L Hepes pH 7.4, and then applied to a 1.0 x 5.0 cm column of DEAE-cellulose (DE52, Whatman, Maidstone, England) equilibrated in the same buffer. After elution of unabsorbed material, a linear gradient (40 mL) of 0-0.6 mol/L NaCl in 20 mmol/L Hepes pH 7.4 was used for elution. The column was run at 12 mL/h and 1 mL fractions were

collected. Annexin V was the major protein peak, eluting at approximately 0.1mol/L NaCl. Protein concentrations were measured using the method of Bradford (Bradford. 1976). The isolated annexin V was subjected to SDS-PAGE and was present as a single polypeptide of 32.5 kDa. All annexin V used was >99% pure.

FITC labelling of Annexin V

0.5 mg of Annexin V was labelled with FITC overnight at 4°C in 0.1 mol/L $NaHCO_3$ pH 9.5 using an 8 mol/L excess of FITC in a total volume of 0.5 mL. This mixture was then applied to a 1.0 x 5.0 Sephadex G-25 column (Pharmacia) and eluted with 20 mL of cold PBS pH 7.4, at a rate of ~1 mL/min. FITC-Annexin V was eluted as a single peak, well separated from unincorporated FITC. The final FITC-Annexin V preparation was adjusted to 1 μg/10 μL in PBS pH 7.4 with 2 moles FITC incorporated per mole Annexin V.

2.2.10 AnnexinV-FITC labelling of neutrophils

Cultured neutrophils were washed twice in PBS pH 7.2, then resuspended in 0.75 mL of binding buffer (1 mmol/L Hepes pH 7.4; 140 mmol/L NaCl; 2.5 mmol/L $CaCl_2$) (Vermes *et al*, 1995). FITC-Annexin V was added to 1 μg/mL and samples were incubated in the dark for 10 min before flow cytometric analysis. The binding of FITC-Annexin V was Ca^{++} dependent as assessed by determination of binding in media devoid of $CaCl_2$. Fluorescence distributions represent a total of 5000 gated events.

2.2.11 Statistical analysis

All experimental data is expressed as mean ± SD. Where sample numbers were not equal, paired data samples were analysed using the students t-test, with statistical significance defined at $P \leq 0.05$ or $P \leq 0.01$.

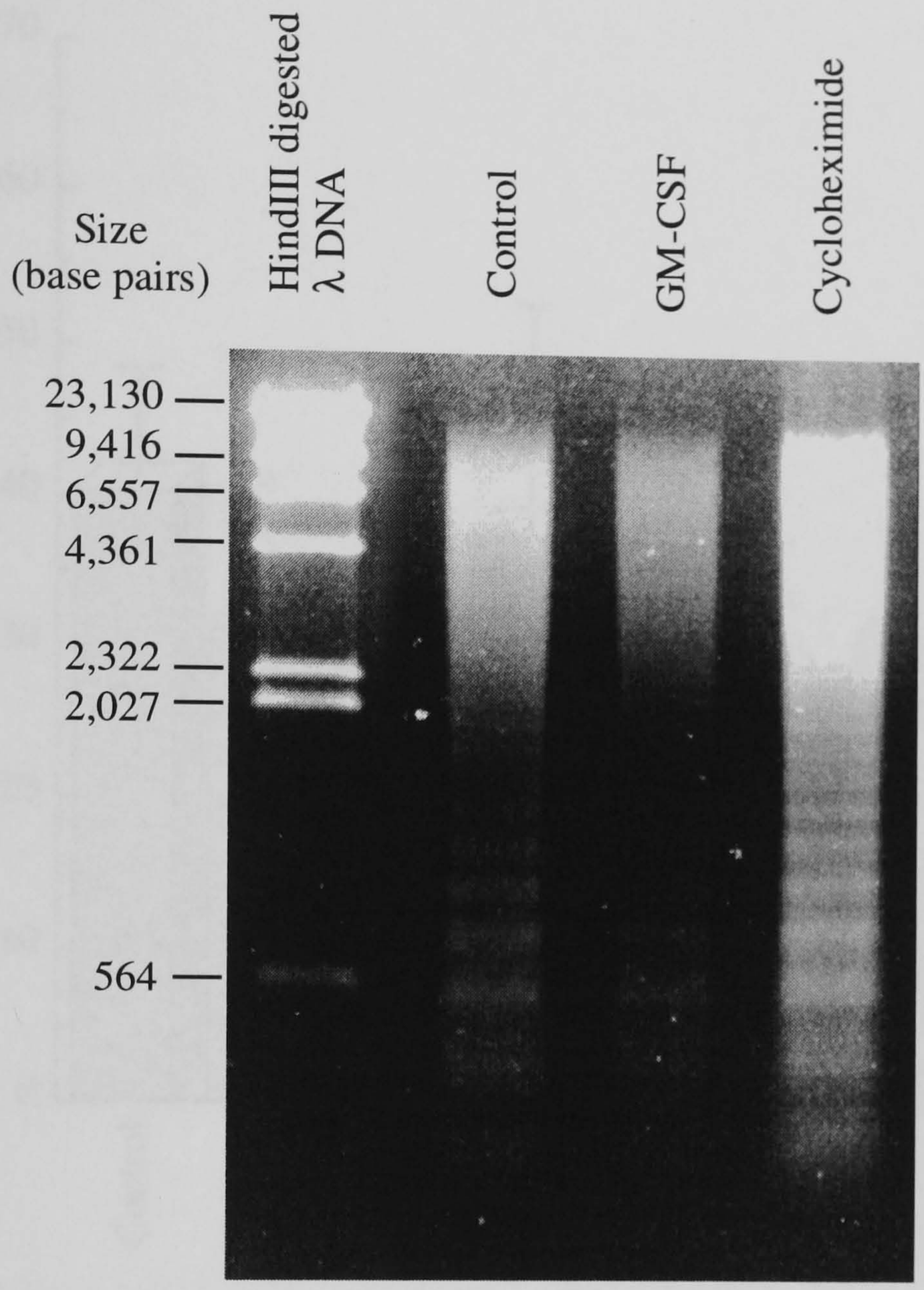
2.3 Results

2.3.1. Internucleosomal chromatin cleavage of cultured neutrophils

The cleavage of chromatin into multi-nucleosome sized fragments indicates that cell death has occurred via apoptosis (section 1.5.2). Neutrophils were cultured for 13 h before extraction of fragmented chromatin and analysis by agarose gel electrophoresis as shown in Figure 2.1. DNA laddering was seen in all culture conditions, confirming death via apoptosis in cultured neutrophils. The degree of DNA fragmentation was less marked for GM-CSF treated neutrophils, whilst treatment with the apoptosis accelerator CHX gave a greater degree of fragmentation.

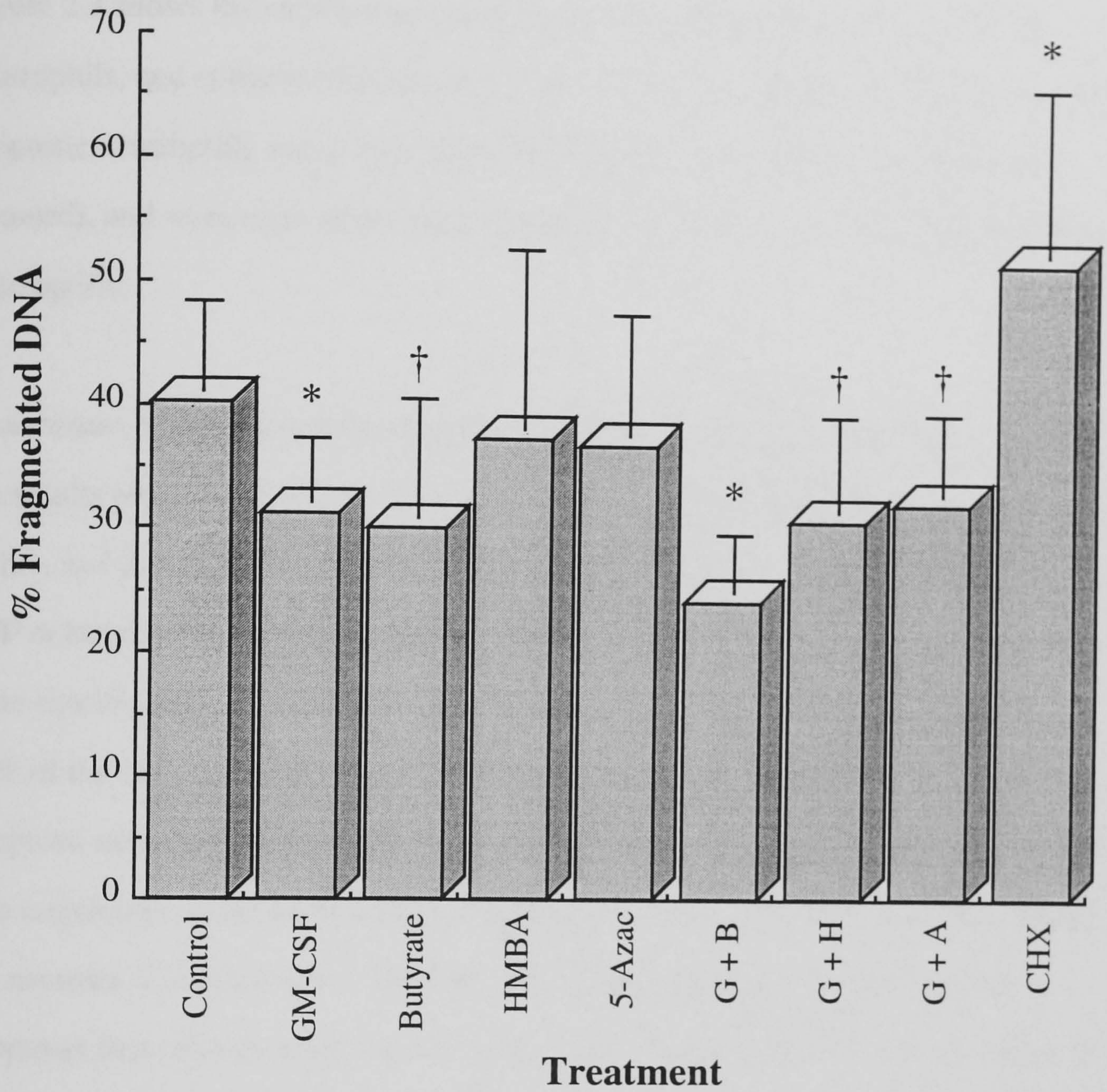
The percentage of fragmented DNA from neutrophil cultures was determined to quantify the extent of DNA fragmentation during 20 h of culture. Figure 2.2 shows that control cultured neutrophils had 40.1 % (± 7.2, n=10) fragmented DNA after 20 h culture. The relatively large error appears to be a result of donor variability. GM-CSF significantly decreased the extent of DNA fragmentation, in agreement with the ability of GM-CSF to delay neutrophil apoptosis. The transcriptional activator butyrate, but not HMBA nor 5-azacytidine, significantly lowered the proportion of fragmented DNA. Inhibition of protein synthesis with CHX significantly increased DNA fragmentation, as may have been expected with this accelerator of neutrophil apoptosis. The effect of butyrate treatment, at the optimal apoptosis delaying concentration of 0.4 mmol/L (Stringer, 1994 Thesis), was enhanced by the addition of 50 U/mL GM-CSF. GM-CSF treatment resulted in 31.1% (± 5.0), butyrate treatment resulted in 30.1% (±9.6), whilst in combination DNA fragmentation was only 24.3% (± 4.7). HMBA or 5-azacytidine treatment had no significant effect on the potency of GM-CSF.

Figure 2.1 Gel electrophoresis of fragmented DNA from 13 h aged neutrophils



Neutrophils were isolated and cultured as in Materials and Methods, with no additions to the media (control), or with GM-CSF (50 U/ml), or cycloheximide (10 μg/ml). Following culture, fragmented DNA from equal numbers of neutrophils was isolated and separated by agarose gel electrophoresis as described in Materials and Methods.

Figure 2.2 Quantification of fragmented DNA from 20 h cultured neutrophils



Neutrophils were isolated and cultured as described in Materials and Methods, with no additions to the media (control), or supplemented as indicated. Culture in the presence of two agents have single letter abbreviations: G = GM-CSF; B = butyrate; H = HMBA; A = 5-Azac. Following culture for 20 h fragmented DNA was quantified as described in Materials and Methods. Results are means (with SD) of at least 3 and up to 10 separate experiments. Significant difference from control is indicated by † ($p \leq 0.05$) or * ($p \leq 0.01$).

2.3.2 Morphological changes during neutrophil culture

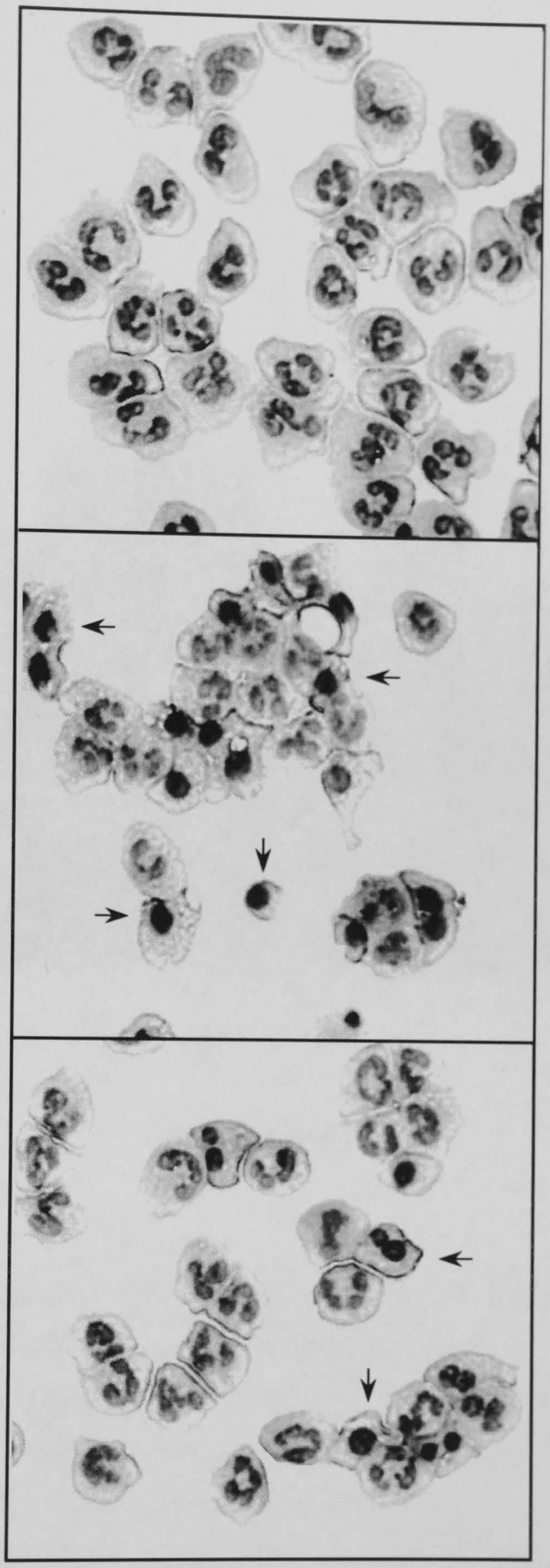
Figure 2.3 shows the contrasting morphology of freshly isolated, non-apoptotic neutrophils, and cultured neutrophils which have become apoptotic. After 12 h culture apoptotic neutrophils with condensed chromatin are clearly visible (examples are arrowed), and were more abundant in untreated neutrophils than in GM-CSF treated neutrophils.

Assessment of apoptosis following 20 h culture was determined by morphology and the results obtained shown in Figure 2.4. Control cultured neutrophils showed 60.0% (± 8.1, n=10) apoptosis by morphology. Apoptosis was significantly reduced by GM-CSF or butyrate treatment, but not by HMBA or 5-azacytidine. Apoptotic neutrophils were significantly more abundant in CHX containing suspensions, with more than 75% of the cells showing apoptotic morphology after 20 h. The exact percentage of apoptotic neutrophils from CHX treatments is likely to be higher, as a loss of viability was commonly observed by 20 h: some apoptotic neutrophils were lost from culture via necrosis. Combination of GM-CSF and butyrate gave better protection from apoptosis than was seen with either agent alone (GM-CSF 46.8 ± 4.3%; butyrate 45.6 ± 5.2%; GM-CSF + butyrate 33.6 ± 6.5%). HMBA in combination with GM-CSF slightly diminished the protective effect of GM-CSF (52.4 ± 3.6%), whereas combination of GM-CSF with 5-azacytidine slightly improved the efficacy of GM-CSF protection (39.0 ± 2.2%).

2.3.3 Preservation of oxidative burst during neutrophil culture

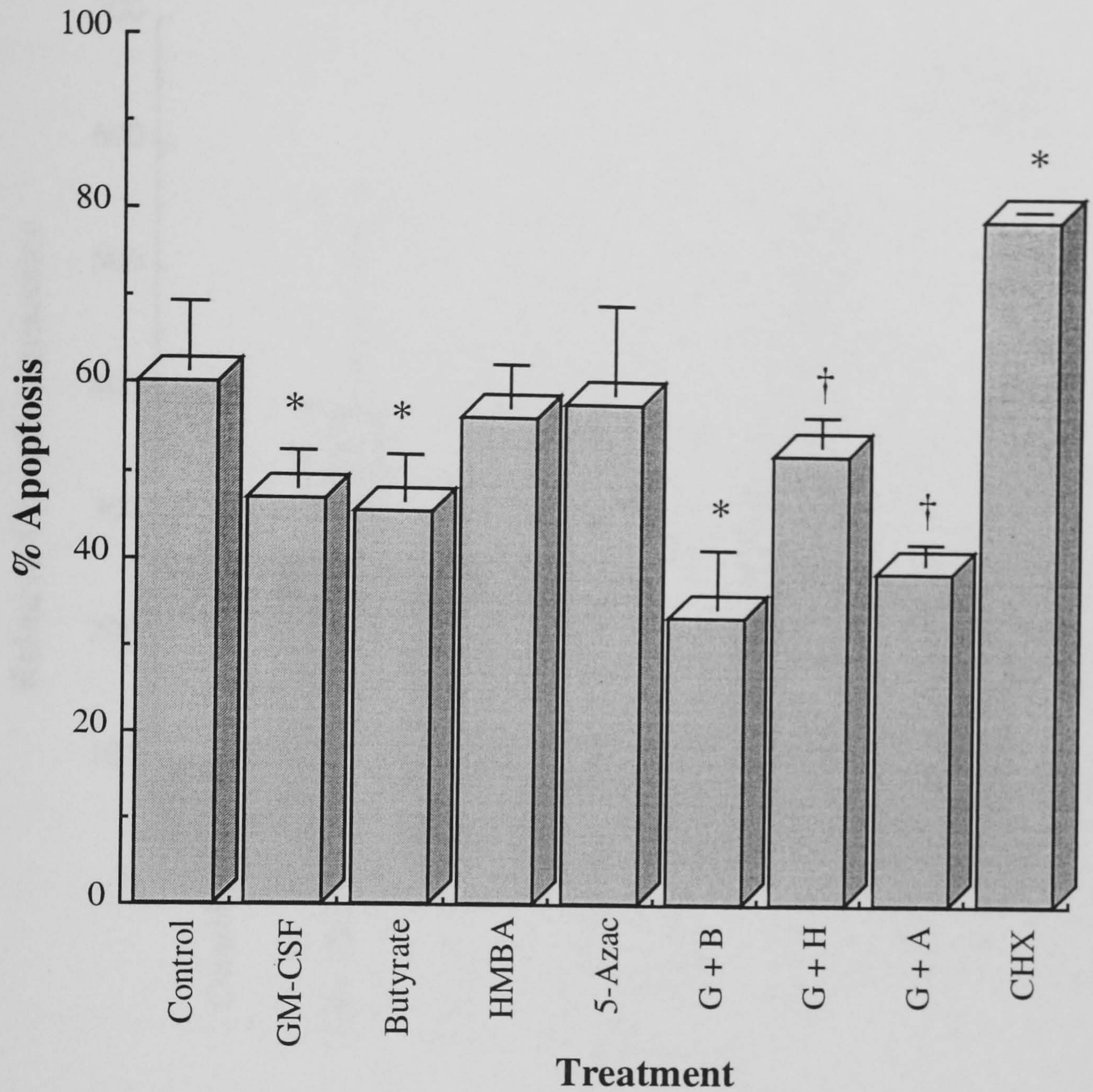
Treatment with either GM-CSF or butyrate gave a significantly greater oxidative burst activity after 20 h culture than control cultured neutrophils, in response to either PMA or fMLP (Figure 2.5). Neither HMBA or 5-azacytidine show any significant difference from control. GM-CSF in combination with butyrate gave a higher oxidative burst

Figure 2.3 Morphology of freshly isolated and 12 h cultured neutrophils



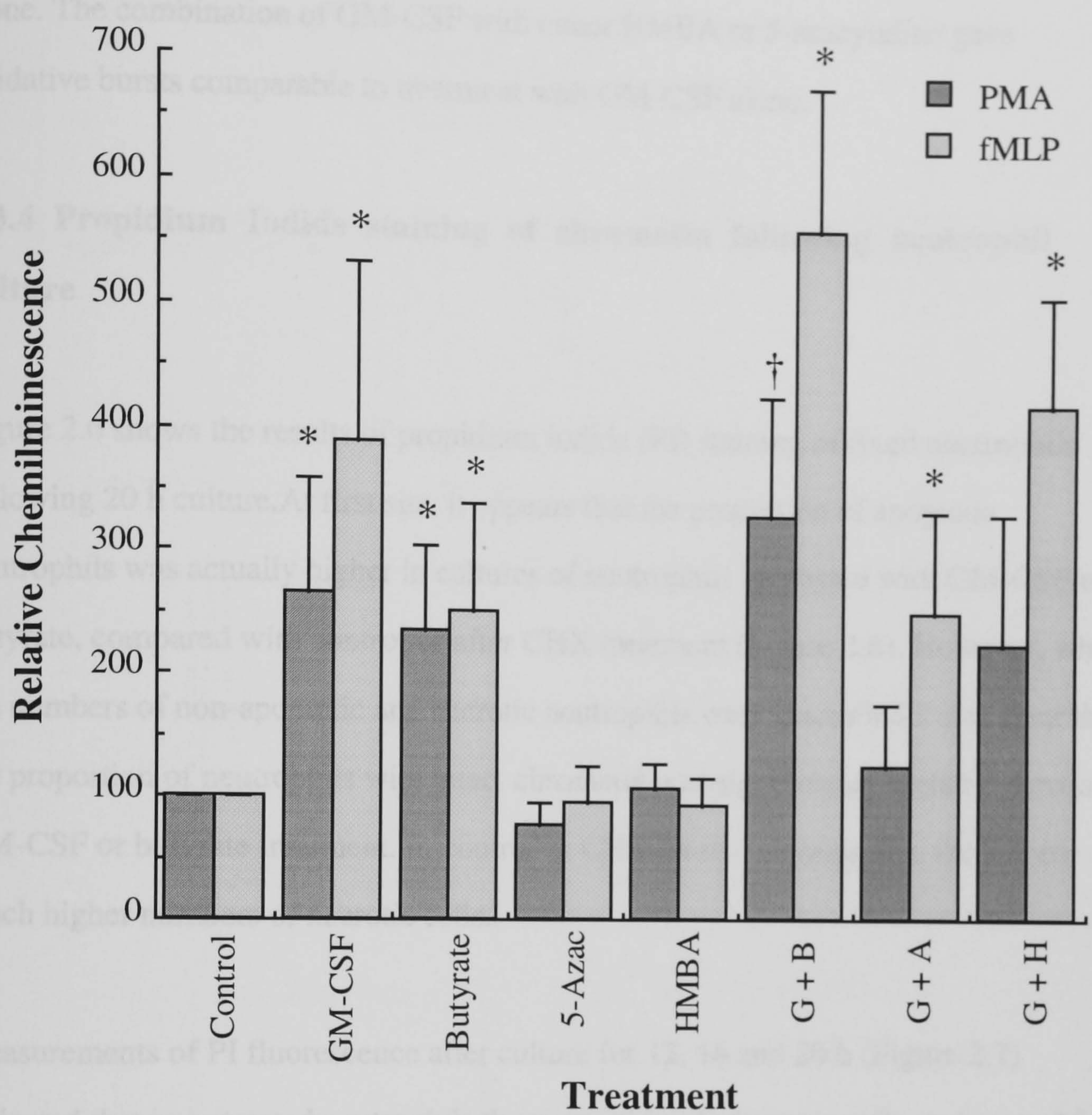
Neutrophils were isolated and cultured as described in Materials and Methods. Aliquots of freshly isolated or 12 h cultured neutrophils were cytocentrifuged onto glass slides, before staining and microscopic examination. Freshly isolated neutrophils have a distinctive multilobed nucleus. Apoptotic neutrophils are easily distinguishable (examples arrowed) with condensed nuclei (which is stained more intensely), and loss of cell volume.

Figure 2.4 Assessment of apoptosis by morphology after 20 h culture



Neutrophils were isolated and cultured as in Materials and Methods, with no additions to the media (control), or supplemented as indicated. Culture in the presence of two agents have single letter abbreviations: G = GM-CSF; B = butyrate; H = HMBA; A = 5-Azac. Following culture for 20 h aliquots from each culture were cytocentrifuged onto glass slides, stained and assessed for apoptosis. Results are means (with SD) of at least 3 and up to 10 separate experiments. Significant difference from control is indicated by † ($p \leq 0.05$) or * ($p \leq 0.01$).

Figure 2.5 Relative oxidative burst activity of 20 h cultured neutrophils



Neutrophils were isolated and cultured as described in Materials and Methods, with no additions to the media (control), or supplemented as indicated. Culture in the presence of two agents have single letter abbreviations: G = GM-CSF; B = butyrate; H = HMBA; A = 5-Azac. Following culture for 20 h, aliquots of cells were prepared for measurement of oxidative burst in response to either fMLP or PMA. Results shown are means of maximum chemiluminescence (with SD) of between 3 and 22 separate experiments. Significant difference from control is indicated by † ($p \leq 0.05$) or * ($p \leq 0.01$).

activity after 20 h culture than was seen following treatment with either of these agents alone. The combination of GM-CSF with either HMBA or 5-azacytidine gave oxidative bursts comparable to treatment with GM-CSF alone.

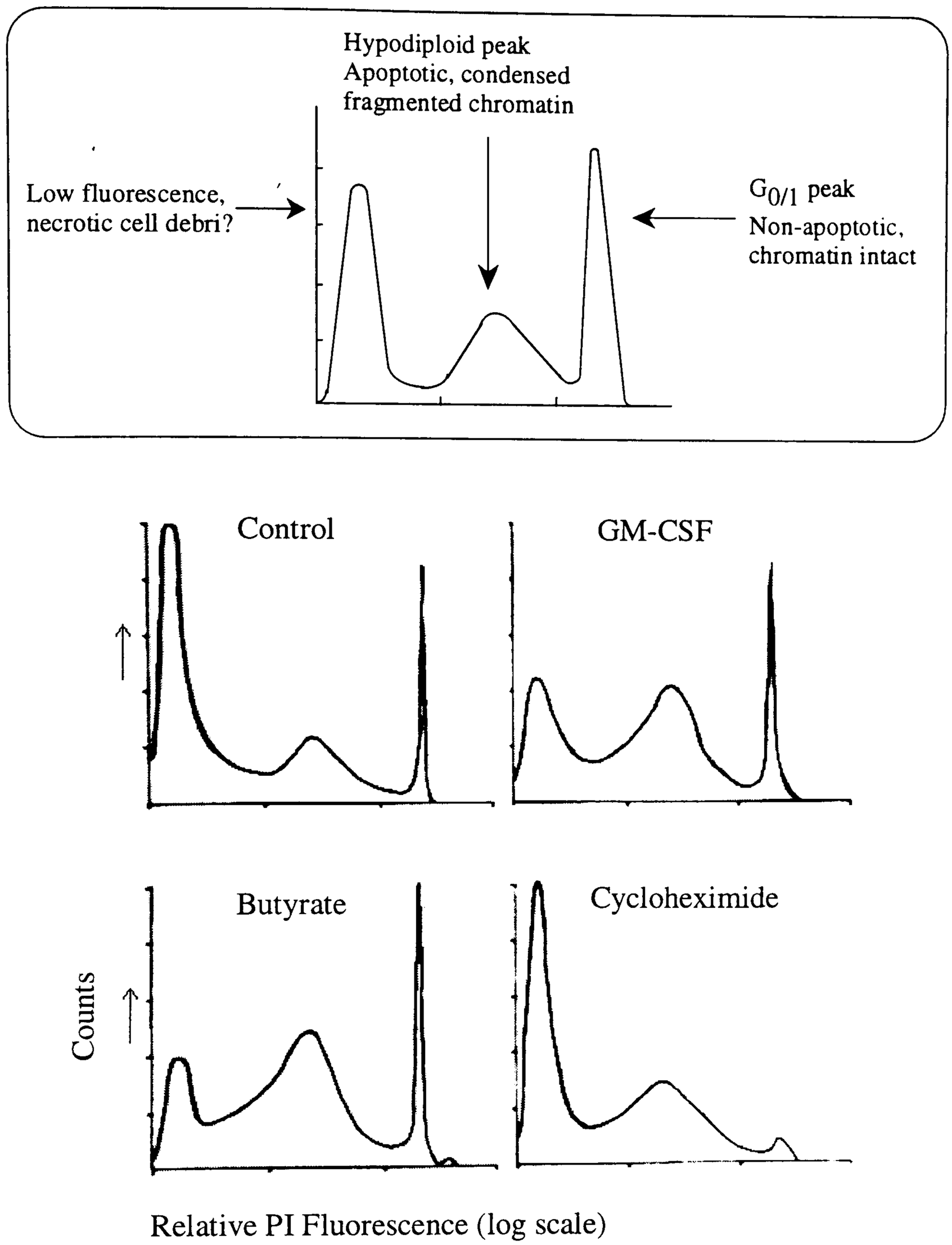
2.3.4 Propidium Iodide staining of chromatin following neutrophil culture

Figure 2.6 shows the results of propidium iodide (PI) staining of fixed neutrophils following 20 h culture.At first site, it appears that the proportion of apoptotic neutrophils was actually higher in cultures of neutrophils incubated with GM-CSF or butyrate, compared with control or after CHX treatment (Figure 2.6). However, when the numbers of non-apoptotic and necrotic neutrophils were quantified it was clear that the proportion of neutrophils with intact chromatin was significantly higher following GM-CSF or butyrate treatment. In control or CHX treated suspensions, there were much higher numbers of necrotic cells.

Measurements of PI fluorescence after culture for 12, 16 and 20 h (Figure 2.7) indicated that in untreated neutrophils the proportion of apoptotic cells rose up to 16 h, but then declined as these cells then became necrotic and showed very low PI fluorescence. This was more marked for CHX treated neutrophils: in these cultures there was a large number of apoptotic cells at 12 h, but these cells then became necrotic (Figure 2.7). Both GM-CSF (Figure 2.7) and butyrate (not shown) treatments resulted in fewer apoptotic neutrophils after 12 h culture than for controls, but again the proportion of apoptotic neutrophils decreased by 20 h culture and some necrosis was seen when assessed by this assay.

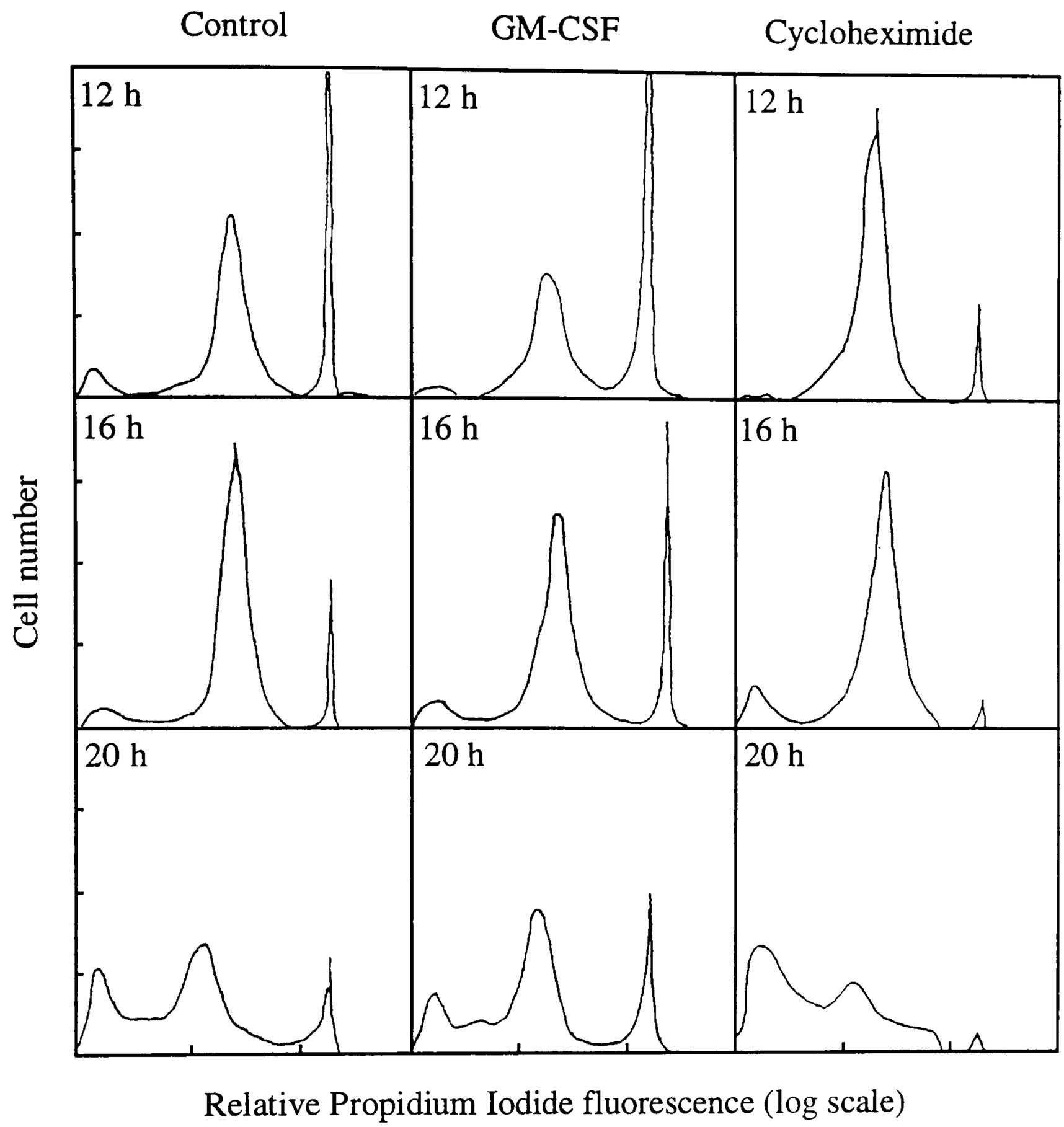
Figure 2.8 shows that the proportion of non-apoptotic neutrophils decreased more slowly over time with GM-CSF or butyrate treatments, compared to control incubations. Furthermore CHX treatment accelerated the decrease in non-apoptotic

Figure 2.6 Propidium Iodide staining of chromatin in 20 h cultured neutrophils



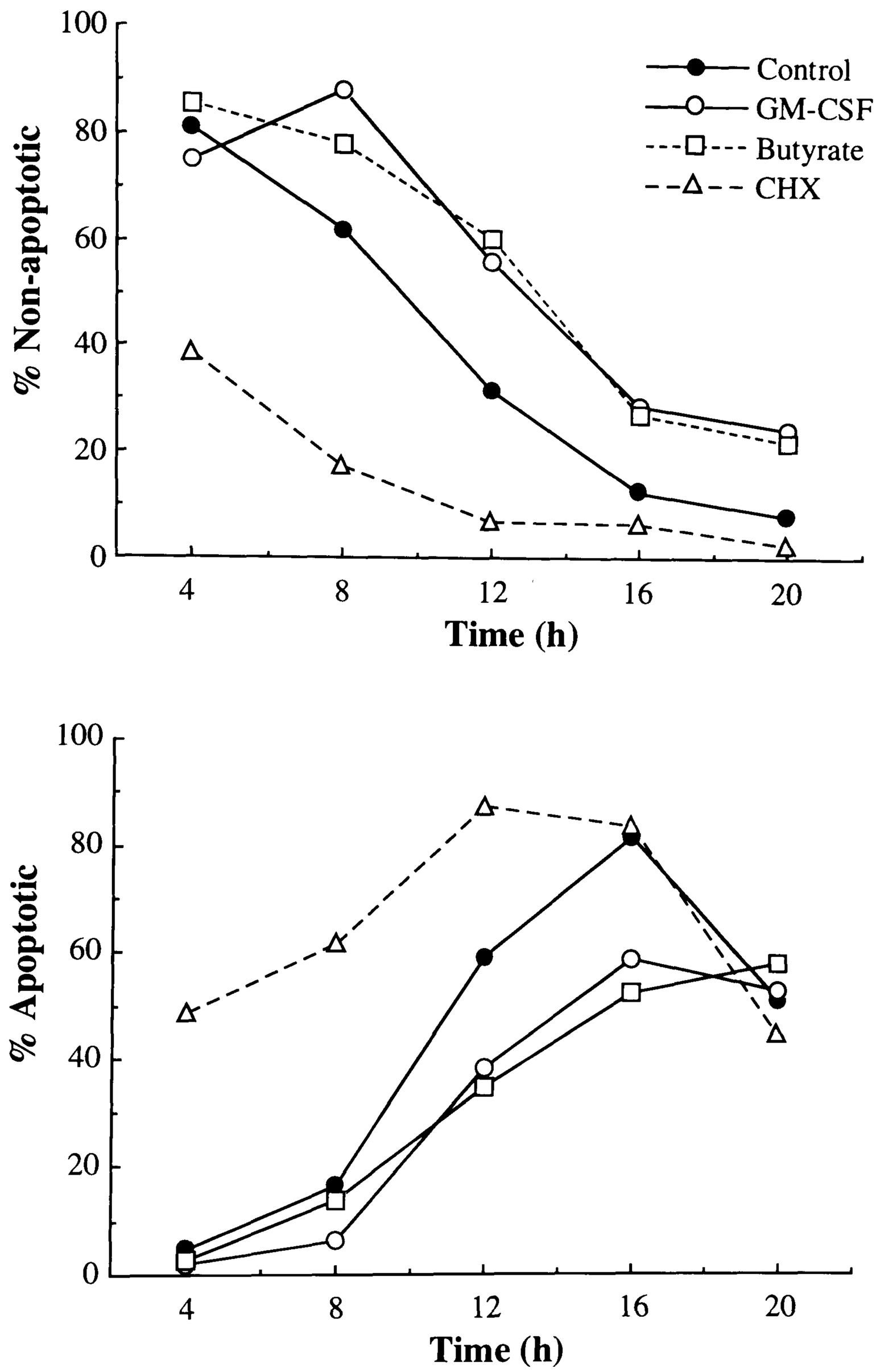
Neutrophils were isolated and cultured as described in Materials and Methods, with no additions to the media (control), or supplemented as indicated. Following culture for 20 h aliquots of cells were fixed and stained for flow cytometric analysis of propidium iodide (PI) fluorescence. Non-apoptotic neutrophils have high propidium iodide fluorescence ($G_{0/1}$), apoptotic cells have diminished fluorescence (hypodiploid), and necrotic cells have low fluorescence. This figure is representative of at least 3 separate experiments.

Figure 2.7 Propidium Iodide staining of cultured neutrophils



Neutrophils were isolated and cultured as described in Materials and Methods, with no additions to the media (control), or supplemented as indicated. Following culture for the indicated times aliquots of cells were processed for flow cytometric analysis of propidium iodide stained chromatin. The results shown are typical of two separate experiments. Butyrate treated cells were also examined, with results obtained very similiar to GM-CSF, so butyrate results are not shown.

Figure 2.8 Time course of $G_{0/1}$ (non-apoptotic) and hypodiploid (apoptotic) neutrophils over 20 h



Neutrophils were isolated and cultured as described in Materials and Methods. At fixed times during culture, aliquots of cells were removed and processed for propidium iodide staining and flow cytometric analysis. Results shown are representative of two separate experiments.

neutrophils over time. Measurement of PI fluorescence showed that the proportion of apoptotic neutrophils increased up to 16 h in control, GM-CSF or butyrate treated suspensions, and then the number of apoptotic cells declined between 16 and 20 h, as they became necrotic. CHX treatment resulted in a more rapid increase in the number of apoptotic neutrophils over time, but by 12 h culture these apoptotic cells became necrotic.

Table 2.1 shows the results of measurements of non-apoptotic neutrophils after 20 h culture, by PI fluorescence. Both GM-CSF and butyrate treatments resulted in approximately twice as many non-apoptotic cells after 20 h culture compared to control. Neither HMBA or 5-azacytidine had any effect on the rate of apoptosis. CHX treated cultures showed a significantly higher rate of apoptosis compared to controls. The combination of GM-CSF and butyrate was slightly more effective than either treatment alone.

2.3.5 Assessment of phosphatidylserine exposure by annexinV-FITC binding

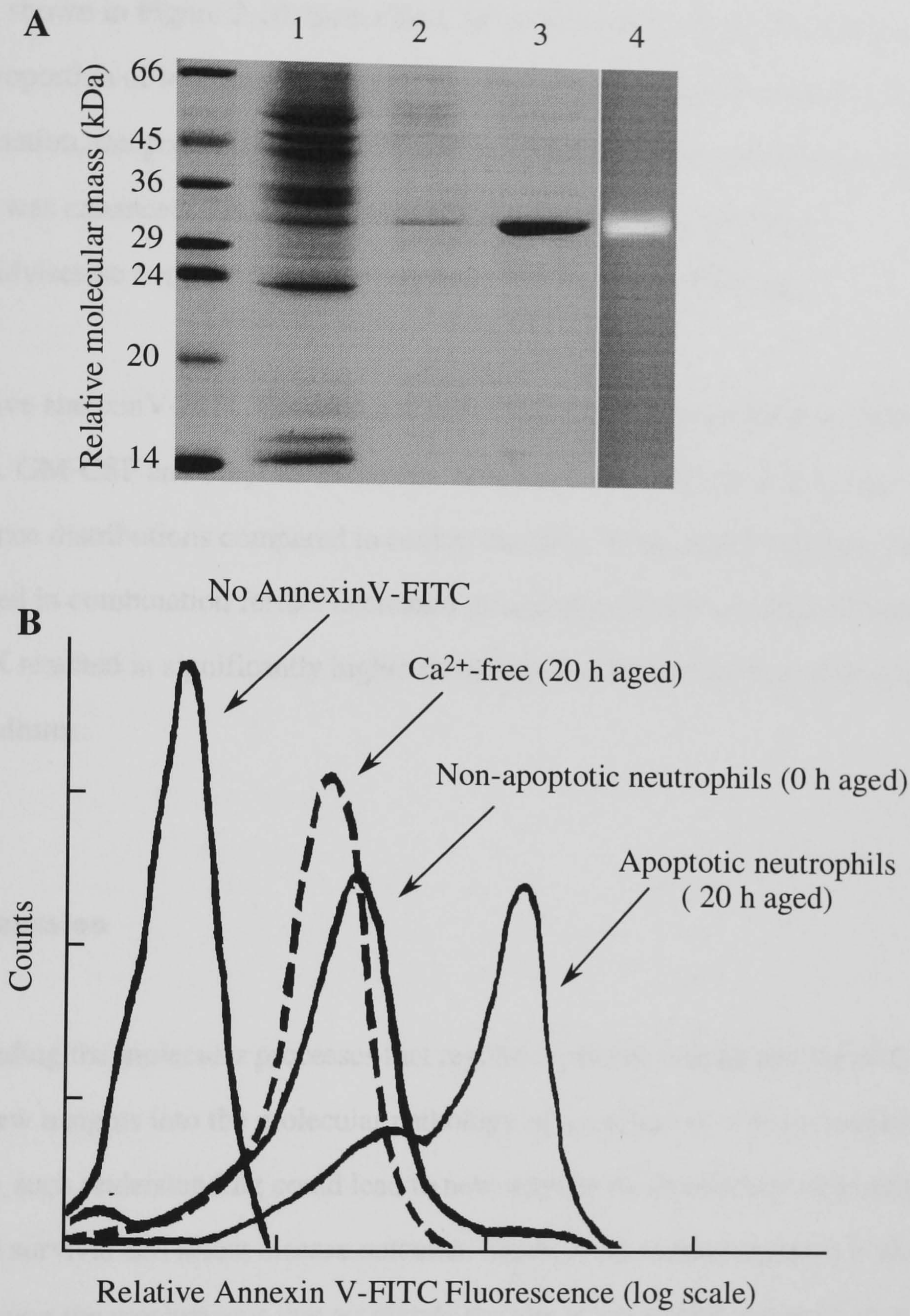
The purification of annexinV from whole chicken liver is shown in Figure 2.9a, yielding a single polypeptide of approximately 32.5 kDa. The recovery of annexin V was ~3.9 mg from 50 g fresh chicken liver, which compared favourably to that of Bousted *et al* (1988; 1993), who recovered ~2 mg from 50 g frozen chicken liver. This polypeptide was labelled with FITC as described in Materials and Methods, and the fluorescence of the annexinV-FITC is also shown in Figure 2.9a. The binding of annexinV-FITC to neutrophils is dependent on Ca^{2+}, and binding of annexinV-FITC was clearly greater for apoptotic neutrophils than for freshly isolated neutrophils (Figure 2.9b).

Table 2.1 Non-apoptotic neutrophils after 20 h culture assessed by Propidium Iodide staining of fixed neutrophils

	Control	GM-CSF	Butyrate	HMBA	5-Azac	B + G	CHX
% $G_{0/1}$	22.6	44.8 †	41.1 *	20.2	22.9	47.8 †	13.9 †
s.d.	6.9	17.1	6.8	--	--	11.7	4.6
n =	7	6	6	1	1	4	3

Neutrophils were isolated and cultured as described in Materials and Methods, with no additions to the media (control), or supplemented as indicated (B + G represents treatment with both GM-CSF and butyrate during culture). After 20 h culture, cells were fixed and stained as described in Figure 2.6. The mean percentage of neutrophils in the $G_{0/1}$ peak are shown (non-apoptotic), with a significant difference from control indicated by † ($p \leq 0.05$) or * ($P \leq 0.01$).

Figure 2.9 AnnexinV-FITC preparation and characterization



A AnnexinV was isolated and purified from chicken liver as described in Materials and Methods. Samples from the purification stages are shown following separation by SDS-PAGE. 1: whole liver homogenate, 2: $CaCl_2$ precipitate, 3: Pure AnnexinV following ion-exchange chromatography. 4: FITC-labelled annexinV fluorescence.

B AnnexinV-FITC binding to neutrophils. AnnexinV-FITC was incubated with neutrophils as described in Materials and Methods, then cellular fluorescence was analysed by flow cytometric analysis.

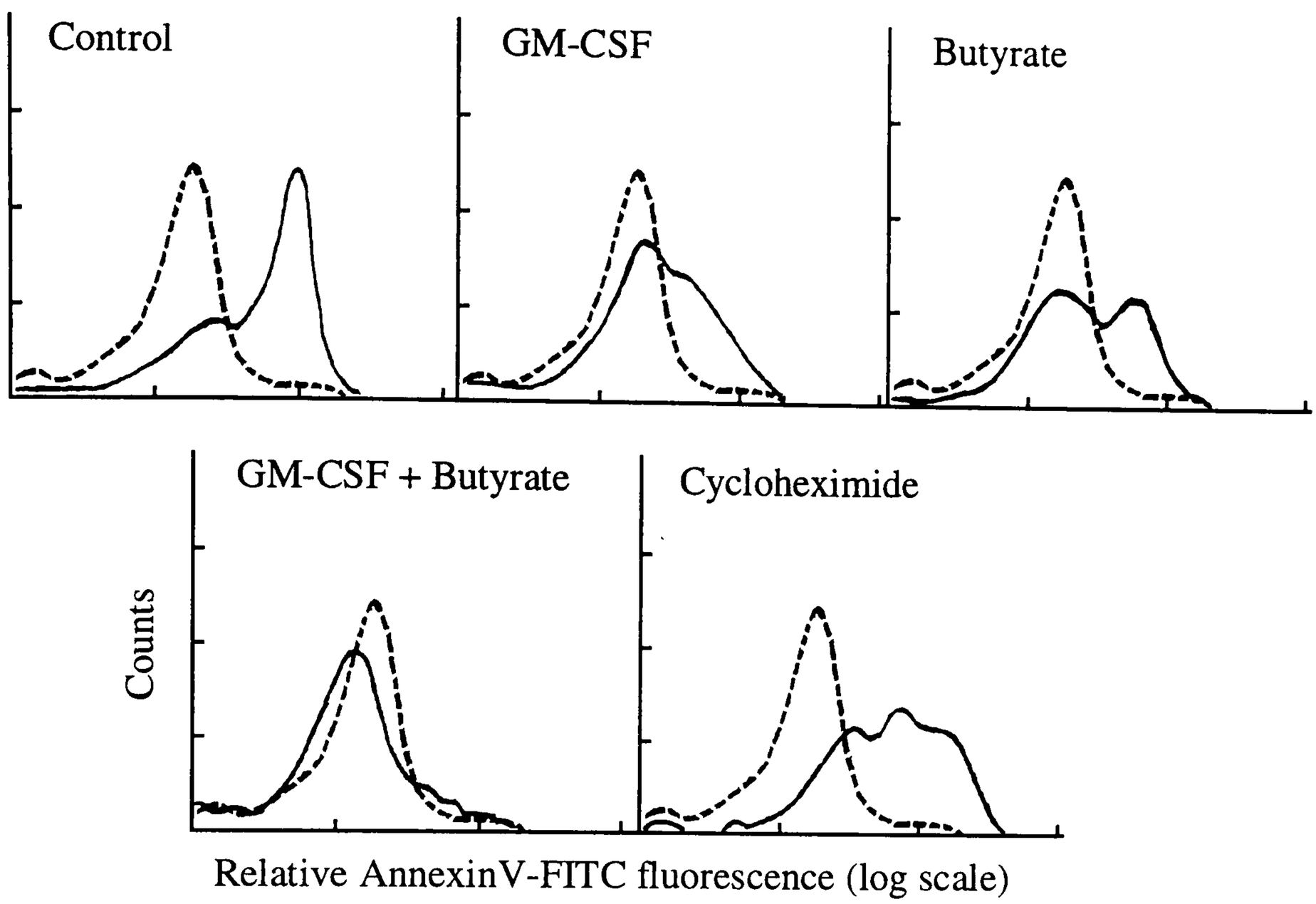
The fluorescence distributions of 20 h cultured neutrophils incubated with annexinV-FITC are shown in Figure 2.10. Both GM-CSF or butyrate treated cells showed a greater proportion of low annexinV-FITC binding cells compared to control cultures. In combination, the potency of GM-CSF and butyrate in delaying phosphatidylserine exposure was enhanced. Treatment with CHX clearly gave an increase in phosphatidylserine exposure after 20 h compared to control incubations.

The relative annexinV-FITC fluorescence from 20 h cultured neutrophils is shown in Table 2.2. GM-CSF and butyrate treatments both resulted in significantly lower fluorescence distributions compared to control cultures. When added together, the two agents used in combination further decreased phosphatidylserine exposure. Treatment with CHX resulted in significantly higher fluorescence distributions than measured in control cultures.

2.4 Discussion

Understanding the molecular processes that regulate neutrophil death and survival will provide new insights into the molecular pathology of a number of human conditions. Moreover, such understanding could lead to new ways to therapeutically manipulate neutrophil survival and affect disease outcome. Much of the current research is aimed at determining the mechanisms that accelerate the rate of neutrophil apoptosis in order to limit the inflammatory damage associated with diseases involving neutrophil infiltration. Examples of such conditions include the wide-scale tissue damage in post-operative sepsis (Watson *et al*, 1997b), following major elective surgery (Jimenez *et al*, 1997), and lung damage by inflammatory neutrophils (Watson *et al*, 1997). Conversely, in neutropenic patients the risk of morbidity and mortality from infection is greatly increased, and so delayed apoptosis may be of benefit. This is particularly important in the neutropenia experienced during cytotoxic therapy where infections in

Figure 2.10 AnnexinV-FITC binding to 20 h cultured neutrophils



Neutrophils were processed for annexinV-FITC binding and flow cytometric analysis following culture for 20 h in the conditions indicated; fluorescence distributions of freshly isolated cells are shown in the dotted line. Results shown are representative of at least 4 separate experiments.

Table 2.2 Relative annexinV-FITC binding to 20 h cultured neutrophils

	Control	**GM-CSF**	**Butyrate**	**B + G**	**CHX**
Relative AnnexinV-FITC Fluorescence	100	65.7 *	62.6 *	45.9 *	136.1 *
s.d.	--	11.0	12.5	9.1	11.4
n =	4	4	4	4	4

Following culture for 20 h in the conditions indicated neutrophils were processed for flow cytometric analysis of AnnexinV-FITC binding as described in Materials and Methods. Relative binding was calculated by standardising control values to 100. Significant difference from control is indicated by * ($p \leq 0.01$).

patients may necessitate suspension of treatment (Ribas *et al*, 1996). The use of granulocyte colony-stimulating factor has been successfully used to minimise neutropenia in these patients (Morstyn *et al*, 1988; Crawford *et al*, 1994). The relative importance of i) the accelerated release of neutrophils from the bone marrow into the bloodstream or ii) the delayed apoptosis of bloodstream neutrophils, in the alleviation of neutropenia is uncertain (Maksoud *et al*, 1994), although a combination from both processes is likely to be involved. Therefore, a fuller understanding of the mechanisms controlling neutrophil apoptosis may allow the development of treatments to benefit patients suffering a wide range of conditions.

In this Chapter, I have shown neutrophil apoptosis can be measured by a number of different parameters, such as DNA fragmentation and chromatin condensation, functional capacity, morphology, and exposure of phosphatidylserine residues on the outer leaflet of the plasma membrane. Furthermore, I have shown that the delayed apoptosis resulting from GM-CSF treatment (Table 1.2) is easily measured by these parameters. These parameters represent distinct molecular processes associated with death via apoptosis, and I have shown that all the methodologies I have used to assess apoptosis gave comparable, though not perfectly matching, results.

However, necrotic death cannot be distinguished from apoptotic death by measuring preservation of the oxidative burst, but examination of any of the other parameters used in this Chapter is sufficient to confirm death occurs via apoptosis. Therefore, it is preferable to determine neutrophil apoptosis by more than one parameter, but once death is confirmed to be via apoptosis, any of the parameters I have measured are suitable to determine the rate of apoptosis. This is exemplified by the results obtained by assessing apoptosis by propidium iodide staining of chromatin, which showed more apoptotic neutrophils in GM-CSF treated suspensions after 20 h culture than control suspensions (Figure 2.6) despite the diminished rate of apoptosis found following treatment with this agent. Closer examination of the fluorescence

distributions showed that although fewer apoptotic neutrophils were detected in control incubations, this was due to the loss of many of these apoptotic, fragile neutrophils due to cell lysis during processing for flow cytometry.

Using these methodologies I have examined the role of active gene expression in regulating entry into neutrophil apoptosis by measuring apoptosis following treatment with three transcriptional activators (Butyrate, HMBA and 5-azacytidine) or with the protein synthesis inhibitor CHX, in comparison to control and GM-CSF treated neutrophils. The ability of GM-CSF to delay apoptosis in neutrophils was confirmed (e.g. Collota *et al*, 1992), as was the increased rate of apoptosis following inhibition of protein synthesis (e.g. Whyte *et al*, 1997). Activation of protein synthesis with sodium butyrate resulted in a significantly slower entry into apoptosis, in agreement with previous work in this laboratory (Stringer, 1994; Stringer *et al*, 1996). This was confirmed by measuring DNA fragmentation, morphological changes associated with apoptosis, preservation of the oxidative burst, PI staining of chromatin, and annexinV-FITC binding to exposed phosphatidylserine residues on the neutrophil surface. However, the transcriptional activators HMBA and 5-azacytidine had no effect on neutrophil apoptosis as assessed by these parameters. Apoptosis was also assessed by measuring surface levels of the low affinity receptor for IgG immune complexes FcγRIIIb (CD16). These results (shown in detail in Chapter 3 where the control of surface CD16 expression is determined) paralleled the results shown in this Chapter.

The lack of effect of both HMBA and 5-azacytidine on neutrophil apoptosis is surprising as the concentrations used resulted in a similar upregulation of protein synthesis as was observed for GM-CSF or butyrate treatments (Stringer, 1994). However, this apparent inconsistency in the theory that maintenance of protein synthesis will delay neutrophil apoptosis may be explained by the different modes of action of butyrate, and HMBA or 5-azacytidine. Whereas butyrate increases gene expression by hyperacetylation of histones (Kruh, 1982), HMBA and 5-azacytine act

by demethylation of DNA on cytosine residues (Doerfler, 1983; Kolata, 1985; Razin *et al*, 1986; Bernstein & Kappes, 1988). These different mechanisms may result in the expression of different classes of neutrophil genes, such that butyrate treatment enhances expression of the key gene(s) needed to delay neutrophil apoptosis, whereas neither HMBA nor 5-azacytidine alter expression of the key gene(s). A further difference in the response to treatment with butyrate, as opposed to either HMBA or 5-azacytidine, is that butyrate treatment also leads to higher levels of DNA methylation on cytosine residues (Boffa *et al*, 1994), in direct contrast to HMBA or 5-azacytidine treatment. In this manner, butyrate may also be exerting its action on neutrophil apoptosis by silencing key regulatory genes, due to the increased DNA methylation. However, there is no known mechanism by which DNA methylation inhibits transcription, merely a correlation between DNA methylation and levels of transcription (Kolota, 1985).

The use of butyrate and GM-CSF together showed that these two treatments were more effective in delaying neutrophil apoptosis when used in combination. Butyrate was used at its maximal potency of 0.4 mmol/L, but GM-CSF was used at 50 U/mL, which is not the concentration for maximal efficacy in delaying apoptosis (e.g. Colotta *et al*, 1992). GM-CSF can inhibit apoptosis more effectively at higher concentrations, but at 500 U/mL the extra effect is only slight (Colotta *et al*, 1992). The enhancement of butyrate-delayed apoptosis by GM-CSF indicates that there may be (at least) two distinct processes effected by each of these treatments, with each process alone able to slow entry into apoptosis, and together having a greater effect. The difference in action between GM-CSF-delayed and butyrate-delayed apoptosis may be enhancement of synthesis of some different gene products, or due to GM-CSF stimulated alterations in signal transduction pathways.

The methodologies established in this Chapter show that they can successfully be used to determine the effects of agents which accelerate or delay neutrophil apoptosis.

Moreover, this experimental system can be used to probe the molecular processes that control neutrophil death and survival. These experiments thus provide an experimental platform for the following chapters to investigate the effects of new agents on neutrophil survival and the mechanisms by which such agents exert their effects.

Having shown that butyrate delays neutrophil apoptosis in addition to enhancing gene expression, but without priming neutrophils, I have further examined differences in the actions of butyrate and GM-CSF in processes associated in neutrophil apoptosis. Chapter 3 shows the preservation of surface CD16 expression by both of these agents, and examines how this is achieved by a priming agent (GM-CSF) and a non-priming agent (butyrate).

In Chapter 4, I show the effects of the superantigens produced by *Staphylococcus aureus* on neutrophil apoptosis. These superantigens have previously been shown to effect other neutrophil functions (Hensler *et al*, 1991; 1993), but their effects on apoptosis has not been examined.

The ability of sodium butyrate to delay neutrophil apoptosis implies a role for histone acetylation in the control of neutrophil apoptosis. The acetylation of histones was thus measured in neutrophils treated to delay apoptosis with butyrate and GM-CSF in Chapter 5. The effect of trichostatin A, a more specific inhibitor of histone deacetylase than butyrate (Yoshida *et al*, 1990), is also examined, with regard to its effects on both neutrophil apoptosis and histone acetylation.

Finally, the expression of individual gene products are measured in Chapter 6. The Bcl-2 family of proteins are implicated in the regulation of apoptosis in many cell types (Section 1.5.3.2), though there has been relatively little research into the role of this family of proteins in the control of neutrophil apoptosis (section 1.6.5). The

expression of members of this family are shown, and measured in neutrophils treated to delay apoptosis with a variety of agents, including GM-CSF and butyrate.

CHAPTER 3 REGULATION OF CD16 SURFACE EXPRESSION IN NEUTROPHILS TREATED TO DELAY APOPTOSIS

3.1 Introduction

As neutrophils age they undergo apoptosis and lose much of their surface CD16 (FcγRIIIb) an abundant cell surface-receptor on non-apoptotic neutrophils (Dransfield *et al*, 1994; Homberg *et al*, 1995). The CD16 molecule is present on the cell surface in high numbers (100,000-200,000 per cell) and binds to IgG immune complexes with low affinity. The role of CD16 in neutrophil activation is much debated in the literature (Huizinga *et al*, 1989; Huizinga *et al*, 1989a; Hundt & Schmidt 1992), but we have recently shown that CD16 is needed for the activation of primed neutrophils by soluble immune complexes (Edwards *et al*, 1997).

Surface CD16 is linked to the plasma membrane by an easily cleaved glycerophosphatidyl-inositol anchor, but can be removed from the cell surface by a metalloprotease (Middlehoven *et al*, 1997). Activation of the neutrophil causes shedding of surface CD16 (Huizinga *et al*, 1988), with surface levels of CD16 maintained by translocation of preformed receptors from intracellular storage compartments (Tosi & Zakem, 1992). The intracellular compartments which contain CD16 also contain CD14 (the LPS receptor) and alkaline phosphatase, indicating that they are secretory vesicles (Detmers *et al*, 1995). CD16 is also reported to be actively synthesized by neutrophils (Jack & Fearon, 1988; Jost *et al*, 1990).

The mechanism(s) by which surface CD16 levels are maintained when neutrophils are rescued from apoptosis are undefined, but may involve i) decreased rates of shedding, ii) increased mobilisation of intracellular stores, iii) increased biosynthesis, or iv) a combination of these events. The work presented in this chapter investigates

the mechanisms by which GM-CSF (which primes and activates the neutrophil, whilst also delaying apoptosis) and sodium butyrate (which delays apoptosis without priming or activation) preserve surface CD16 expression.

3.2 Materials and Methods

3.2.1 Materials

All materials used for the isolation and culture of neutrophils are listed in section 2.2.1. Leu 11a (anti-CD16) and FITC labelled goat anti-mouse antibodies were from Becton and Dickinson (Cowley, UK), whilst [^{35}S]-methionine, ECL detection kit and Amplify were from Amersham (UK). 5D2 monoclonal anti-CD16 antibody and antisera to CD16 from mouse ascites were generous gifts from Dr A Verhoeven, with 3G8 antibody from Medarex Inc. (NJ). Pansorbin® was from Calbiochem, Dynabeads (coated with sheep anti-mouse IgG) were from Dynal (UK). [^{32}P]dCTP was from ICN, random primed labelling kit from Boehringer-Mannheim, Nuctrap columns from Stratagene, Zetaprobe GT nylon membrane from Bio-Rad and Trizol was from Gibco-BRL.

3.2.2 Neutrophil isolation

Neutrophils were isolated as described in section 2.2.2.

3.2.3 Neutrophil culture

Neutrophils were cultured as described in section 2.2.3, in the absence (control) or presence of rhGM-CSF (50 U/mL), sodium butyrate (0.4 mmol/L), or LPS (10 ng/mL). For [^{35}S]-methionine labelling, neutrophils were pre-incubated with 60 μCi/mL for 15 min at 2 x 10^7 cells/mL before the addition of rhGM-CSF or sodium

butyrate. At various times as indicated, cells were removed and processed as described below.

3.2.4 CD16 surface expression measurement by flow cytometry

Following culture of neutrophils (in the conditions and times indicated), expression of surface CD16 (FcγRIIIb) was measured by flow cytometry using a standard, indirect immunofluorescence technique, as described previously (Edwards *et al*, 1990). Cells were suspended in: PBS (10 mmol/L potassium phosphate; 0.9% (w/v) NaCl, pH 7.4); 1% bovine serum albumin (BSA) (globulin free); 0.1% sodium azide pH 7.2, and incubated with the monoclonal antibody Leu 11a, as a first layer antibody. FITC-labelled goat anti-mouse antibody was used as a second layer antibody. Both were used at saturating concentrations and nonimmune mouse IgG of the appropriate subclass was used as a specific first layer control. Stained cells were fixed in 1% (w/v) paraformaldehyde in PBS and analysed using a Becton Dickinson Ortho Diagnostics Cytron analyser. Fluorescence distributions represent a total of 5,000 gated events.

3.2.5 Dual labelling for surface CD16 and Propidium Iodide

Following culture of neutrophils (in the conditions and times indicated), dual labelling of neutrophils with propidium iodide (to measure chromatin structure) and anti-CD16 antibodies (to detect FcγRIIIb surface expression) was carried out as described previously (Dransfield *et al*, 1994), with some modifications. Briefly, neutrophils were labelled with first (Leu 11a) and second layer antibodies as described above. Cells were then washed twice with: cold PBS; 1% BSA; 0.1% azide and fixed for 15 min in ice-cold 70% ethanol. Cells were then washed and resuspended in propidium iodide solution (1.5μg/ml in PBS) and incubated for at least 1 h in the dark at 4°C before cytometric analysis using a Becton Dickinson

Ortho Diagnostics Cytron Analyser. Fluorescence distributions represent 5,000 gated events.

3.2.6 Total/shed CD16 measurements by western blotting

Neutrophils were cultured at 10^7 cells/100 μL for this assay. Neutrophils lysates and supernatants were subjected to SDS-PAGE using the method of Laemmli (1970), with 10 % acrylamide gels under non-reducing conditions. Supernatants (100 μL) were loaded onto gels with 0.2 vol of 5x non-reducing sample buffer. Electrotransfer of proteins to nitrocellulose (Hybond ECL, Amersham) was achieved using Bio-Rad transfer apparatus, following the manufacturers instructions. Following transfer, the membrane was blocked with blocking buffer (3 % BSA (fraction V, Sigma); 0.025 % (v/v) Tween-20; PBS pH 7.4) for 1 h at room temperature. Membranes were then incubated with primary Ab (either 3G8 or anti-CD16 antisera from mouse ascites, which gave near identical results) in blocking buffer overnight at 4°C, and then washed with: PBS; 0.1 % (v/v) Tween-20 for 2 x 1 min, 1 x 15 min and 2 x 5 min. Anti-mouse HRP-conjugated Ab (Sigma) was then incubated with membranes in blocking buffer for 1 h at room temperature followed by washes in: PBS; 0.1 % (v/v) Tween-20 for 2 x 1 min, 1 x 15 min, and 4 x 5 min. Bound Ab was detected by using an ECL kit. Relative amounts of CD16 were calculated both from exposure to Hyperfilm ECL (Amersham) with care taken to avoid overexposure of film, and using a Bio-Rad Molecular Imager GS-363 with imaging screen-CH. Both systems gave comparable results.

3.2.7 Surface/internal CD16 measurements by flow cytometry

Flow cytometric determination of surface and internal CD16 levels were accomplished by using a modification of a previously described method (Tosi & Zakeem, 1992). Briefly, neutrophils recovered after various treatments were fixed in

1 % paraformaldehyde at room temperature for 10 min. Fixed neutrophils were then permeabilized in: 0.1 % (w/v) BSA; PBS; 0.1 mol/L glycine with 0.04 % (w/v) saponin for all steps of immunofluorescent staining. CD16-FITC mAb (Becton & Dickinson) or an isotype matched control FITC mAb were added at saturating concentration for 30 min at room temperature. Cells were washed three times in: 0.1 % (w/v) BSA; PBS; saponin, then washed once in PBS before resuspension in PBS for flow cytometric analysis. Parallel aliquots of fixed neutrophils were treated identically but without saponin for non-permeabilized controls. Fluorescence distributions represent a total of 5,000 gated events. Internal CD16 expression was calculated by subtracting surface fluorescence values from permeabilized fluorescence values after calculation of the number of CD16 molecules bound per cell using the Quantum Simply Cellular microbead kit (Sigma) following manufacturers instructions.

3.2.8 Surface/internal CD16 measurements by western blotting

Determination of surface and internal CD16 by flow cytometry proved unsatisfactory for neutrophils cultured overnight, and so a new technique utilising western blotting was devised. Neutrophils were recovered from incubations, and aliquots ($4x10^6$) from each incubation were treated as follows. Cells, from each incubation, were washed in RPMI 1640, and then were split into 2 equal aliquots. One set of aliquots was treated with pronase E (Sigma) at 0.25 mg/ml in RPMI 1640 for 10 min at 37°C. The remaining aliquots were treated identically except that pronase E was not included. Following two washes in RPMI 1640, all aliquots were split again. for surface CD16 determination by flow cytometry (as described above. to determine efficiency of surface CD16 removal by pronase), with the remaining cells ($1x10^6$) subjected to SDS-PAGE using 10% acrylamide gels under non-reducing conditions and western blotting as described above (3.2.6). Relative amounts of CD16 were calculated as above. This methodology gives relative values of total CD16 from cells

not treated with pronase E, and relative values of internal CD16 from pronase E treated cells. The pronase E treatment typically removed >90% of surface CD16 as determined by flow cytometry. Surface CD16 could be replenished following pronase E treatment by stimulating cells for 10 min with 10 nmol/L fMLP (Sigma).

3.2.9 *De novo* CD16 biosynthesis

Neutrophils at 10^7 cells/ml were pre-incubated with 60 μCi/ml [^{35}S]-methionine for 15 min at 37^{0}C. GM-CSF or butyrate were then added (or no addition for control) and cells were incubated for 18 h, as above. Immunoprecipitation from cell pellets was as previously described (Perregeux, 1992). Briefly, cell pellets were lysed in: 10 mmol/L Tris pH 7.2; 5 mmol/L EDTA; 0.15 mol/L NaCl; 1% (v/v) Nonident P40; 1 mmol/L PMSF; and aprotinin (Sigma), chymostatin (Sigma), pepstatin A (Sigma), leupeptin (Sigma), and antipain (Sigma) all at 3μg/ml. Following lysis for 2 h at 4^{0}C, 25 μl of Pansorbin® (*Staphylococcus aureus* bacteria coated with protein A, Calbiochem) was added on ice for 10 min to remove all non-specific binding proteins. Pansorbin® was removed by centrifugation and then either 3G8, anti-CD16 mouse ascites or 5D2 antibodies were added to the supernatants at saturating concentrations (up to 5 μg/mL) and incubated for 2 h at 4^{0}C. 75 μL of Pansorbin® was then added for 30 min. Pansorbin® was then pelleted and washed 5 times with: 10 mmol/L Tris pH 8.0; 10 mmol/L EDTA: 0.4%(w/v) deoxycholic acid; 1% (v/v) Triton X-100; 0.1% (w/v) SDS and then once with 50 mmol/L Tris pH 6.8. The Pansorbin® pellet was then resuspended in SDS-PAGE sample buffer, boiled for 5 min and the resulting suspension centrifuged to remove Pansorbin®. The supernatant was subjected to SDS-PAGE on a 10% acrylamide gel. Following electrophoresis the gel was fixed in 40% methanol/10% acetic acid for 1 h, washed in 10% ethanol for 10 min, then placed in Amplify for 1 h. The gel was then vacuum dried at 60^{0}C, before exposure to Fuji RX X-ray film at -70°C. Films were developed after

exposures of up to 3 months. Radioactivity in gels was also determined using a GS-363 Molecular Imager (Bio-Rad) with GS-250 imaging screen-CS.

This methodology proved unsuccessful for detection of *de novo* CD16. so alternative methods were tried, firstly by incubating cells in methionine-free medium to improve incorporation of radio-labelled methionine, but with no improvement. The final methodology attempted (as shown in Results) utilised Dynabeads coated with antibody to mouse IgG's, to remove the 5D2 mAb, as it was found that Pansorbin® was binding to CD16 non-specifically. Cell pellets were lysed as above. then incubated with either 5 μg/mL 5D2 mAb (>1000 fold excess to CD16), for 2 h as above, before addition of Dynabeads (10^7) for 3 h, or for 3 h with 10^7 Dynabeads pre-coated with excess 5D2 mAb. Beads were then removed magnetically following manufacturers instructions, washed 6 times with: 5 mmol/L EDTA pH 7.5; 10 mmol/L Hepes pH 7.5; 150 mmol/L NaCl; 0.2 % (w/v) BSA; 0.2 mmol/L PMSF; 3 μg/mL protease inhibitors as above; 0.5 % (v/v) Triton X-100, then boiled for 5 min in SDS-PAGE sample buffer before final removal of beads. Immunoprecipitates from up to 3.3×10^7 neutrophils were separated by SDS-PAGE and radioactivity detected as above.

To test efficiency of radio-labelling, whole cell lysates from 10^6 neutrophils after 18 h culture were examined for radioactivity by SDS-PAGE, as described above. Efficiency of immunoprecipitation of CD16 was tested by western blotting (as above) of samples from 10^6 neutrophils, immunoprecipitates from 10^6 neutrophils. and cell lysate of 10^6 neutrophils after immunoprecipitation of CD16.

3.2.10 Measurement of CD16 mRNA

Following incubation of neutrophils (for the times and in the conditions indicated). total RNA was isolated from equal numbers of neutrophils using Trizol. following

the manufacturers instructions. For northern blotting, RNA samples from equal numbers of neutrophils were electrophoresed on 1.2 % (w/v) agarose gels containing 1 % (v/v) formaldehyde; 20 mmol/L 3-*N*-morpholinopropane sulfonic acid; 1 mmol/L EDTA; 5 mmol/L sodium acetate (pH 8.0) for 16 h at 4-5 mA. After electrophoresis, gels were stained with ethidium bromide and RNA visualised by UV illumination to check equal loading of lanes, and integrity and positions of 28S and 18S rRNA. The RNA was then transferred by capillary blotting to Zetaprobe GT nylon membranes in 20 x SSC (3 mol/L NaCl; 0.3 mol/L sodium citrate, pH 7.0). The filter was then baked at 80^0C for 30 min and stored at room temperature in Saranwrap until use. The filters were then probed with the following: CD16 (a PCR-generated probe to CD16 as described in: Peltz *et al*, 1989; Ory *et al*, 1989); and β-actin (ATCC 65128). The cDNA inserts were excised using appropriate restriction endonucleases and isolated from vector DNA by electrophoresis in low melting point agarose. Then 50-100 ng of each probe was radio-labelled using a random-primed labelling system using 25 μCi [^{32}P]-CTP (Quayle *et al*, 1995). Labelling proceeded for 20 h at 16^0C and then unincorporated CTP was removed from the labelled probe using Nuctrap columns. The amount of radioactivity incorporated was typically $>10^8$ cpm/μg DNA. The filters were pre-hybridised for 30 min at 65^0C in: 0.25 mol/L $Na_2HPO_4.7H_2O$; 7 % (w/v) SDS, pH 7.2, and then hybridised with the probe for 16-20 h in the same buffer at 65^0C. The filters were then washed in: 20 mmol/L $Na_2HPO_4.7H_2O$; 5 % (w/v) SDS, pH 7.2 for 2 x 30 min at 65^0C, followed by 2 x 10 min washes in: 20 mmol/L $Na_2HPO_4.7H_2O$; 0.1 % (w/v) SDS, at the same temperature. The filters were then blotted dry, wrapped in Saranwrap and radioactivity was quantified using a GS-363 Molecular Imager with GS-250 imaging screen-BI. The probes were removed by heating the filters to 95^0C in 0.1 x SSC (15 mmol/L NaCl; 1.5 mmol/L Na citrate, pH 7.0) containing 0.5 % SDS for 2 x 20 min. After checking for removal of the probe, the filters were then re-probed.

3.2.11 Statistical analysis

All data is expressed and analysed as described in section 2.2.11

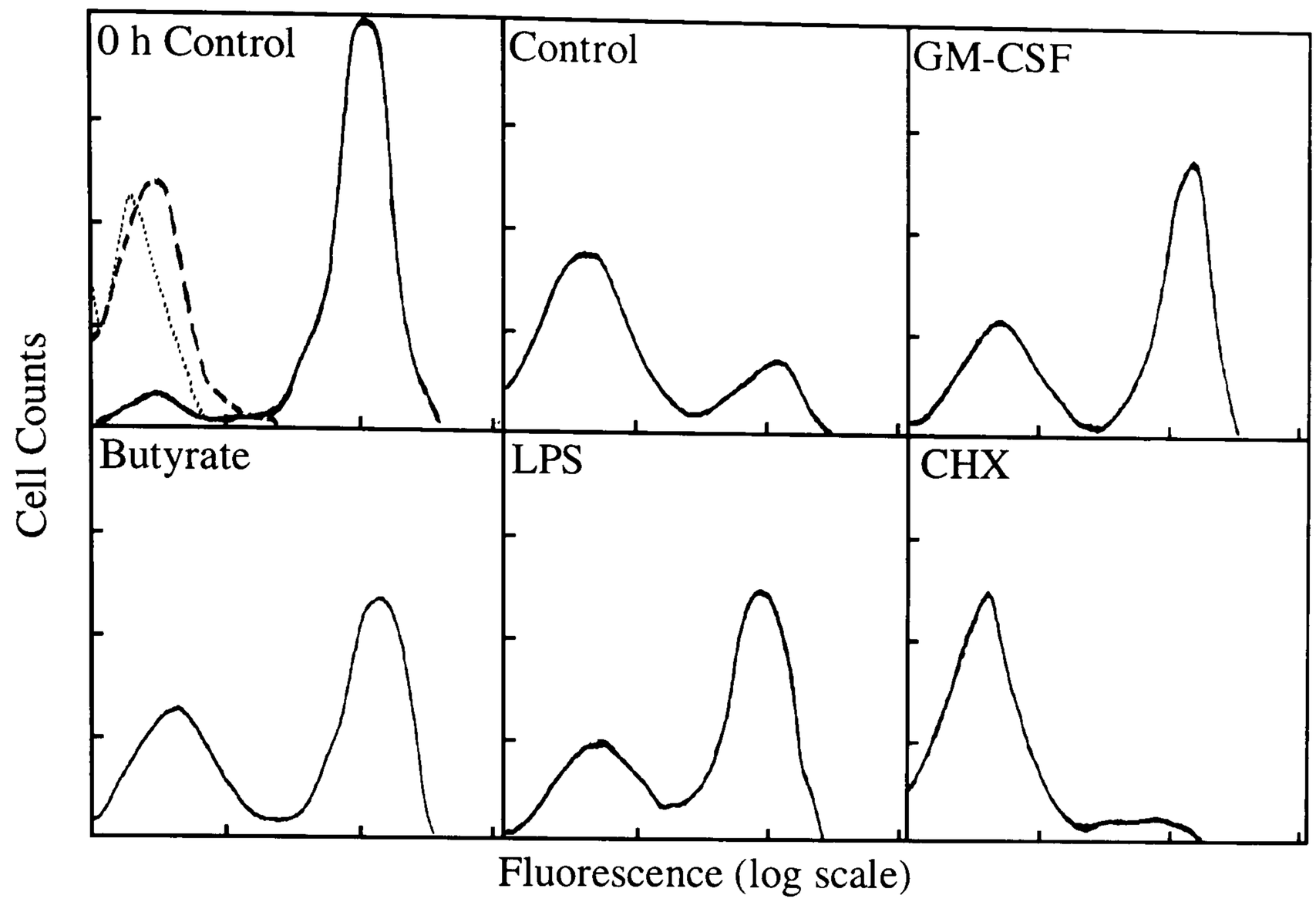
3.3 Results

3.3.1 Maintenance of surface CD16 expression by delaying apoptosis

Figure 3.1 shows the surface expression of CD16 on freshly isolated neutrophils, and on neutrophils cultured for 20 h with agents which either delay apoptosis (GM-CSF, butyrate and LPS) or accelerate apoptosis (CHX). Treatment with all apoptosis delaying agents tested preserved surface CD16 expression, as compared to control cultures, whilst CHX treatment resulted in a greater loss of surface CD16 from the whole population of neutrophils. Table 3.1 shows the percentage of cultured neutrophils with preserved levels of surface CD16 after 20 h culture, indicating that both GM-CSF and butyrate treatments significantly increased the number of CD16+ neutrophils after 20 h culture, whilst CHX significantly lowered the numbers of CD16+ neutrophils, as would be expected for these agents. In one experiment to test the ability of LPS to preserve surface CD16 expression, surface CD16 levels were maintained similarly to GM-CSF or butyrate treated neutrophils.

The loss of CD16 surface levels was only seen on neutrophils that had become apoptotic, as determined by dual labelling for CD16 surface expression and chromatin condensation by PI (Figure 3.2). It is clear that freshly isolated neutrophils showed high surface CD16 and high PI fluorescence (normal chromatin). After 20 h culture, a second population of apoptotic neutrophils with low PI fluorescence (condensed chromatin) and low surface CD16 was apparent. Delaying apoptosis with

Figure 3.1 Surface CD16 expression measured by flow cytometry



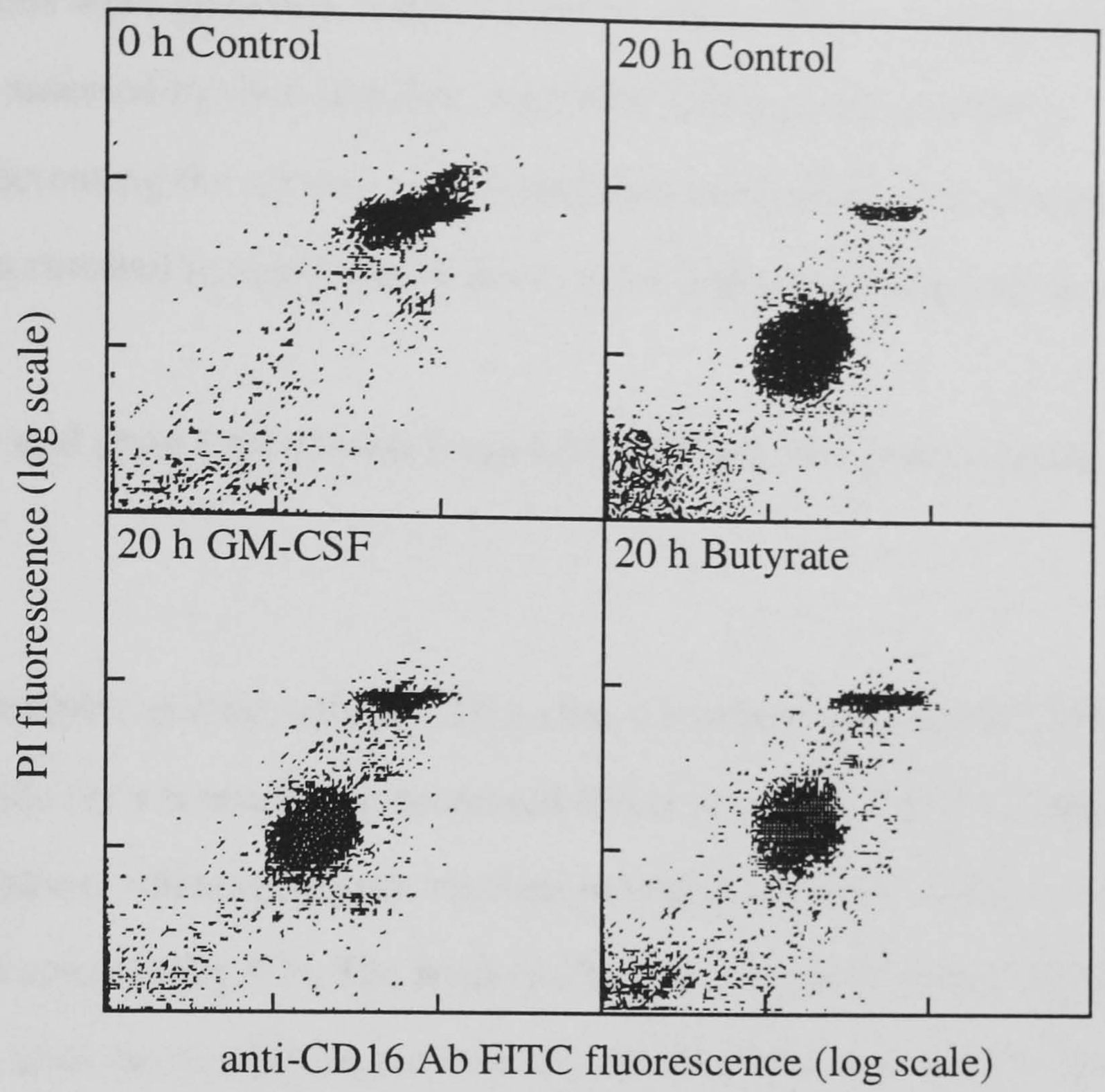
Following isolation and culture of neutrophils, surface CD16 expression was determined by two layer immunofluorescence and flow cytometric analysis. Dotted line represents unlabelled cell autofluorescence, dashed line is fluorescence from isotype matched control antibody, with solid lines representing surface CD16. Samples were analysed after incubation at 37^{0}C for 20 h (except for 0 h control panel).

Table 3.1 Percentage CD16+ cells after 20 h culture

	Control	GM-CSF	Butyrate	LPS	CHX
% C16 +	36.8	67.3 †	66.6 †	68.2	25.5 *
s.d.	10.9	18.3	16.1	--	10.4
n =	3	3	3	1	3

Neutrophils were isolated and cultured, then labelled for measurement of surface CD16 as described in Figure 3.1. Percentage of CD16+ cells are shown from 3 separate experiments. LPS is shown from one experiment only to confirm that CD16 surface expression is maintained by this apoptosis delaying agent. Significant difference from control is indicated by † ($p \leq 0.05$), or * ($p \leq 0.01$)

Figure 3.2 Fluorescence distributions of dual (CD16/PI) labelled neutrophils



Neutrophils were isolated and cultured, and then labelled for measurement of surface CD16 and PI fluorescence, as described in Materials and Methods. Results shown are representative of at least 5 separate experiments.

Table 3.2 Percentage non-apoptotic cells after 20 h culture determined by dual labelling of surface CD16 and chromatin with PI

	Control	GM-CSF	Butyrate	CHX
% non-apoptotic	15.96	32.79 *	27.59 *	7.20 *
s.d.	13.03	20.94	14.56	4.49
n =	13	13	13	5

Neutrophils were incubated and processed as described in Figure 3.2. Percentages of non-apoptotic cells as determined by high surface CD16 and high PI fluorescence are shown. Significant difference from control is indicated by * ($p \leq 0.01$).

either GM-CSF or butyrate resulted in the same two populations of neutrophils, but fewer neutrophils were apoptotic. Table 3.2 shows the numbers of non-apoptotic neutrophils as assessed by dual labelling, with GM-CSF and butyrate both significantly increasing the number of non-apoptotic neutrophils after 20 h culture: CHX treatment resulted in significantly fewer neutrophils remaining non-apoptotic.

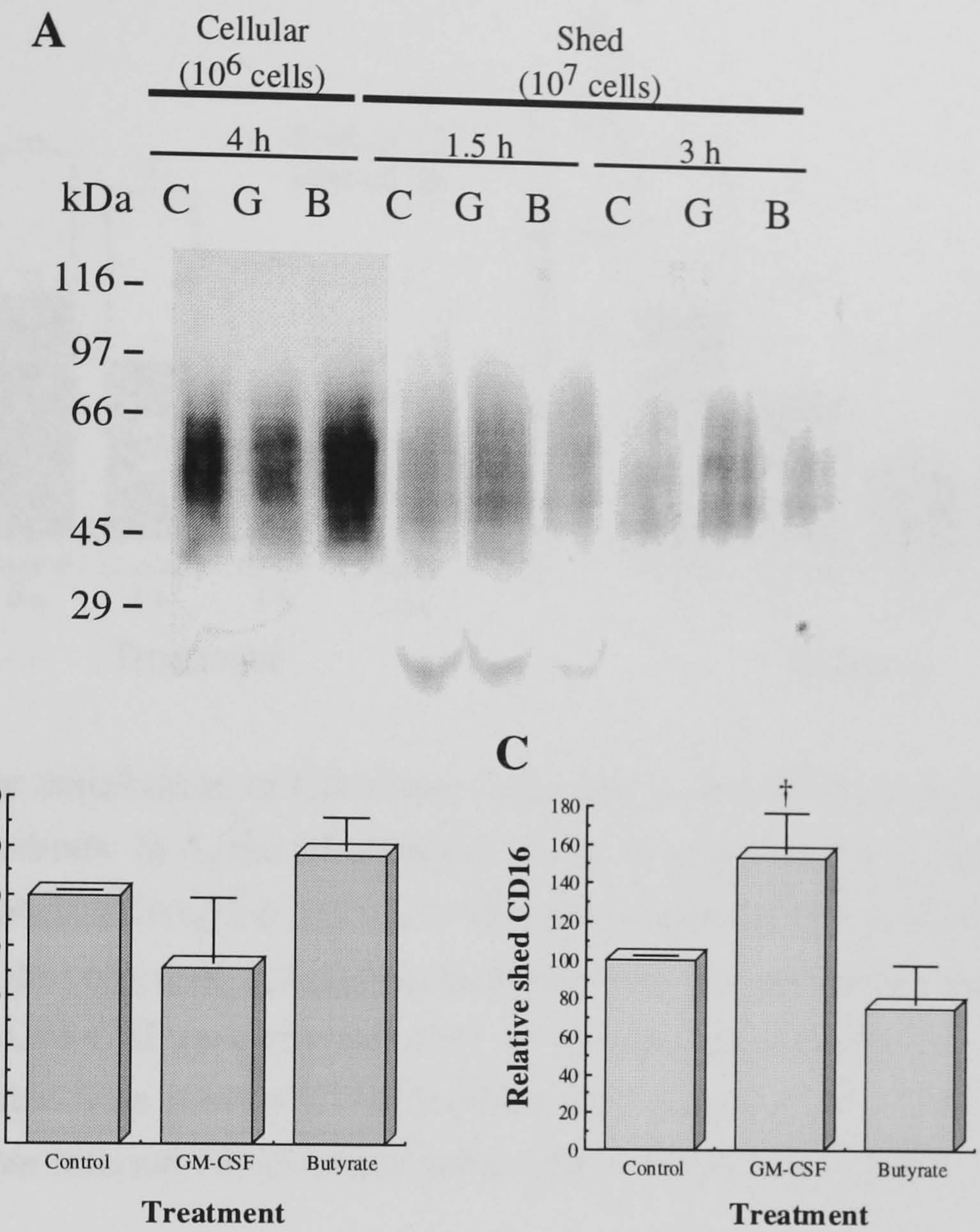
3.3.2 Cellular and shed CD16 levels from GM-CSF and Butyrate treated neutrophils

Western blot analysis of total cellular CD16 after 4 h culture showed that GM-CSF treatment of cells for 4 h resulted in decreased levels of cellular CD16 compared to control suspensions, whereas butyrate resulted in slightly elevated levels of cellular CD16 after 4 h culture (Fig 3.3). The level of CD16 in GM-CSF treated neutrophils was 71.3 % (± 25.8, n=3), while in butyrate treated neutrophils it was 116.1 % (± 12.6, n=3), compared to controls (100 %). This pattern of results was repeated in each of three experiments, but the differences did not reach statistical significance's due to the relatively large variations. Measurements of shed CD16 in culture supernatants indicated that GM-CSF significantly accelerated the shedding of this receptor for periods of up to 3 h, whereas butyrate treatment slightly decreased the rate of shedding (Fig 3.3).

3.3.3 Surface and internal levels of CD16 in cultured neutrophils

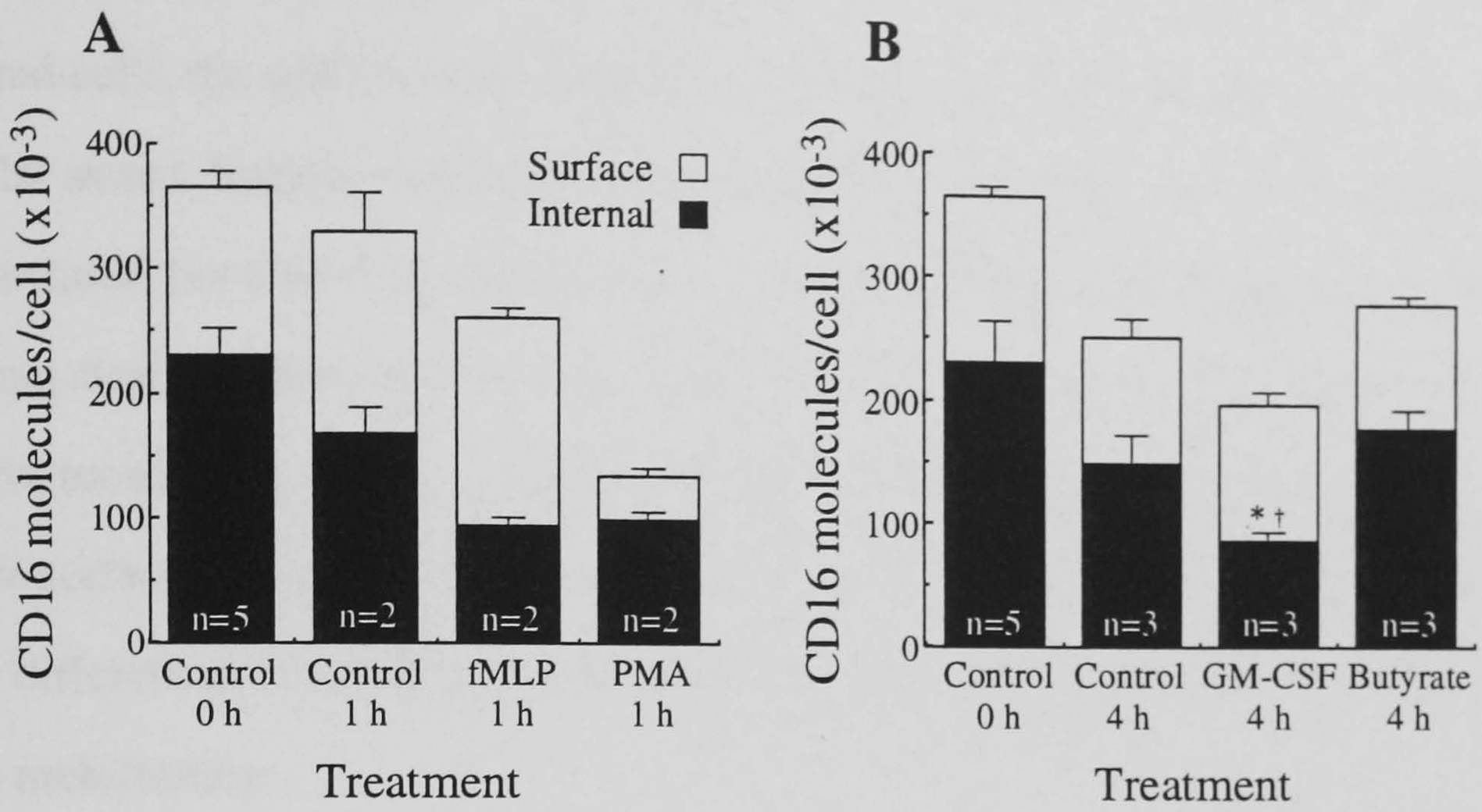
Determination of surface and internal levels of CD16 was initially determined by flow cytometry. The methodology used was proved satisfactory, as numbers of surface CD16 molecules were as expected (100,000-200,000 per cell), internal CD16 levels were depleted after 1 h culture, and activation of neutrophils with fMLP, or stronger activation with PMA, resulted in further depletion of internal CD16 stores, to replace CD16 shed from the surface (Fig 3.4a). Figure 3.4b shows the cellular

Figure 3.3 Immunoblot analysis of cellular and shed CD16



Neutrophils were isolated and cultured as described in Materials and Methods. Proteins were extracted and separated by SDS-PAGE then transfered to nitrocellulose. Following blocking, membranes were incubated with anti-CD16 antisera and then a secondary HRP conjugated antibody, before detection. The blots shown are representative of 3 separate experiments (A), with lanes labelled C = control, G = GM-CSF, and B = butyrate. In B (4 h incubations, total CD16) and C (1.5 h incubations, shed CD16) relative CD16 levels are shown from 3 separate experiments in which samples were processed as for part A. Densitometric measurements were used to calculate CD16 levels relative to the control sample. † indicates significant difference from control ($p \leq 0.05$).

Figure 3.4 Surface and internal CD16 determined by flow cytometry



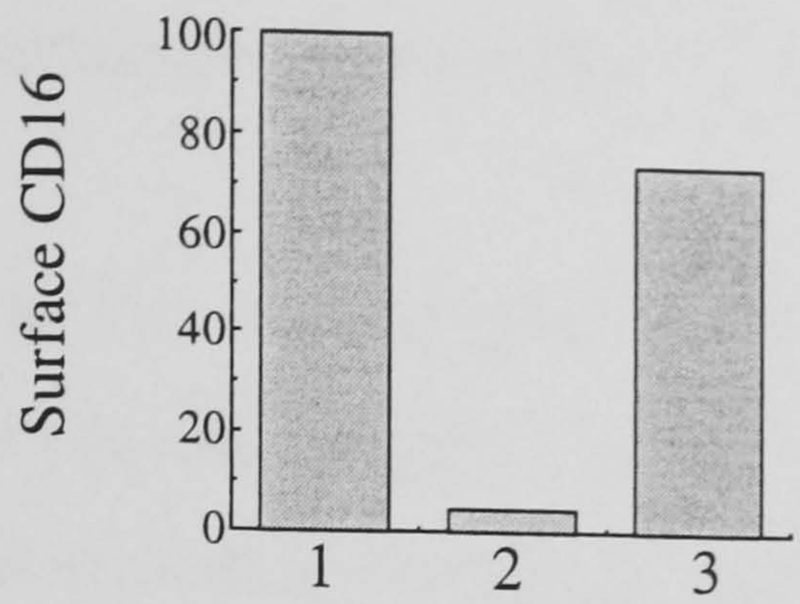
Cellular distribution of CD16 was examined as described in Materials and Methods. In A, the reliability of the assay was tested by treating neutrophils with agents known to stimulate redistribution of CD16. B shows the results of neutrophils incubated with the apoptosis delaying agents GM-CSF and butyrate, after 4 h incubations. Significant difference from internal CD16 levels at 0 h is indicated by * ($p \leq 0.01$), and from internal CD16 in untreated cells at 4 h by † ($p \leq 0.05$).

distribution of CD16 following 4 h culture. Whilst surface levels of CD16 in control suspensions were maintained to levels approximate to those determined on freshly-isolated cells, the surface expression was achieved via mobilisation of the sub-cellular stores. Butyrate treatment caused no discernible differences from control suspensions, but GM-CSF treated cells showed a significant difference from control. Internal stores decreased from ~230,000 (±46,000) at 0 h, to ~148,000 (±36,000) in control incubations and to ~85,000 (±11,000) in GM-CSF treated suspensions. Thus whilst surface levels of CD16 were very similar for all treatments at 4 h there were clear differences in the extent to which surface CD16 had been shed and replaced by store mobilisation.

The same flow cytometric approach to measure surface and internal CD16 was unsuitable for 20 h cultured cells, as the mild detergent treatment used to permeabilize cells for antibody entry actually lysed the aged cells. I therefore developed an alternative method based on immunoblotting. Figure 3.5 shows removal of surface CD16 by a 10 min pronase treatment, to less than 10 % of initial levels. Stimulation of pronase treated cells with fMLP for 10 min was able to replenish surface CD16 levels back to ~75 % of initial levels. Figure 3.6 shows results of surface and internal CD16 measurements by this immunoblotting technique, utilising the near total removal of surface CD16 by pronase. Neutrophils cultured for 3 h showed very similar levels and distribution of CD16 in control, GM-CSF and butyrate treated suspensions, as those obtained with the flow cytometric technique after 4 h. Thus, whilst surface levels of CD16 were nearly identical in all suspensions, there were marked differences in the extents of store mobilisation: GM-CSF and LPS resulted in significantly higher levels of store mobilisation.

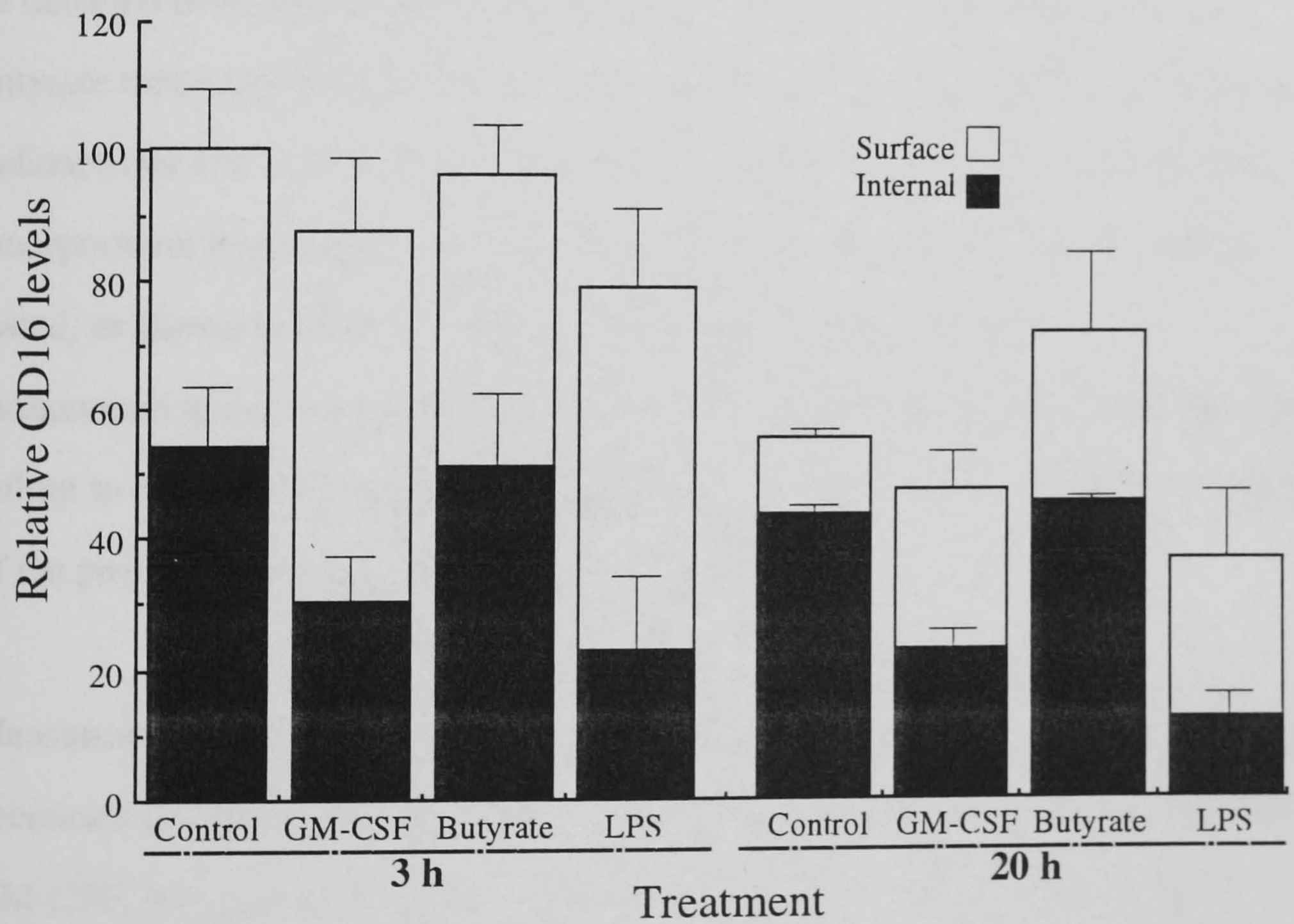
Internal CD16 levels were similar in both control and butyrate treated cultures at 20 h, but GM-CSF suspensions were significantly lower, at ~ 50 % the levels of internal CD16 of control and butyrate suspensions ($p \leq 0.01$). LPS showed significantly

Figure 3.5 Removal of surface CD16 with Pronase



Surface CD16 was measured by 2 layer immunofluorescence, as described in Materials and Methods. Column 1 shows surface CD16 from freshly isolated neutrophils, column 2 from neutrophils treated for 10 min with pronase (0.25 mg/mL), column 3 from neutrophils treated with fMLP for 10 min following pronase treatment. Pronase treatment removed 92.4% (± 3.65, n=4), whilst fMLP restored surface CD16 as shown in column 3 (representative of 2 experiments).

Figure 3.6 Surface and internal CD16 determined by immunoblot analysis



Following isolation and culture of neutrophils (in the conditions indicated) surface and internal CD16 levels were determined, as described in Materials and Methods by an immunoblotting/pronase technique. All values are relative to the total CD16 level in 3 h control neutrophils. Significant differences are discussed in the text.

lower internal levels of CD16 compared to GM-CSF treatments ($p \leq 0.01$)., being ~ 25 % of the control and butyrate treated values.

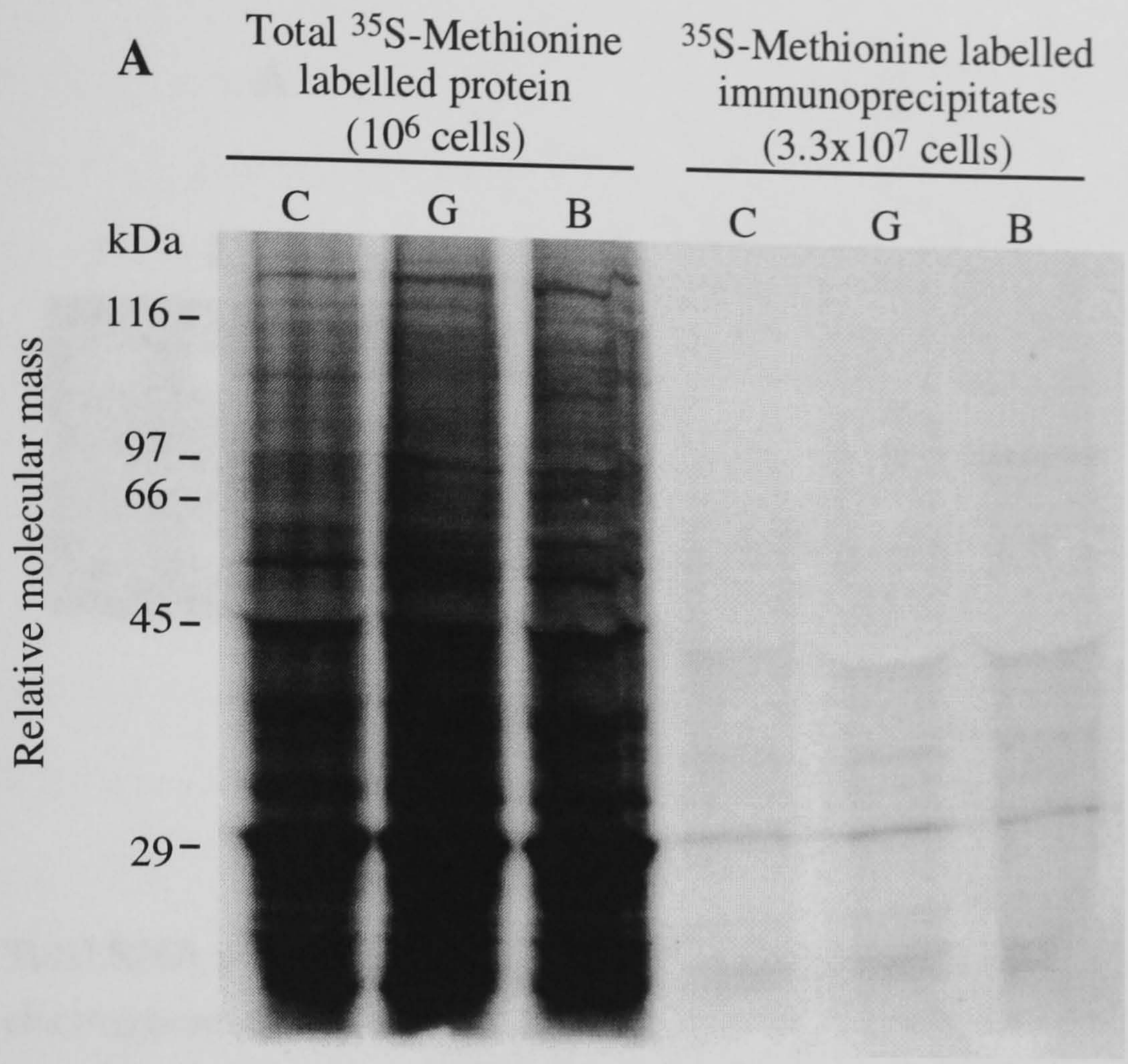
In cultures incubated for 20 h, surface levels of CD16 were decreased. as compared to those measured at 3 h incubation (Fig. 3.6). In control, 20 h suspension. surface levels were only about 25 % of those at time zero, whilst for GM-CSF. butyrate and LPS, surface levels were about 50 % of the corresponding 3 h levels.

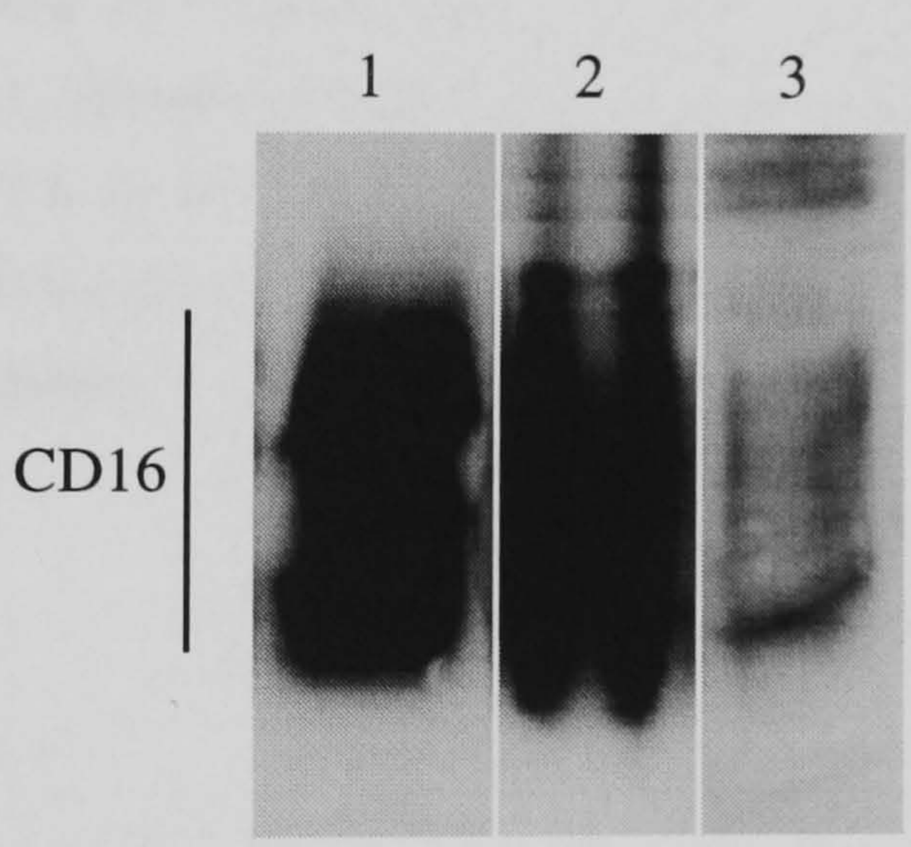
3.3.4 *De novo* CD16 synthesis and mRNA measurements

Figure 3.7 shows a typical result of numerous attempts to detect [^{35}S]-methionine labelled CD16 following immunoprecipitation. *De novo* synthesized CD16 could not be detected from 18 h incubated neutrophils from either control, GM-CSF or butyrate treated suspensions under the conditions employed. The incorporation of radioactivity into total newly synthesized proteins was tested, and high levels of incorporation found (Fig 3.7a). The efficiency of immunoprecipitation was also tested, as shown in Figure 3.7b, with ~75 % of all CD16 becoming immunoprecipitated using the techniques described in Materials and Methods. The failure to detect newly synthesized CD16 suggests that there is insufficient synthesis of the protein to be detected under these conditions.

Measurement of mRNA levels by northern blotting showed that CD16 mRNA levels decreased to ~50 % of initial levels by 3 h culture of neutrophils treated with either GM-CSF, butyrate, or in control suspensions (Fig 3.8). mRNA levels were also examined after 20 h culture: Relative to control suspensions. GM-CSF treatment resulted in mRNA levels that were 57.0 % (±25.2, n=3) of control. whilst butyrate treatment resulted in levels that were 115.3 % (±40.9. n=3) of control. No significant differences were found, though GM-CSF treatment gave lower levels of CD16 mRNA in all 3 experiments.

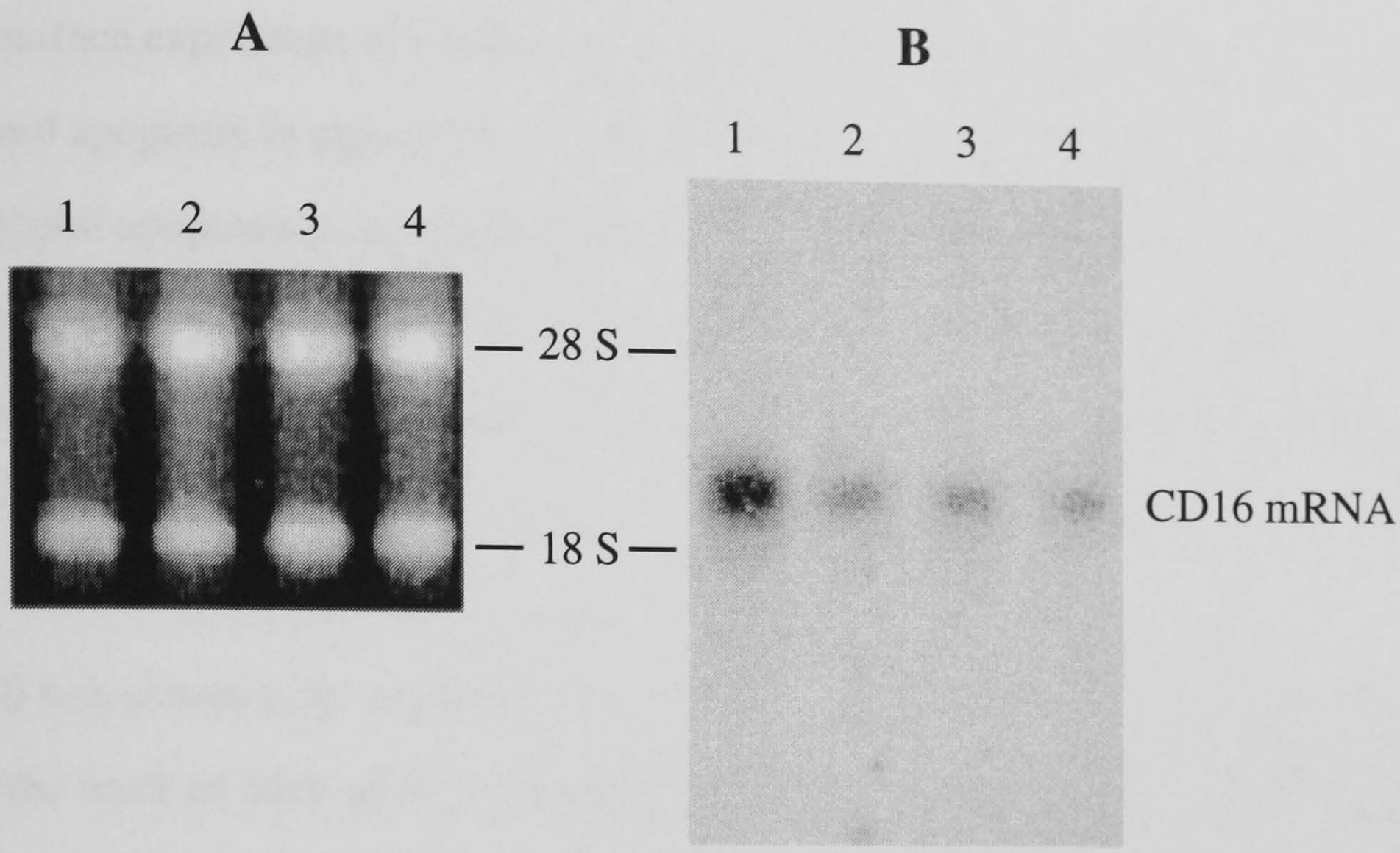
Figure 3.7 Immunoprecipitation of ^{35}S-Methionine labelled CD16





In **A**, neutrophils were cultured for 18 h in media alone (C) or in media supplemented with GM-CSF (G) or butyrate (B). Total protein from 10^6 neutrophils, or CD16 immunoprecipitates from $3.3x10^7$ neutrophils were separated by SDS-PAGE and detected by radiography as described in Materials and Methods. In **B,** parallel samples from 10^6 neutrophils were prepared for immunoblotting for CD16 as described in Materials and Methods. Samples used were: 1 whole cell lysate, 2 immunoprecipitated material, 3 lysate after removal of immunoprecipitated material. In 3 separate experiments 74.3 ± 5.1 % of CD16 was immunoprecipitated by this method.

Figure 3.8 Northern blot analysis for CD16 mRNA



Total RNA was isolated from neutrophils, separated by denaturing agarose electrophoresis, transferred to nitrocellulose and probed for CD16 as described in Materials and Methods. Equal amounts of RNA were loaded per lane as determined by visualisation of 28S and 18S ribosomal RNA with UV illumination (A). Samples analysed were: 1) 0 h neutrophils, 2) 3 h cultured neutrophils, 3) 3 h cultured neutrophils with GM-CSF, 4) 3 h cultured neutrophils with butyrate. The blot shown (B) is representative of two separate experiments.

3.4 Discussion

The surface expression of CD16 was maintained over 20 h by all the agents which delayed apoptosis in agreement with the work of two groups which showed neutrophil apoptosis is associated with a loss of surface CD16 (Dransfield *et al*, 1994; Homburg *et al*, 1995). Accelerating apoptosis with the protein synthesis inhibitor cycloheximide was associated with an increase in the number of neutrophils with low levels of surface CD16.

CD16 was shown to be constantly shed from neutrophil populations, in agreement with the work of other groups (Huizinga *et al*, 1988; Huizinga *et al*, 1990; Tosi & Zakem, 1992; Middlehoven *et al*, 1997). Whether CD16 is constantly shed from resting neutrophils, or is only shed as cells become apoptotic, is uncertain. It is possible that the constant shedding of CD16 observed in a population of neutrophils is a result of CD16 shed only by older neutrophils undergoing apoptosis. However, activation of neutrophils can result in increased shedding of CD16 (Huizinga *et al*, 1988; Tosi & Zakem, 1992; Middlehoven *et al*, 1997) as shown here for GM-CSF treatment (Figs 3.3, 3.4 & 3.6). Sodium butyrate treatment, on the other hand, did not increase the rate of CD16 shedding. The increased shedding of CD16 in GM-CSF treated neutrophils, but not in butyrate treatments, suggests that surface CD16 expression is maintained by mechanisms which do not involve slowing the rate of shedding, but rely on replacement of surface levels by store mobilisation.

The surface levels of CD16 are maintained by replacing shed CD16 from internal stores (Tosi & Zakem, 1992). The internal stores of CD16 are shown here to be constantly depleted in a neutrophil population, with surface levels of CD16 maintained following treatment to delay apoptosis, by continued replacement from internal stores. In control suspensions, the internal levels of surface CD16 remain relatively high, indicating loss of surface CD16 in apoptotic neutrophils results due

to shedding of surface CD16 without concomitant replacement from internal stores. The increased shedding of surface CD16 following GM-CSF treatment is counteracted by increased replacement from internal stores, as indicated by the further depletion of these stores in GM-CSF treated cells. LPS treatment, which mobilises the vesicles containing the LPS receptor CD14 (co-mobilising CD16 which is located in the same vesicles) to the cell surface (Detmers *et al*, 1995), gave a greater depletion of internal stores. Despite this depletion of internal stores, surface levels of CD16 are maintained by LPS for periods up to 20 h. This suggests the constant, unstimulated shedding of surface CD16 occurs at a rate low enough to allow continued replenishment from internal stores, even if internal stores are initially depleted by activation with either GM-CSF or LPS.

I was unable to detect any *de novo* synthesized CD16 under any experimental conditions, even though *de novo* synthesis of CD16 has been reported in human neutrophils (Jack & Fearon 1988; Jost *et al*, 1990). CD16 mRNA levels decreased over time, with GM-CSF treatment actually further lowering CD16 mRNA abundance by 20 h, in agreement with previous work in this laboratory (Moulding *et al,* 1996). The internal stores of CD16 are large (> 200,000 molecules per cell) and so *de novo* synthesis of CD16 may not be necessary even if the lifespan of the neutrophil is extended, necessitating continued surface expression. The immunoprecipitation method successfully precipitated > 75 % of total cellular CD16, and the cells actively synthesized proteins, as determined by [^{35}S]-methionine labelling. mRNA levels for CD16 actually <u>decreased</u> following GM-CSF treatment. These data indicated that *de novo* CD16 biosynthesis is extremely low and below the level of detection in this experimental system.

The maintenance of surface CD16 expression in neutrophils treated to delay apoptosis can be accomplished by the mobilisation of internal stores to the cell surface. In GM-CSF treatment, there is actually an <u>increased</u> rate of CD16 shedding,

but this is matched by increased mobilisation of internal stores. In butyrate delayed apoptosis there is no need for increased mobilisation of internal stores, as CD16 shedding is not accelerated. The finding that internal stores of CD16 are only slightly diminished after 20 h culture in control neutrophils, but are significantly depleted in activated neutrophils (Fig 3.6) suggests that shedding may not be continuous in unstimulated neutrophils. Rather, it appears that CD16 may only be shed upon activation or apoptosis.

The physiological role of shedding of surface CD16 remains uncertain. It has been noted that the uncleaved portion of CD16 may mark the neutrophil for phagocytosis (Homberg *et al*,1995). However, this would suggest that activated neutrophils, which also shed surface CD16 (Huizinga *et al*, 1988) would be similarly recognised, as both shedding processes utilise a metalloprotease (Middlehoven *et al*, 1997) and activated neutrophils are thought not to be recognised as apoptotic and phagocytosed (Haslett *et al*, 1994). It is therefore unlikely that CD16 shedding is a marker for phagocytosis of apoptotic neutrophils, or else the cleavage sites following activation and apoptosis are different, leaving different residual structures in the membrane.

The shedding and replacement of surface CD16 following activation may allow functionally active CD16 to translocate to the cell surface. Soluble immune complexes do not simulate a respiratory burst in unprimed neutrophils, but can activate this event following neutrophil priming (Watson *et al*, 1997d). This stimulation of respiratory burst in primed neutrophils is dependent on CD16, as CD16 deficient individuals, or surface CD16 depleted neutrophils (treated with pronase or PI-PLC), do not produce a respiratory burst (Edwards *et al*, 1997). It is then possible that <u>either</u> a) priming results in 'activation' of the surface CD16 by recruitment of a signalling molecule such as Hck (Edwards *et al*, 1997) or b) the newly-translocated CD16 is already 'activate' being linked to this signalling molecule.

The shedding of CD16 in apoptotic neutrophils may aid the resolution of inflammation. Soluble CD16 is able to bind to IgG molecules (Huizinga *et al*, 1990), so may down-modulate cellular responses to IgG complexes in inflammatory sites by binding these complexes away from the cell surface (Tosi & Zakem, 1992). Therefore, the apoptosis of inflammatory neutrophils may not only allow resolution of inflammation by the removal of these cells from the site of inflammation, but by releasing soluble CD16 may 'mop-up' excess IgG complexes, preventing further activation of inflammatory cells.

CHAPTER 4 EFFECT OF SUPERANTIGENS ON NEUTROPHIL APOPTOSIS

4.1 Introduction

Staphylococcal enterotoxins cause food poisoning and toxic shock, and may lead to multiple organ failure resulting from the massive activation of the immune system (Marrack & Kappler, 1990). There is also evidence which suggests a critical role for *Staphylococcal* enterotoxins in the pathogenesis of rheumatoid arthritis (Origuchi *et al*, 1995). These toxins are superantigens, which activate a subset of T-cells (around 20 % of T-cells have the appropriate V_β region), by binding to MHC class II molecules on antigen presenting cells, and cross-linking to the T-cell receptor (TCR) (Misfeldt, 1990; Herman *et al*, 1991). There is a large production of pro-inflammatory cytokines from both T-cells and monocytes following superantigen activation. T-cells produce IL-1β, interferon-γ (IFN-γ), TNF-α and IL-2 (Jupin *et al*, 1988; See *et al*, 1992; Lagoo *et al*, 1994), whilst monocytes produce both IL-1β and IFN-γ (See *et al*, 1992; See & Chow, 1992).

The effects of superantigens on T cells and monocytes are well studied, but the effects of superantigens on other cells of the immune system are relatively neglected. The indirect activation of these other immune cells may be expected *in vivo* as cytokine secretion by T cells and monocytes will in turn activate other cells. There are a few reports of other immune cells being affected apparently without the involvement of T cells or monocytes. Notably, CD56-positive natural killer (NK) cells have been shown to be directly stimulated by *staphylococcal* enterotoxin B (SEB) (D`Orazio *et al*, 1995). This action on NK cells is not totally unexpected, as a subset of these cells are known to express MHC class II. There have also been reports of neutrophil responses to Toxic Shock Syndrome Toxin-1 (TSST-1), such as expression of heat shock proteins within 30 minutes of stimulation (Hensler *et al*,

1991), and changes in surface protein expression and leukotriene B_4 (LTB_4) synthesis which occur within 10 min of stimulation (Hensler *et al*, 1993). Although the work on neutrophils suggests a direct effect of TSST-1 on neutrophils this was not conclusively shown, as no surface targets for these superantigens have been identified on the neutrophil (Hensler *et al*, 1993). Recently, neutrophils have been shown to express MHC class II molecules after culture for a day or more with IFN-γ, GM-CSF or IL-3 (Gosselin *et al*, 1993; Smith *et al*, 1995), but this does not explain the rapid (10 min) effects of TSST-1 on neutrophils.

The effects of superantigens on neutrophil apoptosis have not been studied to date, though *in vivo* neutrophil apoptosis would most likely be delayed due to the production of cytokines by other immune cells. The work of Hensler and colleagues (1991, 1993) suggests a direct effect of superantigens on neutrophils, including enhanced production of LTB_4. This leukotriene is able to delay neutrophil apoptosis (Hébert *et al*, 1996; Murray *et al*, 1997), providing a possible mechanism for a direct action of superantigens on neutrophils, which will delay apoptosis. Thus, in this Chapter, I have investigated the possibility that superantigens may act directly on neutrophils to delay apoptosis.

4.2 Materials and Methods

4.2.1 Materials

All materials used for the isolation and culture of neutrophils are listed in section 2.2.1. Superantigens SEA, SEB and TSST-1 were purchased from Sigma. Interferon-γ was from Boehringer Mannheim (Lewes, UK). Monoclonal antibody 32.2 (anti-FcγRI) was purchased from Medarex Inc. (NJ), monoclonal antibody Leu 11a (anti-FcγRIII), FITC second layer antibody, and IgG controls were from Becton Dickinson (Cowley, UK). Polyclonal sheep (#90) anti-Interferon-γ antisera was from NIBSC

(UK). Anti-phosphotyrosine Ab RC20 conjugated to HRP was from Transduction Laboratories (Lexington, KY) PVDF membrane was from Millipore (UK). ECL detection kit and Amplify were from Amersham (UK). Pan T Dynabeads and anti-MHC II Dynabeads (antibody coated magnetic beads) were purchased from Dynal (UK). Fluo-3/AM was from Calbiochem. All other specialist reagents used were from Sigma (UK) and were of the highest quality available.

4.2.2 Neutrophil isolation and purification

Neutrophils were isolated as described in section 2.2.2, except FCS was added to isolated neutrophils to 5 % (v/v) rather than 2.5 % (v/v), to minimise cell loss due to necrosis in overnight cultures.

Depletion of T-cells and MHC II expressing cells

Neutrophil preparations were depleted of contaminating T-cells, MHC II expressing cells, or both, by using Dynabeads. The appropriate beads were added to neutrophil preparations at 2 x 10^7 beads/mL after washing the beads 3 times in RPMI 1640, and then incubation at 4^0C for 30 min with rotation, following the manufacturer's instructions, which report removal of 99 % of target cells using these conditions. Incubation was at 4^0C to minimise phagocytosis of the beads by neutrophils. Beads were then magnetically removed from cell suspensions, 6 times. The resulting neutrophil preparations (T-cell and MHC II depleted) contained no remaining beads, and examination of > 1,500 cells (Wrights' staining) showed no contaminating mononuclear cells. Depletion of T-cells alone resulted in just 2 lymphocytes in 1,500 cells. Final neutrophil preparations were > 95 % viable (as assessed by trypan blue exclusion), and were adjusted to 5 x 10^6 cells/mL in RPMI 1640 medium supplemented with 5 % (v/v) FCS and 2 mmol/L L-Glutamine.

4.2.3 PBMC isolation

Heparinized venous blood from healthy volunteers used for the preparation of neutrophils was spun through NIM as above, with peripheral blood mononuclear cells (PBMC) removed at the first step of neutrophil isolation. PBMC were then counted and resuspended in RPMI 1640 medium supplemented with 5% (v/v) FCS and 2 mmol/L L-glutamine.

4.2.4 PBMC conditioned media

PBMC were isolated as described above, and resuspended at $2x10^7$/ml in RPMI 1640 supplemented with 5% (v/v) FCS and 2mmol/L L-glutamine. Incubations were in conical polypropylene tubes at 37^0C with gentle agitation in the absence (control) or presence of SEA (100 ng/ml), SEB (100 ng/ml) or TSST-1 (5 μg/ml). Following 22 h in culture, supernatants were collected and frozen for future use. The supernatants were added to neutrophil suspensions to mimic a 5 % contamination of PBMC. Therefore 12.5 μL conditioned media was added per mL neutrophil suspension. The concentration of superantigen in these neutrophil suspensions is therefore less than 1.25 ng/mL for SEA, and SEB, and less than 62.5 ng/mL for TSST-1.

4.2.5 Neutrophil culture

Neutrophils were cultured as in section 2.2.3, except FCS was added to 5 % (v/v), with no further additions to the media as controls. SEA, SEB, and TSST-1 were added at 0.01, 0.05, 0.1, 0.5, 1.0, or 5.0 μg/mL, GM-CSF at 50 U/mL, and interferon-γ at 100 U/mL. PBMC conditioned media was added at 12.5 μL/mL. Interferon γ-neutralising antisera was added to incubations at a 1:1000 dilution.

4.2.6 NADPH oxidase activity

Chemiluminescence was assayed as described in section 2.2.7.

4.2.7 Morphological assessment of apoptosis

Apoptosis was assessed by morphology as described in section 2.2.6.

4.2.8 Surface expression of FcγRI (CD64) and FcγRIIIb (CD16)

Surface expression of FcγRIIIb was measured exactly as described in section 3.2.4, with FcγRI expression measured by substituting monoclonal Ab 32.2 for the Leu 11a Ab. Fluorescence distributions represent a total of 5,000 gated events.

4.2.9 Intracellular free Ca^{2+} concentrations

Changes in intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$ were monitored with the fluorescent probe Fluo-3. Neutrophils in RPMI 1640 (2 x 10^7/mL) were loaded by incubation at 37^0C for 30 min with 2 μmol/L Fluo-3 AM. The cells were then washed twice and resuspended at 2 x 10^6/mL in Ca^{2+} containing Hepes buffer (1 mmol/L $CaCl_2$; 145 mmol/L NaCl; 1 mmol/L $Na_2HPO_4.2H_2O$; 0.5 mmol/L $MgSO_4.7H_2O$; 5 mmol/L glucose; 20 mmol/L Hepes, pH 7.4). Fluorescence was then measured at 505 nm excitation and 530 nm emission. Calibration of changes in intracellular free Ca^{2+} levels was as described in (Merrit *et al*. 1990) using a Kd for Fluo-3 of 864 nmol/L at 37^0C.

4.2.10 Phosphotyrosine immunoblotting

Neutrophils were stimulated for 0, 1, 5, 10 and 20 min with the agents indicated, at 37^0C before centrifugation at 13,000g for 30 s at 4^0C and solubilisation in (boiling) reducing SDS-PAGE sample buffer. Proteins from equal numbers of cells were separated by 5-15 % SDS-PAGE, and transferred to PVDF membrane using BioRad electrophoresis and transfer apparatus. The membrane was blocked by incubation at 37^0C in: 3 % (w/v) gelatin; 20 mmol/L Tris pH 7.2; 100 mmol/L NaCl; 0.05 % (v/v) Tween-20; 0.05 % (v/v) Nonident P40. The membrane was incubated with RC20-HRP Ab at 1:2000 dilution in: 0.5 % (w/v) gelatin; 20 mmol/L Tris pH 7.2; 100 mmol/L NaCl; 0.05 % (v/v) Tween-20; 0.05 % (v/v) Nonident P40. Membranes were then washed for 2 x 1 min, 1x 15 min, and 3 x 5 min with: 20 mmol/L Tris pH 7.2; 100 mmol/L NaCl; 0.05 % (v/v) Tween-20; 0.05 % (v/v) Nonident P40, before detection of bound Ab with an ECL kit.

4.2.11 Heat shock and *de novo* protein synthesis

Neutrophils at 10^7 cells/mL were pre-incubated with 60 μCi/mL [^{35}S]-methionine for 15 min at 37^0C. Cells were then cultured for either: 1 h at 37^0C; 30 min at 42^0C; 1 h at 42^0C; or 1 h at 37^0C with TSST-1 added at 1.0 and 5.0 μg/mL. Proteins (from 10^6 cells) were then solubilised in (boiling) SDS-PAGE sample buffer and subjected to SDS-PAGE using the method of Laemmli (1970). Radioactivity in gels was detected as described in section 3.2.9.

4.2.12 Statistical analysis

All data is expressed and analysed as described in section 2.2.11

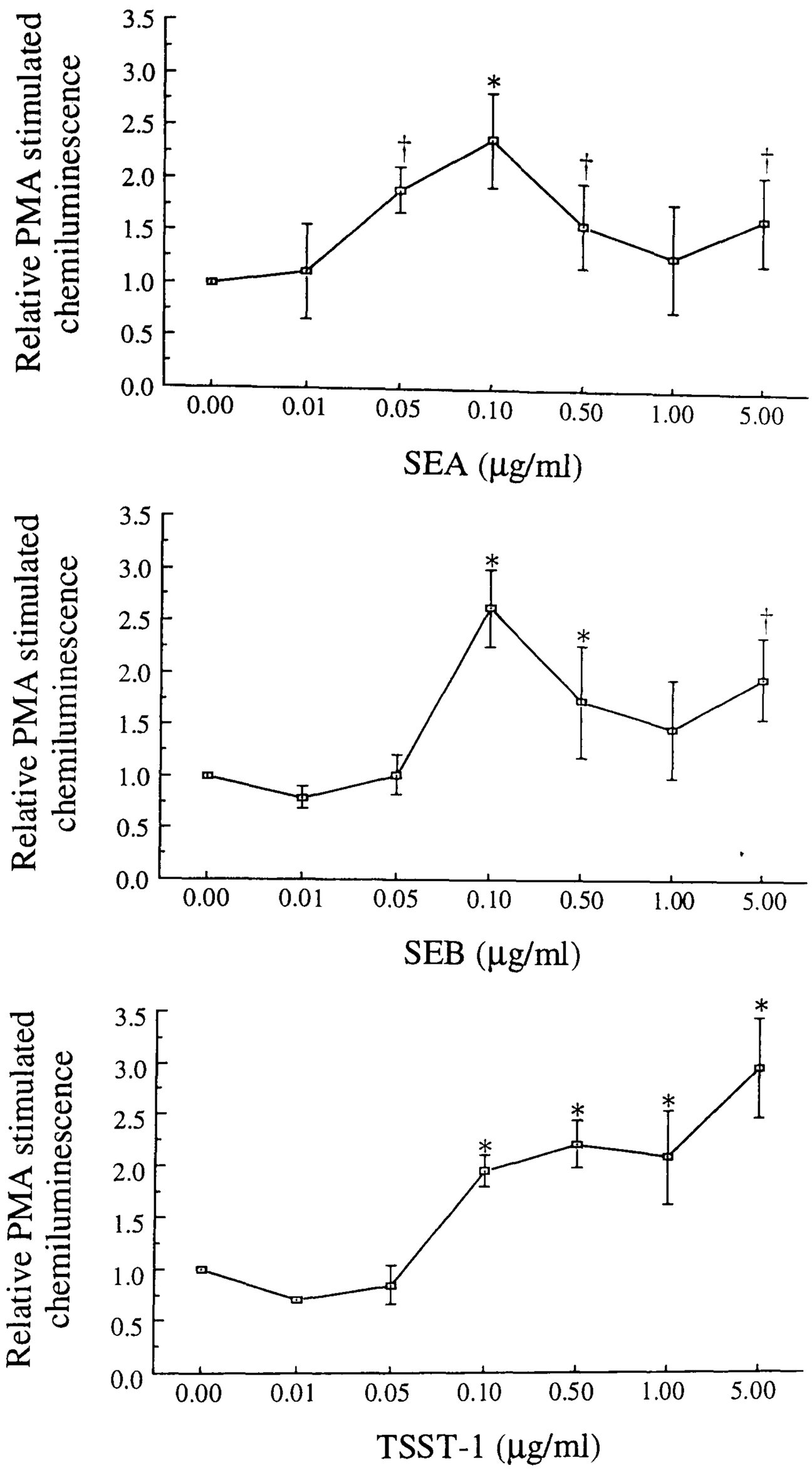
4.3 Results

4.3.1 Delayed apoptosis of superantigen treated neutrophil suspensions

Delayed neutrophil apoptosis is associated with a preservation of functions such as the ability to produce reactive oxygen species (ROS) in response to PMA or fMLP (section 2.3.3). The chemiluminescence of 20 h cultured neutrophil suspensions treated with a range of concentrations of *Staphylococcal* enterotoxin A (SEA), SEB or toxic shock syndrome toxin-1 (TSST-1), relative to untreated control cultures are shown in Figure 4.1. All three toxins gave significant improvement in chemiluminescence after 20 h culture at concentrations above 0.1 μg/mL, and up to 5 μg/mL which was the highest concentration tested. The maximally effective concentration for both SEA and SEB was 0.1 μg/mL, with increased concentrations of toxin showing a decline in effect up to 1.0 μg/mL. TSST-1 showed an increased effect at each concentration above 0.1 μg/mL, with maximal effectiveness at 5.0 μg/mL. The toxins were therefore added to neutrophil suspensions at 0.1 μg/mL for SEA and SEB, and at 1.0 and 5.0 μg/mL for TSST-1 in further experiments.

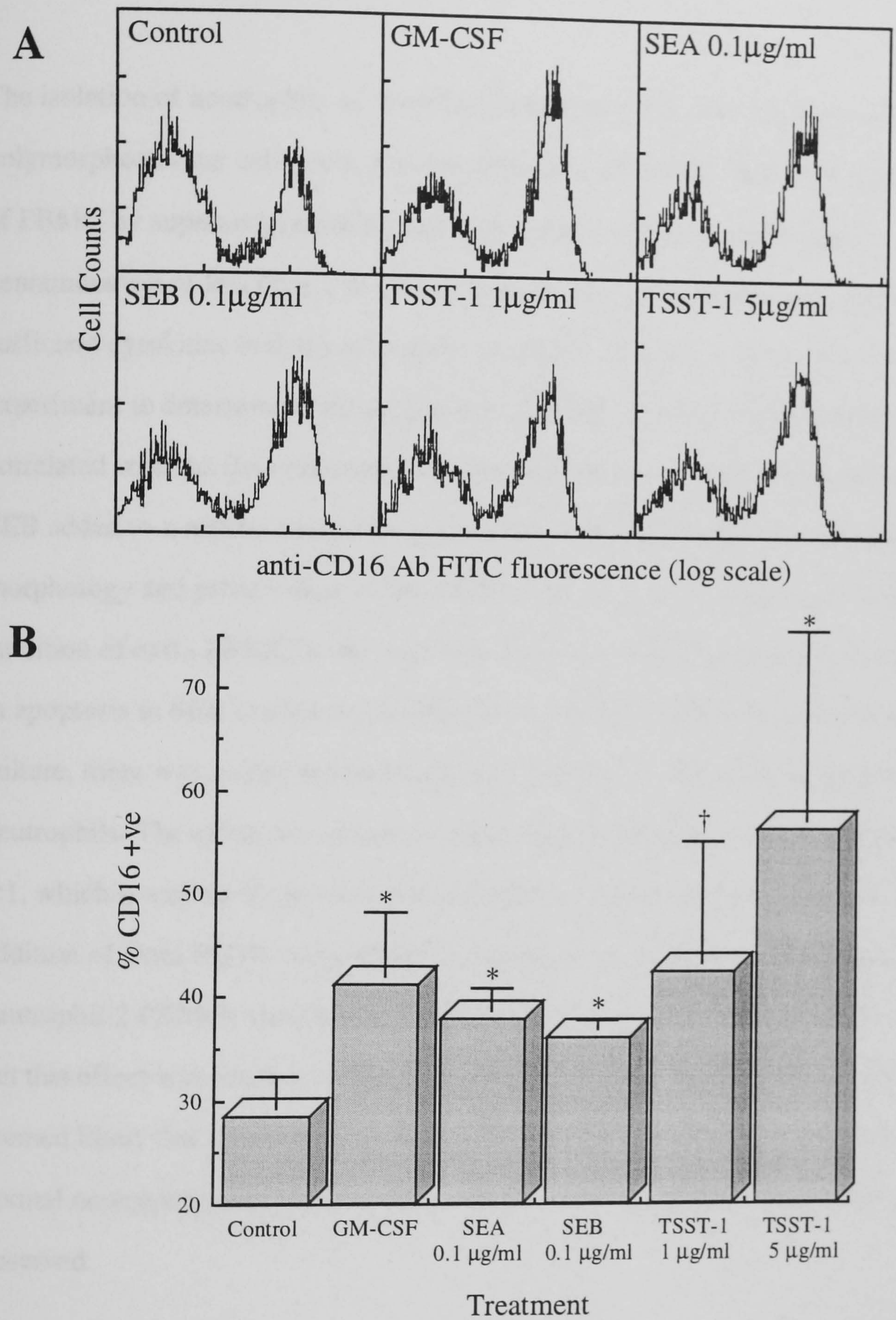
Assessment of protection from apoptosis was also determined by measuring CD16 surface expression in 20 h cultured neutrophils, as shown in Figure 4.2. Each toxin gave a significant protection from apoptosis measured by this assay, with the protection from apoptosis comparable to that found with GM-CSF treatments. TSST-1 gave the best protection from apoptosis, with the higher TSST-1 concentration being the most potent in delaying apoptosis. These results were also confirmed by morphological assessment of apoptosis (data not shown), with the same pattern of results obtained by both measurements.

Figure 4.1 Relative oxidative burst activity following 20 h culture with a range of concentrations of SEA, SEB and TSST-1



Following isolation, neutrophils were cultured in media alone or supplemented with SEA, SEB or TSST-1 to the concentrations shown, as described in Materials and Methods. After 20 h culture aliquots of equal numbers of neutrophils were processed for assessment of oxidative burst activity in response to stimulation with PMA. Results shown are means (± SD) of three separate experiments. Significant difference from control incubations are indicated by † ($p \leq 0.05$) or * ($p \leq 0.01$).

Figure 4.2 Preservation of surface CD16 expression by superantigens in neutrophil cultures



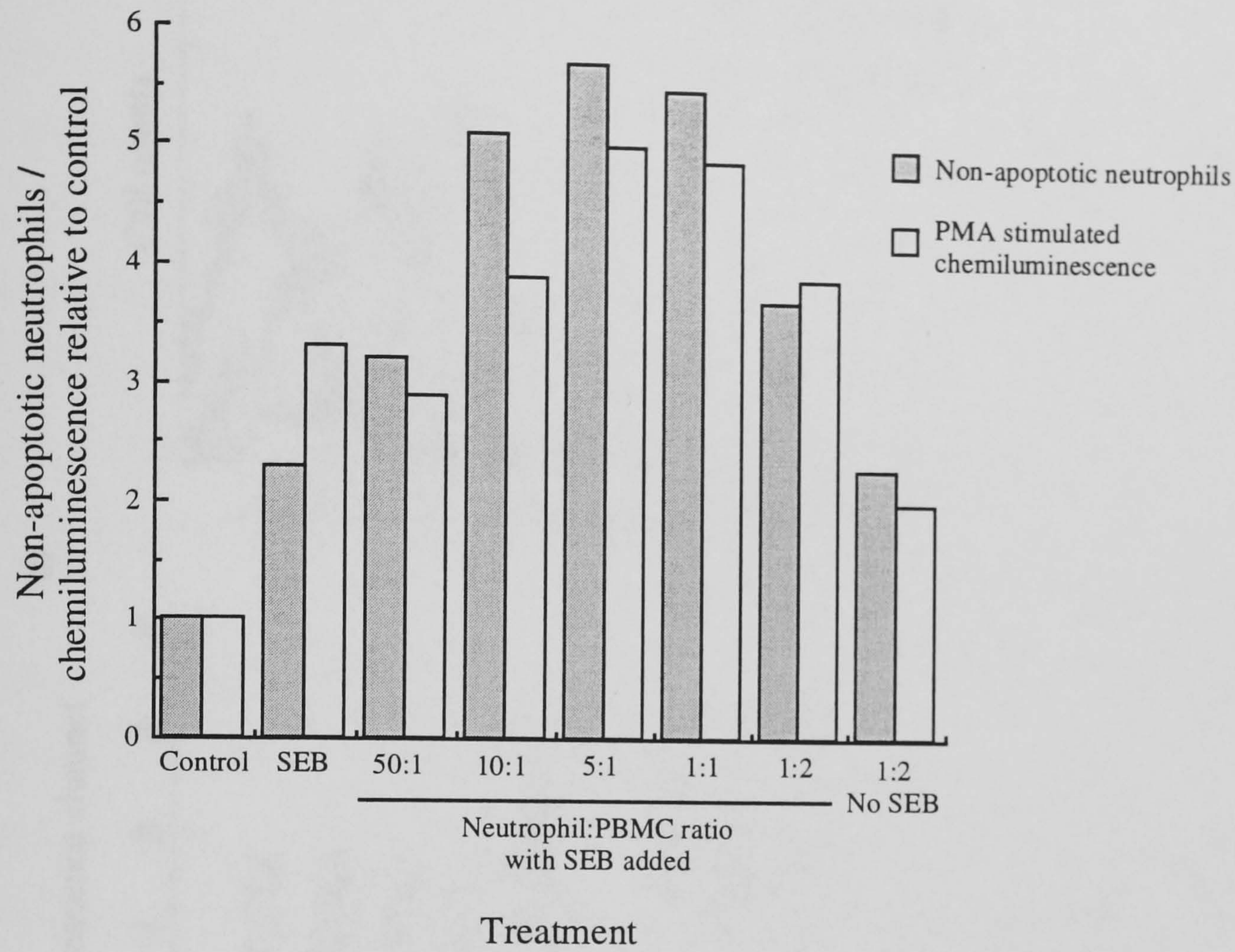
Following isolation, neutrophils were cultured in media alone (control) or supplemented with the agents shown, as described in Materials and Methods. After 20 h culture aliquots of neutrophils were processed for measurement of surface CD16 by flow cytometry. Representative traces are shown in A. B shows means (± SD) of three separate experiments. Significant difference from control incubations are indicated by † ($p \leq 0.05$) or * ($p \leq 0.01$).

4.3.2 Role of PBMC derived cytokines in superantigen mediated delayed apoptosis

The isolation of neutrophils as described in section 4.2.2 routinely gave > 97 % polymorphonuclear cells, or < 3 % contamination of PBMC. However, the activation of PBMC by superantigen yields vast quantities of cytokines, such that a contamination of less than 3 % PBMC in neutrophil preparations may produce sufficient cytokines to delay neutrophil apoptosis. Figure 4.3 shows the results of an experiment to determine if the proportion of PBMC in a neutrophil preparation correlated with the delayed apoptosis observed following superantigen treatment. SEB added to a normal neutrophil preparation delayed apoptosis, as assessed by both morphology and preservation of the ability to produce ROS in response to PMA. Addition of extra PBMC in the ratio 50:1 (neutrophils:PBMC) gave no further delay in apoptosis in SEB treated neutrophils, but as more PBMC were added to the culture, there was a clear enhancement in the ability of SEB to delay apoptosis of neutrophils. The effect was greatest at neutrophil to PBMC ratios of between 5:1 and 1:1, which is similar to the ratio of neutrophils to PBMC in the circulation. The addition of extra PBMC to neutrophil suspensions at the highest ratio tested (1 neutrophil:2 PBMC) showed a delay in neutrophil apoptosis without SEB treatment, but this effect was much less than that found when SEB was included. It therefore seemed likely that cytokine production from PBMC activated by superantigen in normal neutrophil preparations may be responsible for the delayed apoptosis observed.

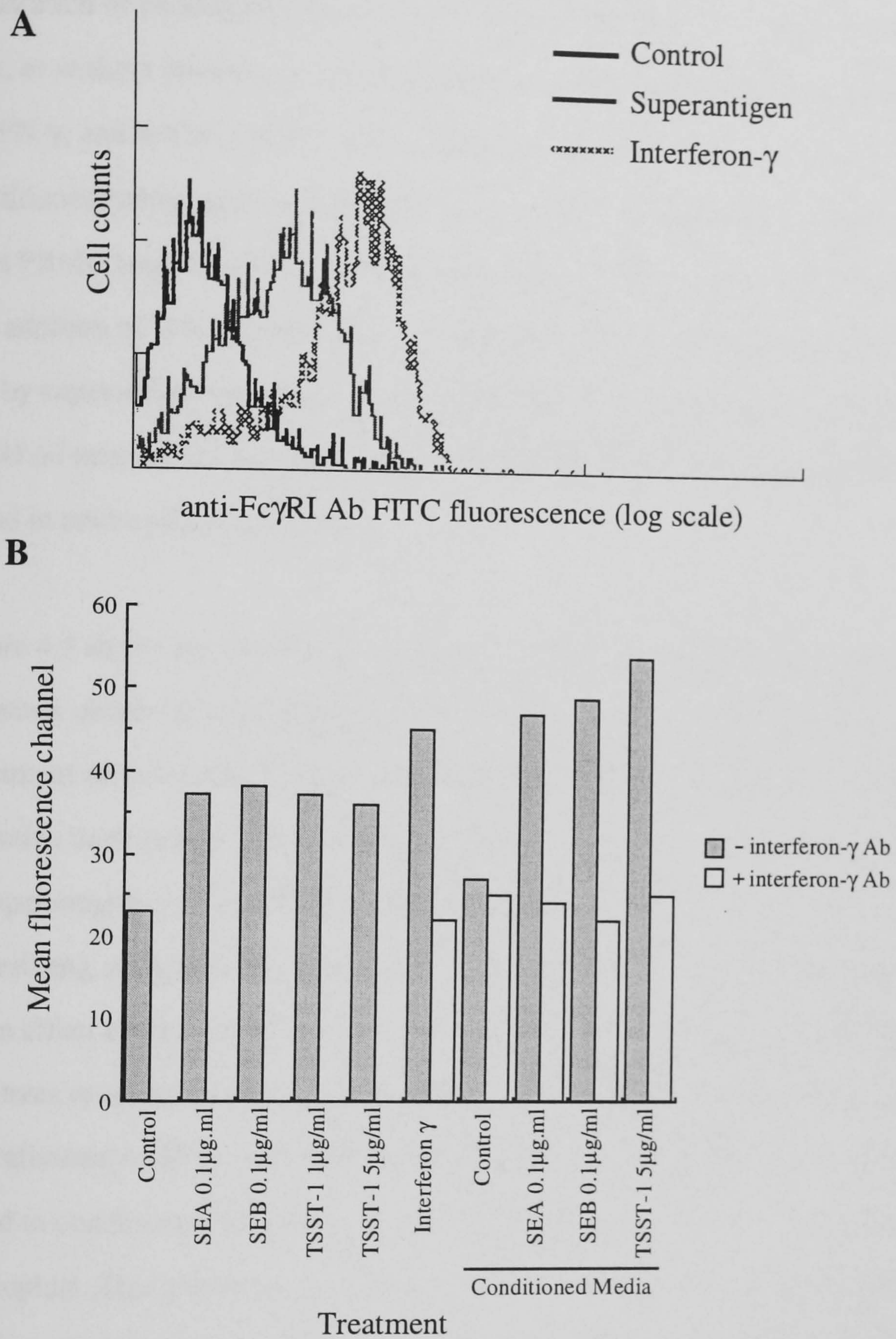
Of the cytokines produced by superantigen activated PBMC, IFN-γ is perhaps the simplest to assay for, as it known to induce surface expression of FcγRI on neutrophils (Petroni *et al*, 1988; Buckle & Hogg, 1989). Overnight culture with each of the superantigens (SEA, SEB 0.1 μg/mL, TSST-1 5 μg/mL) clearly induced FcγRI expression determined by flow cytometry, as shown in Figure 4.4. However, at these

Figure 4.3 Enhanced action of superantigen delayed neutrophil apoptosis by co-culture with PBMC



Isolated neutrophils were cultured for 20 h in media alone (control), or with SEB at 0.1 μg/ml (SEB). PBMC were added to isolated neutrophils at the ratios indicated without altering neutrophil concentration, before addition of SEB at 0.1 μg/ml, or with no SEB added. Apoptosis was assessed morphologically and is expressed relative to the control incubation where the number of non-apoptotic neutrophils is set to a value of 1. Oxidative burst stimulated by PMA is also shown relative to the control culture, as assessed by luminol dependant chemiluminescence.

Figure 4.4 Surface FcγRI expression after 20 h culture

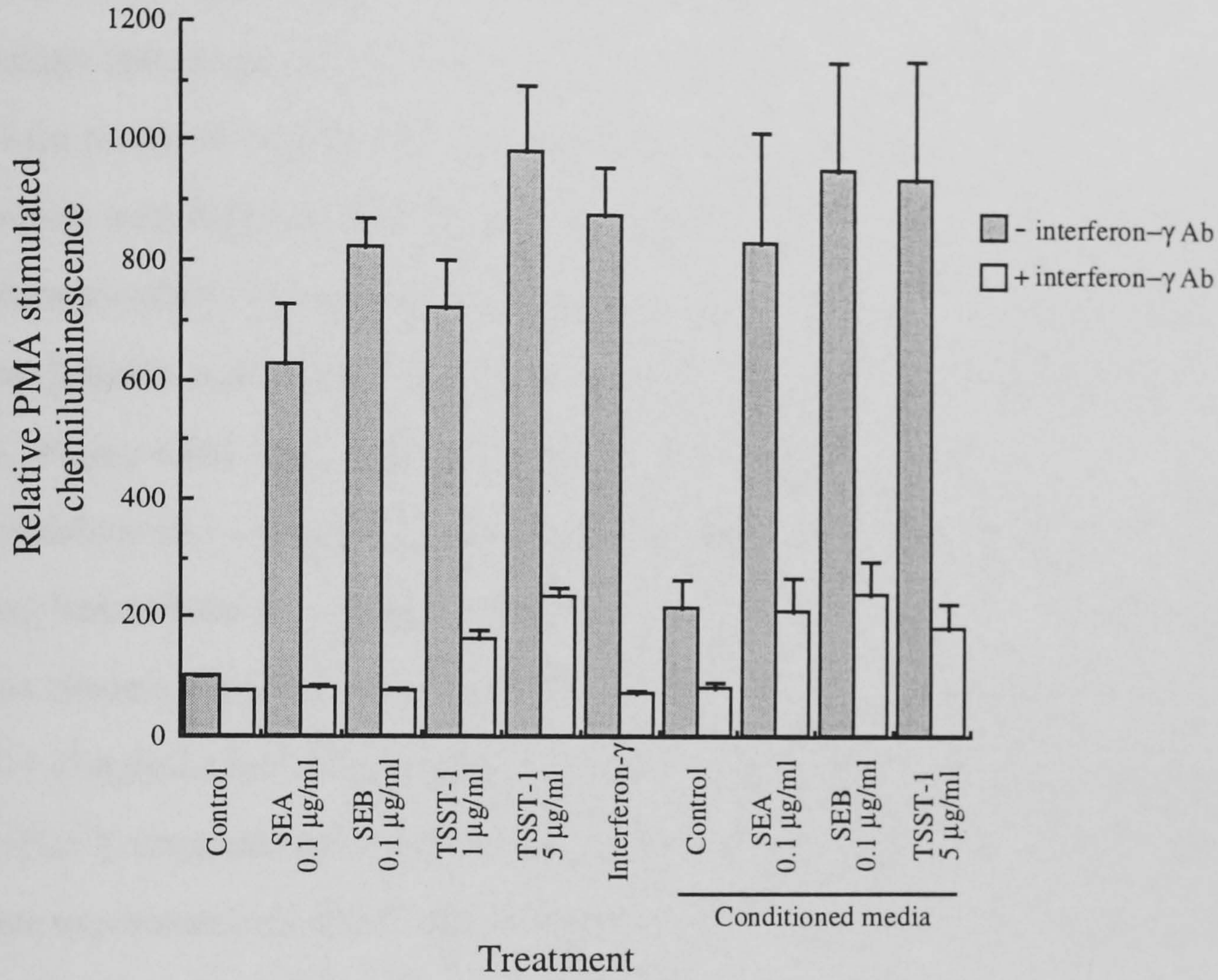


Neutrophils were cultured with media alone (control), or supplemented with the agents shown. After 20 h culture aliquots were processed for analysis of surface FcγRI expression. A, shows representative fluorescence distributions from control, superantigen (SEA,SEB and TSST-1 all gave very similar distributions), or 100 U/ml interferon-γ. B, mean fluorescence channels for 20 h cultured neutrophils are shown from 1 of 2 experiments. Antisera to interferon-γ was included in some incubations to neutralise its action.

concentrations the toxins were less effective than IFN-γ at 100 U/mL. The preparation of conditioned media from PBMC stimulated with each superantigen for 20 h, or without stimuli, provided confirmation that FcγRI expression was induced by IFN-γ, and not by a direct effect of superantigens on the neutrophils. Untreated conditioned media gave no induction of FcγRI on neutrophils, but conditioned media from PBMC treated with any of the superantigens gave a marked induction of FcγRI. The addition of IFN-γ neutralising antisera prevented induction of FcγRI by IFN-γ, and by superantigen activated PBMC conditioned media, indicating the induction of FcγRI on neutrophils was due to IFN-γ secreted from the contaminating PBMC found in neutrophil preparations.

Figure 4.5 shows the results of experiments to determine if the delayed neutrophil apoptosis observed with superantigen treatment is mediated solely by IFN-γ. Treatment of neutrophil cultures with 100 U/mL IFN-γ gave similar preservation of oxidative burst (and protection from apoptosis), as treatment with all superantigens, or superantigen-activated PBMC conditioned media. The inclusion of IFN-γ neutralising antisera in superantigen-treated neutrophil suspensions prevented most of the effect of these agents on neutrophil apoptosis, but some protection from apoptosis remained at higher toxin concentrations. This may be due to inefficient neutralisation of IFN-γ after time in culture. Therefore, the neutralising Ab was added to conditioned media for 20 min before addition of conditioned media to neutrophils. This pre-treatment completely abolished the effects of 100 U/mL IFN-γ. The superantigen treated conditioned media was, however, still able to give some protection from apoptosis after neutralisation of IFN-γ. The delayed apoptosis observed following superantigen treatment was therefore mediated in a large part by IFN-γ, but there was still another mechanism(s) involved. The remaining effect may be mediated either by other cytokines produced from the PBMC, or may be due to a direct effect of the toxins on neutrophils themselves.

Figure 4.5 Effect of interferon-γ neutralising antisera and superantigen-activated PBMC conditioned media on neutrophil function after 20 h culture



Neutrophils were cultured in media alone (control) or supplemented as indicated. After 20 h culture oxidative burst activity in response to PMA was assessed relative to control suspensions. Conditioned media was prepared by incubating PBMC with each of the superantigens for 20 h, as described in Materials and Methods. Interferon-γ neutralising antisera was included in parallel incubations. Results are means (±SD) of 2 separate experiments.

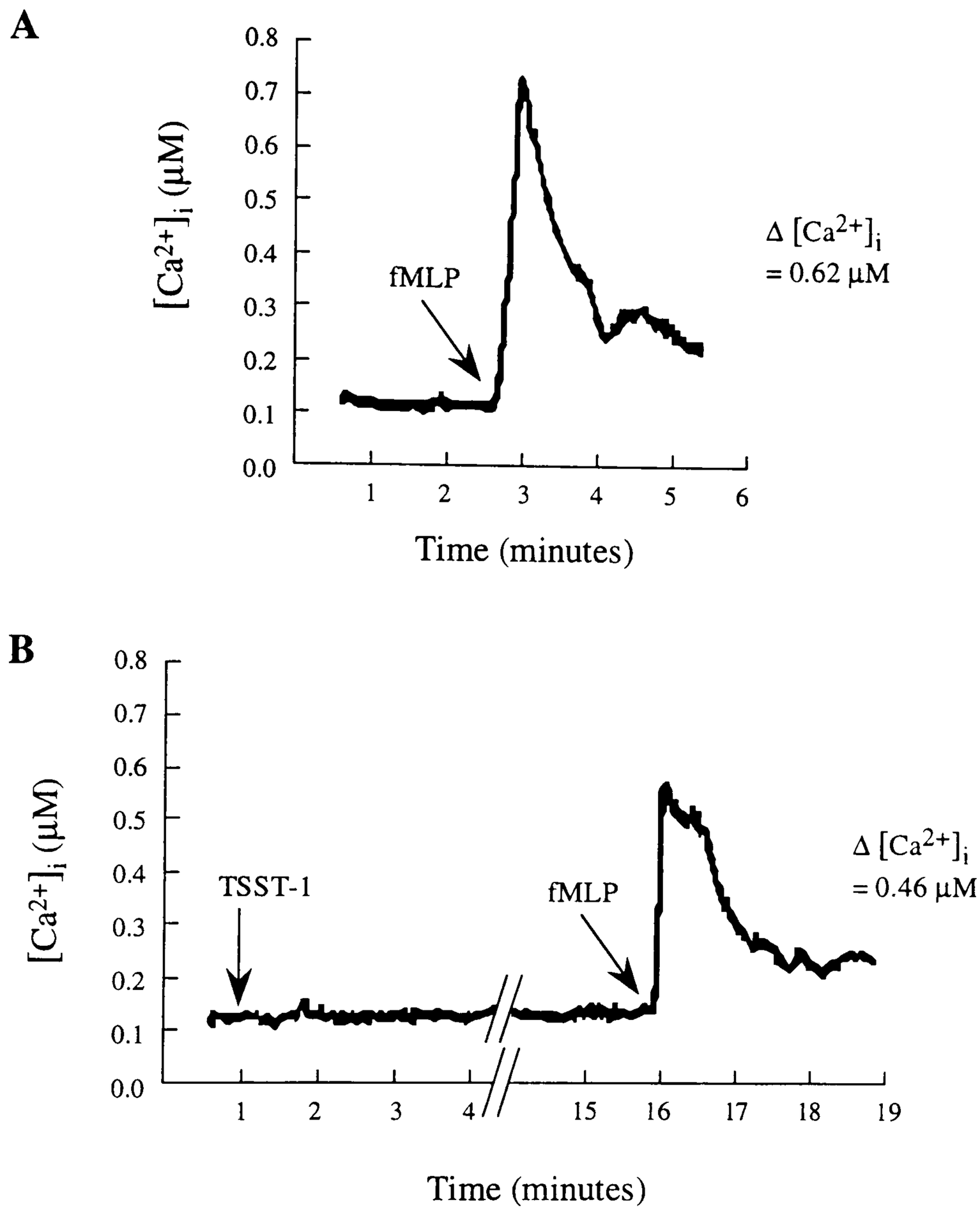
4.3.3 Short term effects of superantigens on neutrophils

A direct effect of superantigens on neutrophils would be expected to cause changes in the signal transduction pathways of neutrophils, leading to changes in neutrophil physiology that can be assessed after much shorter times than apoptosis. Figure 4.6 shows the results of experiments examining $[Ca^{2+}]_i$ following treatment of neutrophils with fMLP or TSST-1. The resting $[Ca^{2+}]_i$ was typically 115 nmol/L, and rose dramatically to 734 nmol/L (± 101) following fMLP treatment, similar to other reports (Scanlon *et al*, 1987). Addition of TSST-1 at 5 μg/mL gave no changes in $[Ca^{2+}]_i$ (Figure 4.6b). SEA, SEB and TSST-1 were all tested at a range of concentrations (0.1 - 5.0 μg/mL) with no changes in $[Ca^{2+}]_i$ observed (data not shown). Intracellular IP_3 measurements were also made following TSST-1 treatment, with no changes observed (data not shown). However, in neutrophils treated with TSST-1 (5 μg/mL) for 15 min before stimulation with fMLP there was a diminished rise in $[Ca^{2+}]_i$ compared to neutrophils not treated with TSST-1 (Figure 4.6b). In two separate experiments the fMLP induced rise in $[Ca^{2+}]_i$ following TSST-1 treatment was only ~75% of that observed in non-TSST-1 treated neutrophils.

Changes in protein tyrosine phosphorylation were investigated following superantigen treatment of neutrophil preparations, as shown in Figure 4.7. GM-CSF treatment induced many changes in protein tyrosine phosphorylation, confirming the sensitivity of this assay. Superantigen treatment induced tyrosine phosphorylation of a number of proteins, notably those with molecular masses of ~40 kDa, ~55 kDa, ~60 kDa (arrowed), and ~70 kDa (arrowed). These rapid changes in protein tyrosine phosphorylation following superantigen treatment suggest a direct action of superantigens on neutrophil suspensions.

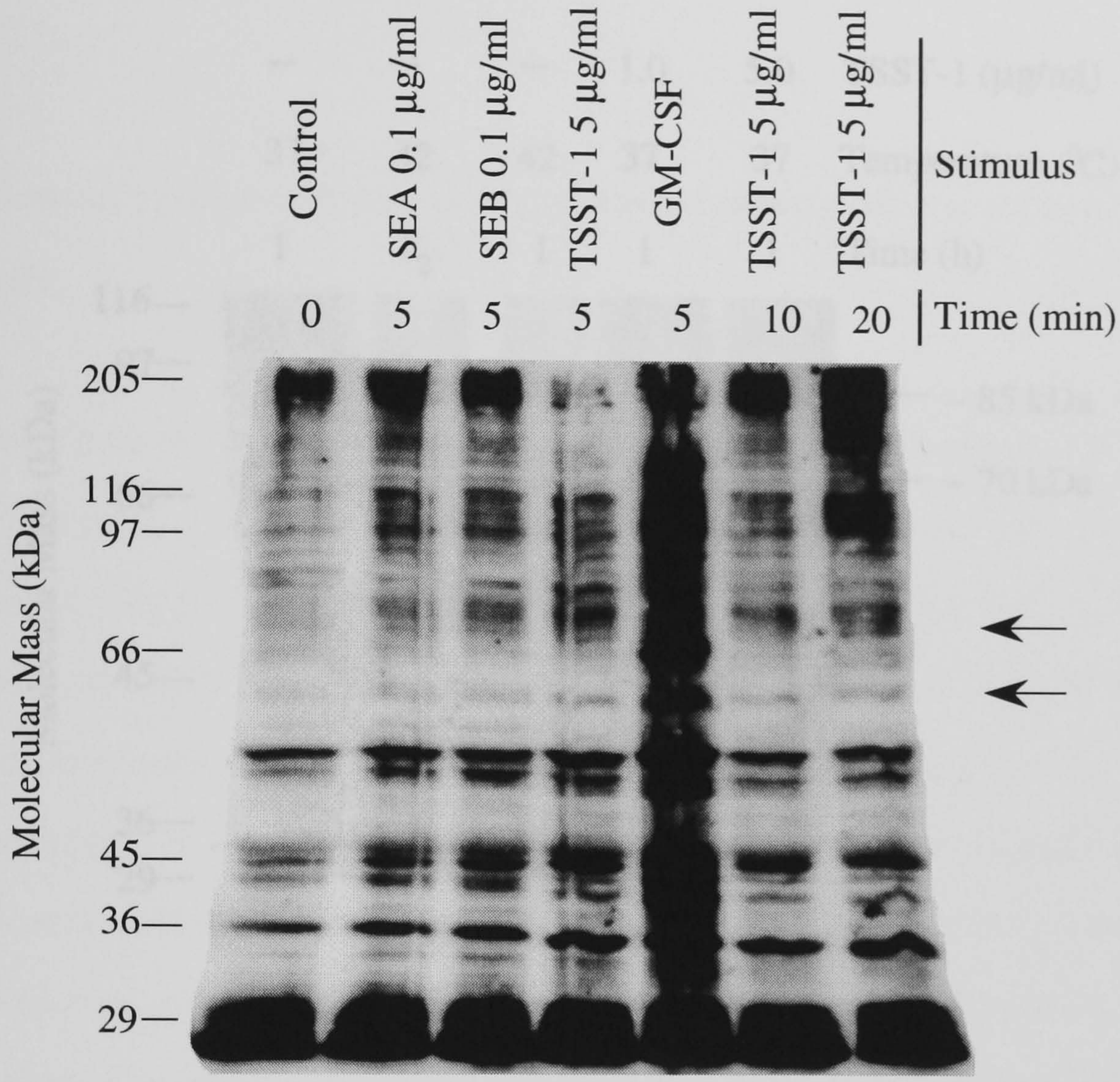
TSST-1 is reported to induce heat shock protein (HSP) expression in neutrophils (Hensler *et al*, 1991). Figure 4.8 shows the results of an experiment investigating

Figure 4.6 Intracellular Ca^{2+} measurements in fMLP and TSST-1 stimulated neutrophils



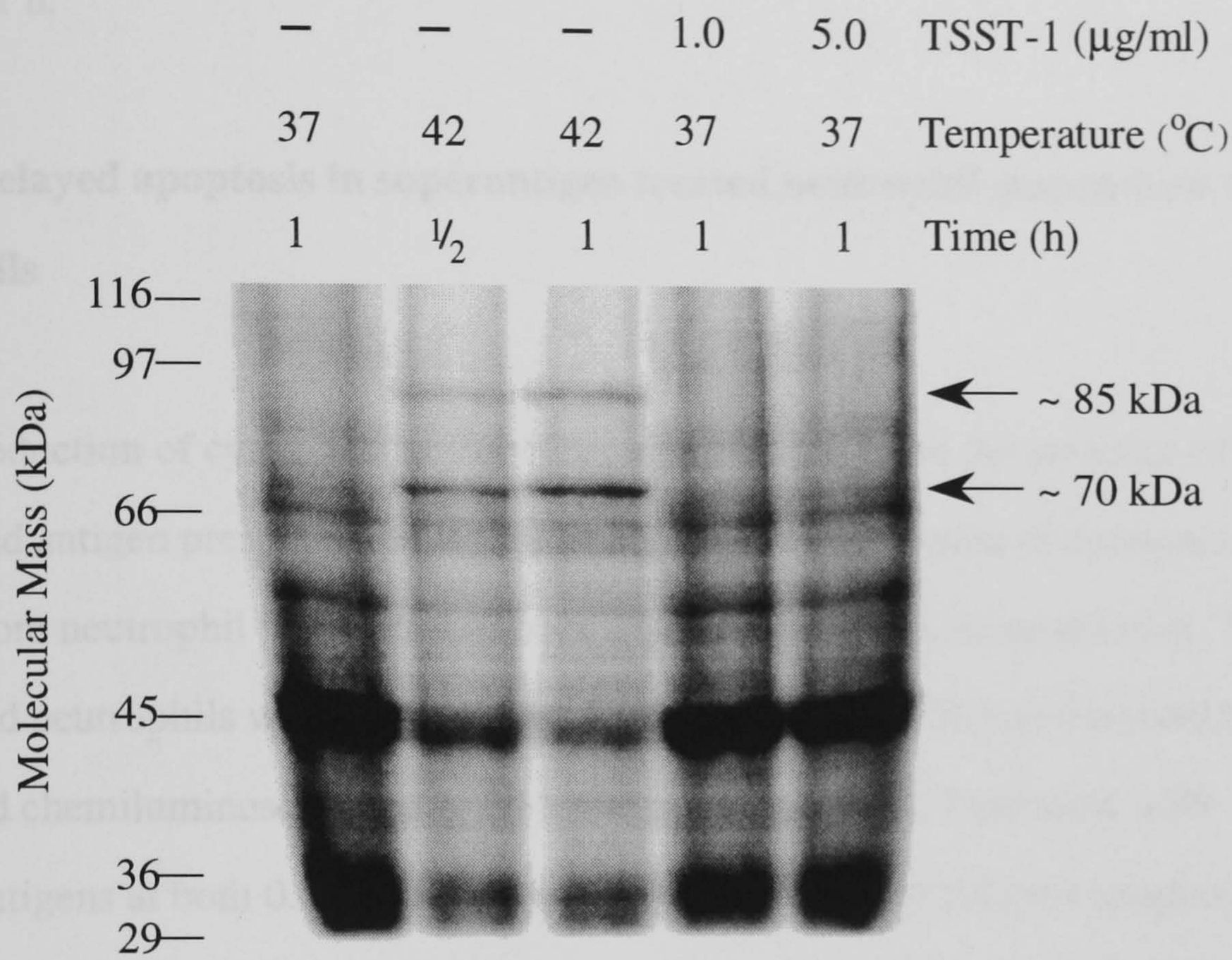
Isolated neutrophils were prepared for measurement of intracellular free Ca^{2+} by measuring fluorescence of Fluo-3, as described in Materials and Methods. Measurements were made following stimulation of neutrophils with TSST-1 at 5 µg/ml, and fMLP at 100 nmol/L. Traces shown are from one of two experiments, which gave very similar results.

Figure 4.7 Phosphotyrosine blot from superantigen stimulated neutrophil suspensions



Neutrophil suspensions were treated with the agents indicated for 0, 5, 10 or 20 min before solubilisation in boiling sample buffer as described in Materials and Methods. Total proteins from equal numbers of cells were separated on 5-15% SDS-PAGE, transferred to PVDF membrane, and probed with anti-phosphotyrosine Ab. The blot shown is representative of two separate experiments, both of which gave similar results. Arrows indicate proteins which have increased tyrosine phosphorylation following superantigen treatment.

Figure 4.8 Induction of heat shock protein synthesis



Neutrophils were incubated in media containing ^{35}S-methionine at the temperatures and times indicated. Neutrophils in identical media were also treated with TSST-1 at 1.0 or 5.0 µg/mL for 1 h. Total protein was then separated by SDS-PAGE and radioactivity detected as described in Materials and Methods.

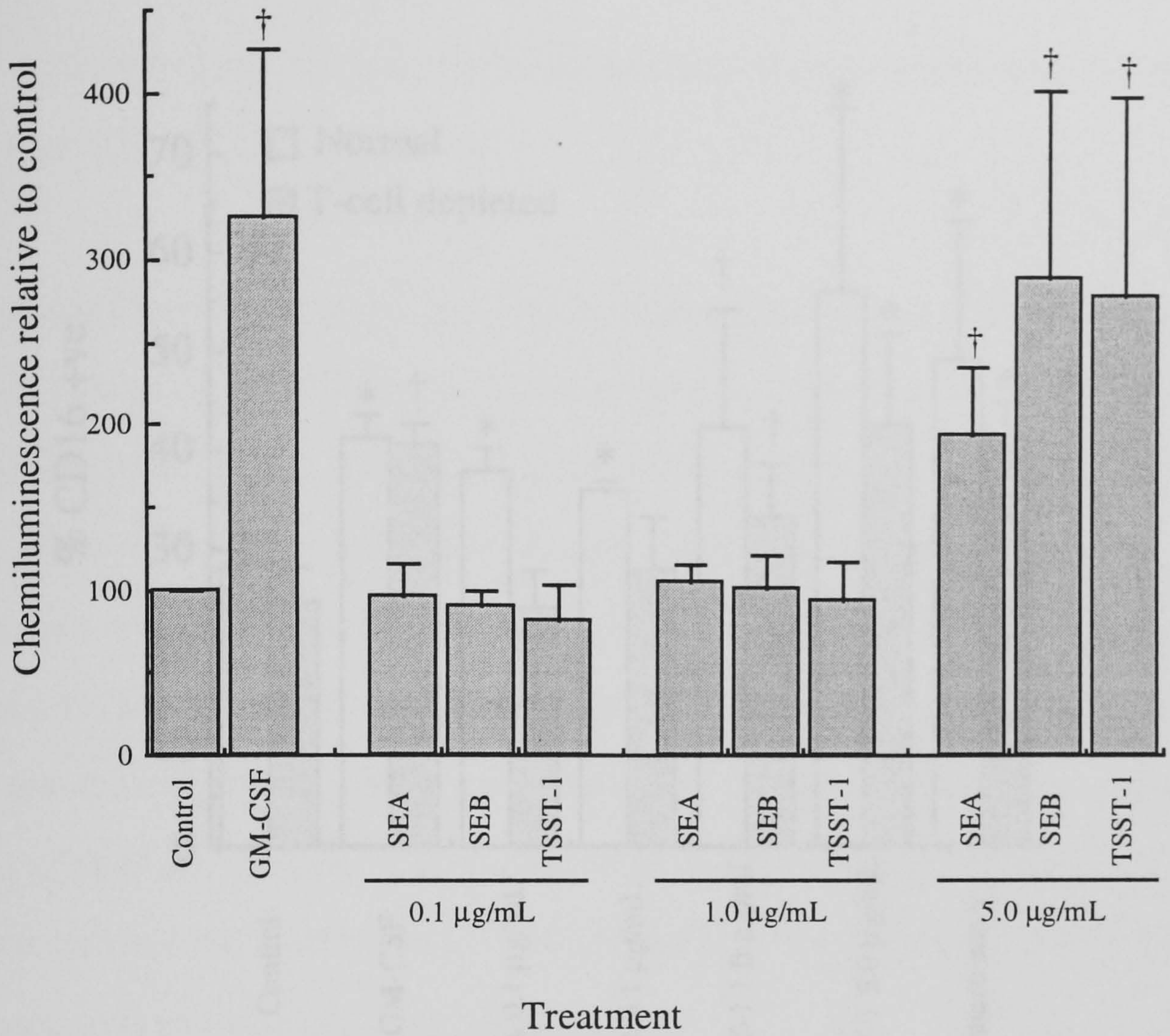
induction of HSPs following treatment of neutrophils for 1 h with TSST-1. No induction of HSPs were found following TSST-1 treatment, with parallel suspensions clearly showing induction of HSPs following heat shock at 42^{0}C for 30 min or 1 h.

4.3.4 Delayed apoptosis in superantigen treated neutrophil suspensions depleted of T-cells

The production of cytokines by PBMC is reported to require the presence of both T-cells and antigen presenting cells (See *et al*, 1992), so depletion of contaminating T-cells from neutrophil preparations should prevent this cytokine production. T-cell depleted neutrophils were rescued from apoptosis by GM-CSF, as assessed by PMA induced chemiluminescence after 20 h culture (Figure 4.9). Treatment with superantigens at both 0.1 and 1.0 μg/mL (which effectively delayed apoptosis in normal neutrophil suspensions) showed no effect on neutrophil apoptosis assessed by preservation of oxidative burst activity (Figure 4.9) in these T-cell depleted suspensions. However, at the highest concentration of superantigen used (5.0 μg/mL) SEA, SEB and TSST-1 were still effective in preserving neutrophil function after 20 h culture.

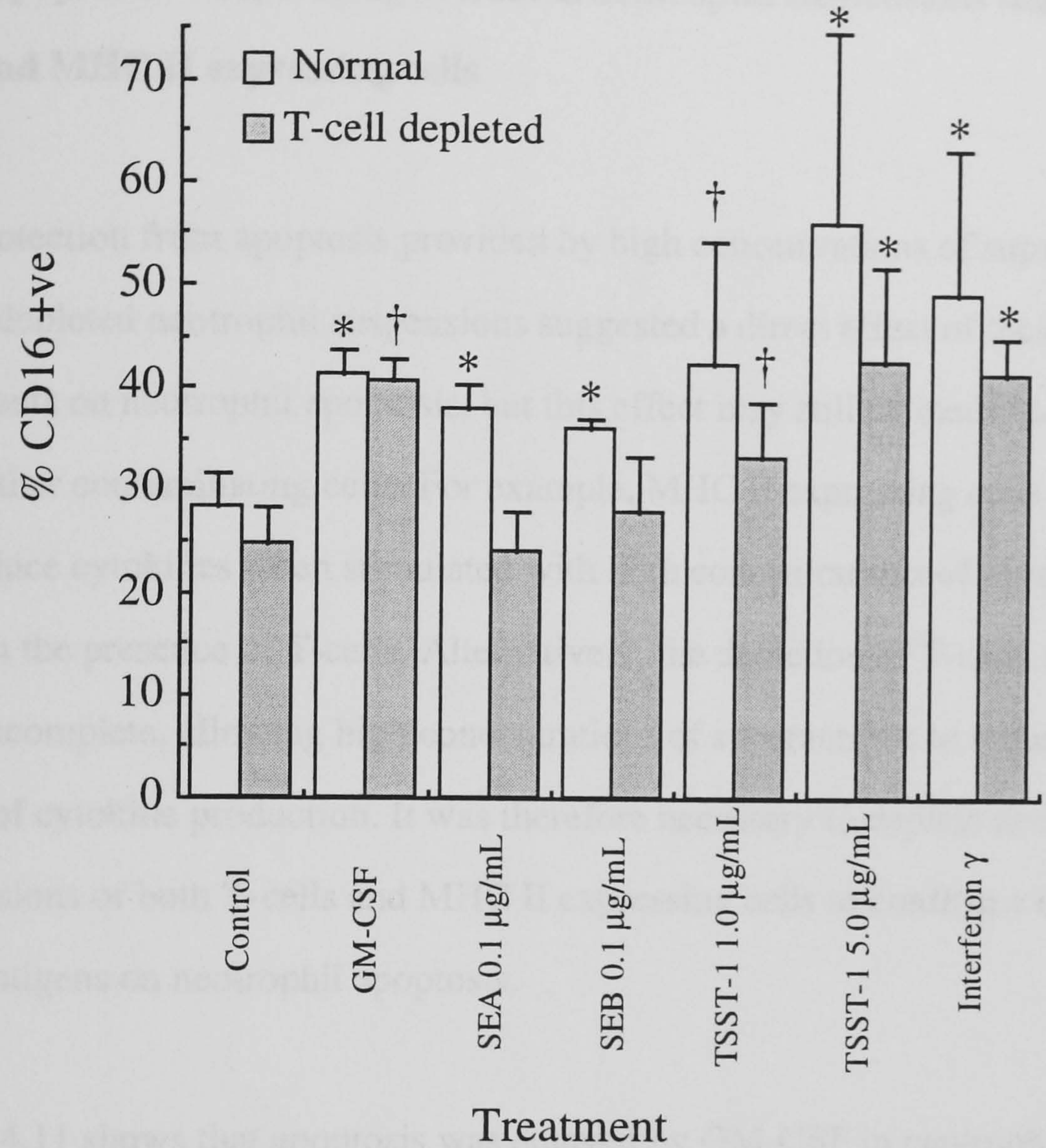
Figure 4.10 shows apoptosis measurements following 20 h culture of neutrophils assessed by the percentage of cells retaining high levels of surface CD16. It is clear that the depletion of T-cells has no adverse effect on the ability of GM-CSF or IFN-γ to delay apoptosis. Superantigen treatment of T-cell depleted neutrophils at 0.1 μg/mL showed a normal rate of apoptosis. However, at higher concentrations of superantigen, the entry into apoptosis was still delayed by a slight but significant extent at 1.0 μg/mL, and more markedly at 5.0 μg/mL. The protection from apoptosis afforded by the higher concentrations of superantigen was not as great in T-cell depleted neutrophils as it was in normal neutrophil preparations, suggesting

Figure 4.9 PMA stimulated chemiluminescence of 20 h cultured T-cell depleted neutrophil suspensions



Neutrophils were isolated and depleted of T-cells as described in Materials and Methods. Following culture for 20 h in the conditions indicated, oxidative burst in response to PMA was measured by luminol dependant chemiluminescence. Values shown are means (± SD) of 4 separate experiments. Significant difference from control is indicated by † ($p \leq 0.05$).

Figure 4.10 Effect of superantigens on CD16 surface expression of 20 h cultured neutrophils, depleted of T-cells



Neutrophils were isolated and cultured with the agents indicated (normal, open bars), or following depletion of T-cells (shaded bars). Cells were then processed for analysis of surface CD16 expression by flow cytometry. Results shown are means (± SD) of three separate experiments. Significant difference from control suspensions are indicated by † ($p \leq 0.05$), or * ($p \leq 0.01$).

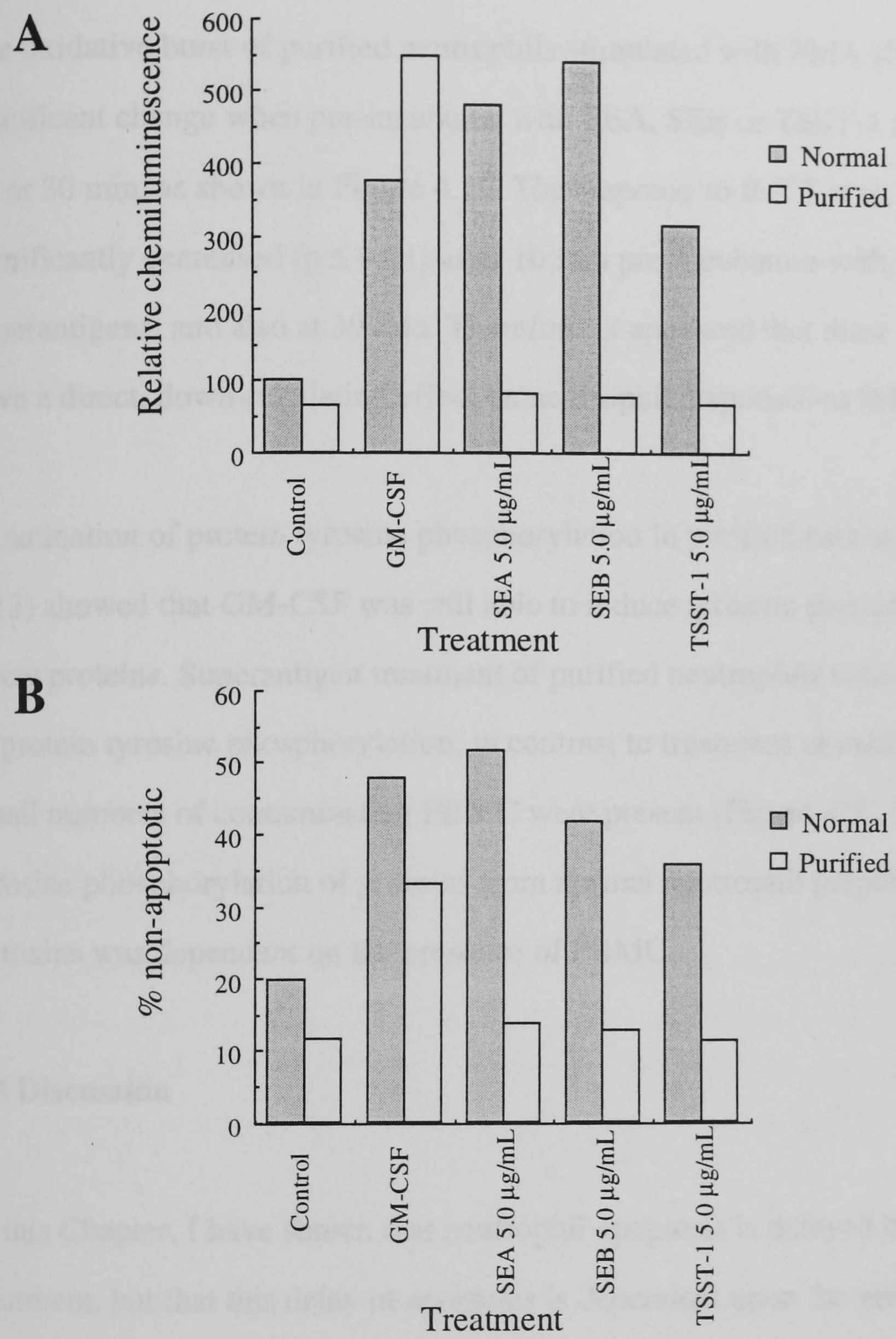
that part of the protection from apoptosis is lost due to a lack of cytokine production in T-cell depleted suspensions.

4.3.5 Apoptosis in superantigen treated neutrophil suspensions depleted of T-cells and MHC II expressing cells

The protection from apoptosis provided by high concentrations of superantigens in T-cell depleted neutrophil suspensions suggested a direct effect of these superantigen treatments on neutrophil apoptosis, but this effect may still be mediated by cytokines from other contaminating cells. For example, MHC II expressing cells may be able to produce cytokines when stimulated with high concentrations of superantigen, without the presence of T-cells. Alternatively, the depletion of T-cells may have been incomplete, allowing high concentrations of superantigen to stimulate high levels of cytokine production. It was therefore necessary to deplete neutrophil suspensions of both T-cells and MHC II expressing cells to confirm a direct effect of superantigens on neutrophil apoptosis.

Figure 4.11 shows that apoptosis was delayed by GM-CSF in neutrophils depleted of both T-cells and MHC II expressing cells, indicating this depletion had not prevented neutrophils from being rescued from apoptosis. Treatment with superantigen at 5.0 μg/mL had no effect on the rate of neutrophil apoptosis in these purified neutrophil suspensions. These results suggest that the depletion of T-cells alone was not sufficient to prevent cytokine production from other contaminating cells at levels able to delay neutrophil apoptosis.

Figure 4.11 Effect of superantigens on apoptosis of neutrophils depleted of both T-cells and MHC II expressing cells



Nuetrophils were isolated and treated with the agents indicated, or were first depleted of both T-cells and MHC II expressing cells, before addition of agents in parallel cultures. Oxidative burst in response to PMA (A) and percentage non-apoptotic neutrophils (B) were assessed after 20 h culture. Results are shown from one of three experiments, which all showed the same pattern of results.

4.3.6 Short term effects of superantigens on neutrophils depleted of T-cells and MHC II expressing cells

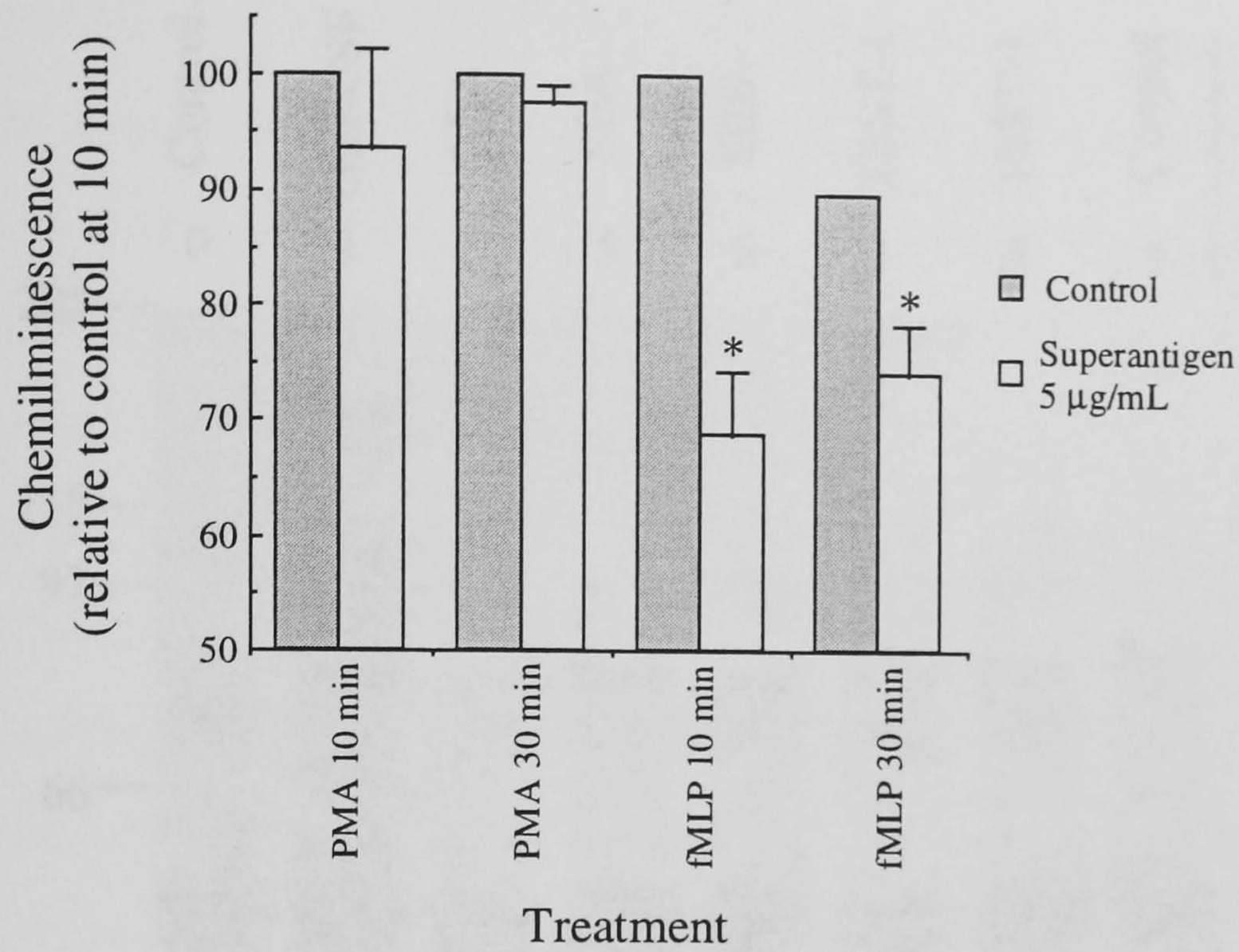
The oxidative burst of purified neutrophils stimulated with PMA showed no significant change when pre-incubated with SEA, SEB or TSST-1 at 5.0 μg/mL for 10 or 30 min, as shown in Figure 4.12. The response to fMLP was, however, significantly decreased ($p \leq 0.01$) after 10 min pre-incubation with any of the superantigens, and also at 30 min. Therefore, it appeared that these superantigens did have a direct, down-regulating effect on neutrophil responses to fMLP.

Examination of protein tyrosine phosphorylation in purified neutrophils (Figure 4.13) showed that GM-CSF was still able to induce tyrosine phosphorylation of many proteins. Superantigen treatment of purified neutrophils resulted in no changes in protein tyrosine phosphorylation, in contrast to treatment of neutrophils when small numbers of contaminating PBMC were present (Figure 4.7). Therefore the tyrosine phosphorylation of proteins from normal neutrophil preparations in response to toxins was dependent on the presence of PBMC.

4.4 Discussion

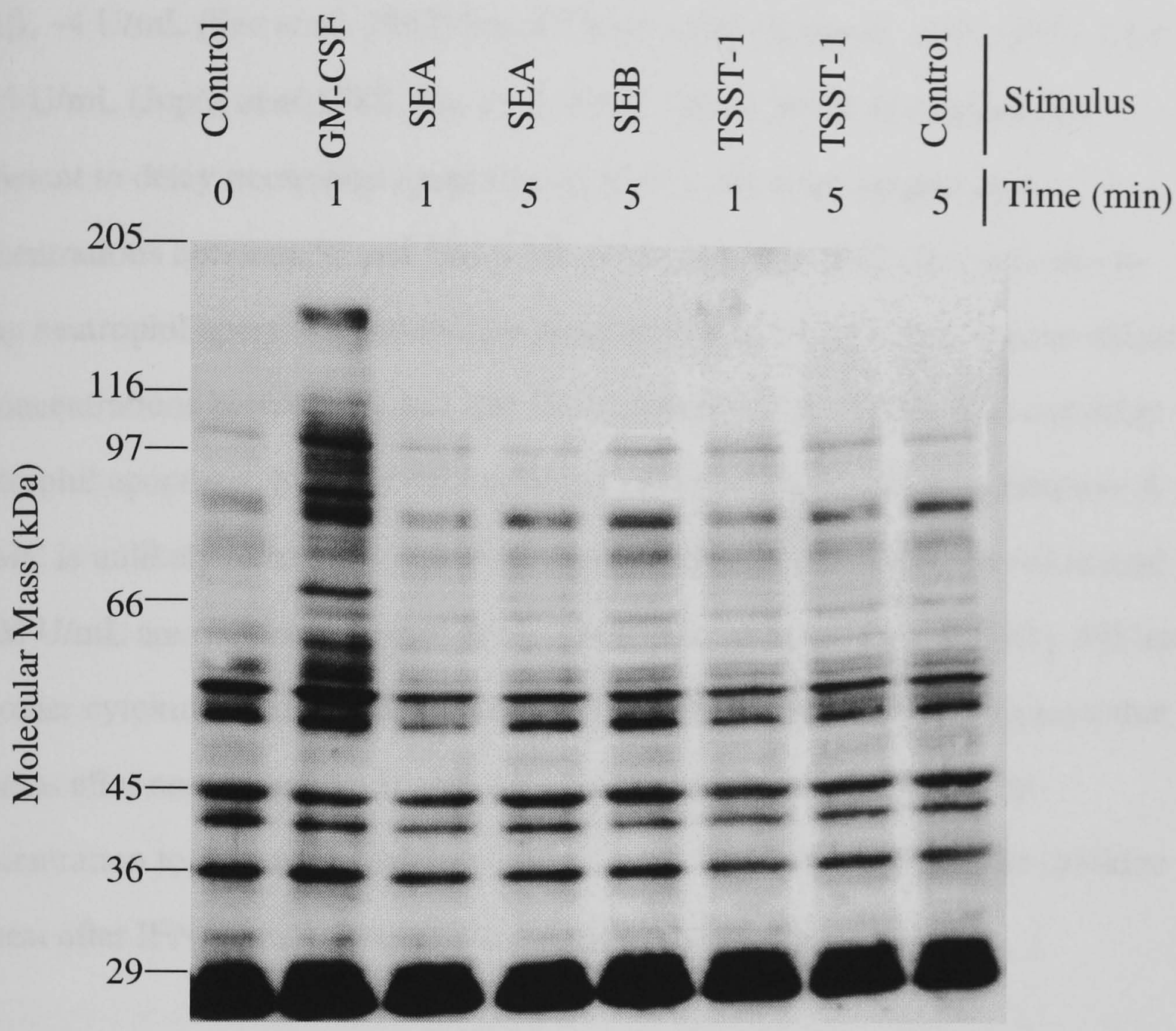
In this Chapter, I have shown that neutrophil apoptosis is delayed by superantigen treatment, but that this delay in apoptosis is dependent upon the presence of a small number of contaminating PBMC in the suspensions. The levels of cytokines produced by contaminating PBMC in normal neutrophil preparations are sufficiently high to delay neutrophil apoptosis. IFN-γ accounts for most of the delayed apoptosis observed, as neutralisation of IFN-γ in superantigen-treated neutrophil suspensions abolished around 80 % of the action of superantigens on neutrophil apoptosis (Figure 4.5).

Figure 4.12 Oxidative burst stimulated by either PMA or fMLP after short incubation with superantigen



Isolated neutrophils were depleted of T-cells and MHC II expressing cells as described in Materials and Methods. Parallel incubations for 10 and 30 min with and without superantigens (SEA, SEB or TSST-1) were assessed for luminol dependent chemiluminescence in response to both PMA and fMLP. Results shown are means (± SD) of measurements following treatment with each of SEA, SEB and TSST-1. fMLP responses following superantigen treatment were significantly lower than untreated neutrophils (* $p \leq 0.01$).

Figure 4.13 Phosphotyrosine blot from superantigen stimulated neutrophil suspensions depleted of T-cells and MHC II expressing cells



Isolated neutrophils were depleted of T-cells and MHC II expressing cells as described in Materials and Methods. Neutrophil suspensions were then treated with the agents indicated (SEA, SEB and TSST-1 all at 5 μg/mL, GM-CSF 50 U/mL, or no stimuli as controls), before solubilisation in boiling sample buffer. Total proteins from equal numbers of cells were separated on 5-15% SDS-PAGE, transferred to PVDF membrane, and probed with anti-phosphotyrosine Ab. The blot shown is representative of two separate experiments. Treatments with superantigens for 10 min were also tested, and gave near identical results to those shown for 5 min.

From published reports, a 3 % contamination of PBMC is likely to result in the production of cytokines to the following levels: IFN-γ, 65-80 U/mL (Jupin *et al*,1988; Lagoo *et al*, 1994); IL-2, 3-250 U/mL (Jupin *et al*,1988; Lagoo *et al*, 1994); IL-1β, ~4 U/mL (See *et al*, 1992; See & Chow, 1992; Driessen *et al*, 1995); TNF-α, 1-2.5 U/mL (Jupin *et al*,1988; See *et al*, 1992). These levels of cytokine are sufficient to delay neutrophil apoptosis, as IFN-γ will delay apoptosis at concentrations between 50 and 500 U/mL (Colotta *et al*, 1992). IL-1β is able to delay neutrophil apoptosis at concentrations as low as 2 U/mL, but is more effective at concentrations between 20 and 200 U/mL (Colotta *et al*, 1992). IL-2 can delay neutrophil apoptosis, but the concentrations produced by a 3 % contamination of PBMC is unlikely to be sufficient to delay apoptosis, as concentrations of around 1,000 U/mL are required (Pericle *et al*, 1992). The neutralisation of IFN-γ still leaves the other cytokines present, which could explain the slight delay in apoptosis that remains after neutralisation (Figure 4.5) IL-1β, which was at a sufficient concentration to give a slight delay of neutrophil apoptosis, could be the cytokine present after IFN-γ neutralisation that resulted in delayed apoptosis.

The depletion of T-cells from the neutrophil suspensions did abolish the delayed apoptosis observed with superantigen concentrations below 5.0 μg/mL, but there was still a delay in apoptosis when this superantigen was used at this higher concentration. This may be explained by the observation of Jupin *et al* (1988), where increasing concentrations of superantigen gave a corresponding increase in the levels of IFN-γ production. Therefore, the depletion of T-cells, if not complete, would still allow the production of IFN-γ at sufficient levels to delay neutrophil apoptosis. Alternatively, the work of Jupin *et al* (1988) suggests that monocytes may be able to produce cytokines (TNF-α and IL-1β) without the presence of T-cells. Therefore, the removal of T-cells alone may not prevent all cytokine production, with cytokines derived from the remaining PBMC being responsible for the delayed neutrophil apoptosis.

The depletion of both T-cells and MHC II expressing cells from neutrophil preparations allowed the action of superantigens on neutrophil apoptosis to be assessed without any contribution from other superantigen-stimulated cells. This clearly demonstrated that superantigens had no direct action on neutrophils that resulted in delayed entry into apoptosis (Figure 4.11). This observation was further strengthened by the measurements of protein tyrosine phosphorylation in response to superantigens. In normal neutrophil preparations there were a small number of proteins which showed enhanced tyrosine phosphorylation, but neutrophils depleted of contaminating T-cells and MHC II expressing cells showed no changes in protein tyrosine phosphorylation (Figures 4.7 and 4.13). The tyrosine phosphorylation seen in normal neutrophil preparations may be proteins derived from the contaminating PBMC, as this technique is very sensitive, and the proteins arrowed in Figure 4.7 are of similar molecular masses as proteins phosphorylated in monocytes treated with superantigen (Trede *et al*, 1994).

The possibility remains that superantigens do have other direct actions on neutrophils, such as the ability of TSST-1 to modulate fMLP receptor expression (Hensler *et al*, 1993), and induce HSP expression (Hensler *et al*, 1991). I have examined the induction of HSP expression by TSST-1 and found no induction under similar conditions to those used by Hensler *et al* (1991). This difference in results may be explained by the purity of neutrophils used. Hensler *et al* (1991) report neutrophil purity of 95 %, which would indicate an approximately two-fold higher contamination of PBMC than in my preparations. Therefore, cytokine production would be considerably higher in Hensler's cultures, and cytokines such as IL-1 and TNF-α are able to induce HSP expression rapidly in neutrophils (König *et al*, 1992).

The reported ability of TSST-1 to modulate fMLP receptor expression (Hensler *et al*, 1993), has no known mode of action. I have shown that the increase in $[Ca^{2+}]_i$ that follows fMLP stimulation of neutrophils is diminished following 15 min pre-

treatment by TSST-1 (Figure 4.6) in normal neutrophil cultures. The purified neutrophils (depleted of T-cells and MHC II expressing cells) also showed diminished oxidative burst in response to fMLP following 10 or 30 min superantigen pre-treatment (Figure 4.12). These results are similar to those of Hensler *et al* (1993), though I have measured responses to fMLP treatment , whereas Hensler *et al* (1993) measured fMLP receptor expression. Therefore, superantigens have no direct effect on neutrophil apoptosis, but may directly effect other neutrophil functions.

CHAPTER 5 BUTYRATE DELAYED APOPTOSIS: INVOLVEMENT OF HISTONE ACETYLATION

5.1 Introduction

Butyrate is able to increase gene expression in a variety of cell types (section 2.1), with the inhibition of histone deacetylase by butyrate being the likely cause of this increased gene expression (Kruh, 1982). Recent research into histone deacetylases has shown that transcriptional repressors can act by tethering a histone deacetylase in close proximity to the repressed gene, with the resulting deacetylated histones displacing transcription factors. This type of transcriptional repression is prevented by specifically inhibiting histone deacetylases (reviewed in Grunstein, 1997). However, butyrate treatment also causes other changes within the cell, such as increased DNA excision repair (Smerdon *et al*, 1982; Dresler *et al*, 1985), and changes in the phosphorylation states of many nuclear proteins (Boffa *et al*, 1981). It is therefore not certain if the delayed neutrophil apoptosis observed in butyrate treated neutrophils, reported previously (Chapter 2), is due to increased histone acetylation, enhanced DNA excision repair or alteration of the phosphorylation of nuclear proteins.

The reported hyperacetylation of histones seen in other cell types requires butyrate concentrations of at least 1 mmol/L and is maximal around 5 mmol/L (Yoshida *et al*, 1990; Schlake *et al*, 1994; Arts *et al*, 1995). The lowest concentrations of butyrate shown to have any effect on other cell types is 0.5 mmol/L which causes a slight increase in histone acetylation in HL-60 cells (Aviram *et al*, 1994), and induces heat shock protein expression in U937 cells (Garcia-Bermejo *et al*, 1995). DNA excision repair is enhanced by butyrate concentrations at around 20 mmol/L (Smerdon *et al*, 1982; Dresler, 1985). Some small changes in nuclear protein phosphorylation are

seen at butyrate concentrations as low as 0.5 mmol/L, but these are maximal at 15 mmol/L (Boffa *et al*, 1981).

Butyrate delays neutrophil apoptosis maximally at a concentration of 0.4 mmol/L (Stringer, 1994), which is below the minimum concentration shown to exert any effect on other cell types. In this chapter, I have therefore investigated the ability of 0.4 mmol/L butyrate to inhibit histone deacetylase activity and cause histone hyperacetylation. The effect of trichostatin A (TSA), a specific inhibitor of histone deacetylase (Yoshida *et al*, 1990) on neutrophil apoptosis, gene expression and histone acetylation is also examined. I have also examined the effect of GM-CSF on histone acetylation.

5.2 Materials and Methods

5.2.1 Materials

Trichostatin A (TSA) was a kind gift from Dr Minoru Yoshida, University of Tokyo. [^{35}S]-Methionine and [^{3}H]-Acetate were from ICN, (UK). Cocktail T (scintillation fluid) was from BDH (UK).

5.2.2 Neutrophil isolation

Neutrophils were isolated as described in section 2.2.2, except that FCS was added to 5 % (v/v) in the final media.

5.2.3 Neutrophil Culture

Neutrophils were cultured as in section 2.2.3, but with FCS at 5 % (v/v), with no further additions to the media as controls. GM-CSF was added to 50 U/mL, butyrate to 0.4 mmol/L. TSA was dissolved in DMSO as a stock of 10 mmol/L. This was

diluted with RPMI and added to neutrophil suspensions to final concentrations of 2, 5, 10, 20, 50, 100, 200, 400, and 500 nmol/L. DMSO was added to a final concentration not in excess of 0.05% (v/v), which had no measurable effects on the parameters assayed in this Chapter.

5.2.4 Morphological assessment of apoptosis

Apoptosis was assessed by morphology as described in section 2.2.6.

5.2.5 NADPH oxidase activity

Chemiluminescence was assayed as described in section 2.2.7

5.2.6 *De novo* protein biosynthesis

[^{35}S]-Methionine was added to neutrophil suspensions at 60 µCi/mL, 15 min before the addition of GM-CSF, butyrate or TSA. Following overnight culture, 10^6 neutrophils were solubilised in boiling SDS-PAGE sample buffer and separated by SDS-PAGE as described in section 3.2.6, under reducing conditions. Gels were then dried and radioactivity quantified with a BioRad Molecular Imager GS 363, using imaging screen-CS.

5.2.7 [^{3}H]-Acetate histone labelling

[^{3}H]-Acetate was added to neutrophil cultures to 100 µCi/mL, for either 1 or 16 h. The 1 h pulse contained no additional agents, whilst the 16 h pulse was in the presence of GM-CSF (50 U/mL), Butyrate (0.4 mmol/L), TSA (100 nmol/L), or with no further additions as a control. Following the pulse (1 or 16 h), cultures were split into two equal aliquots. Nuclear proteins were immediately extracted from one

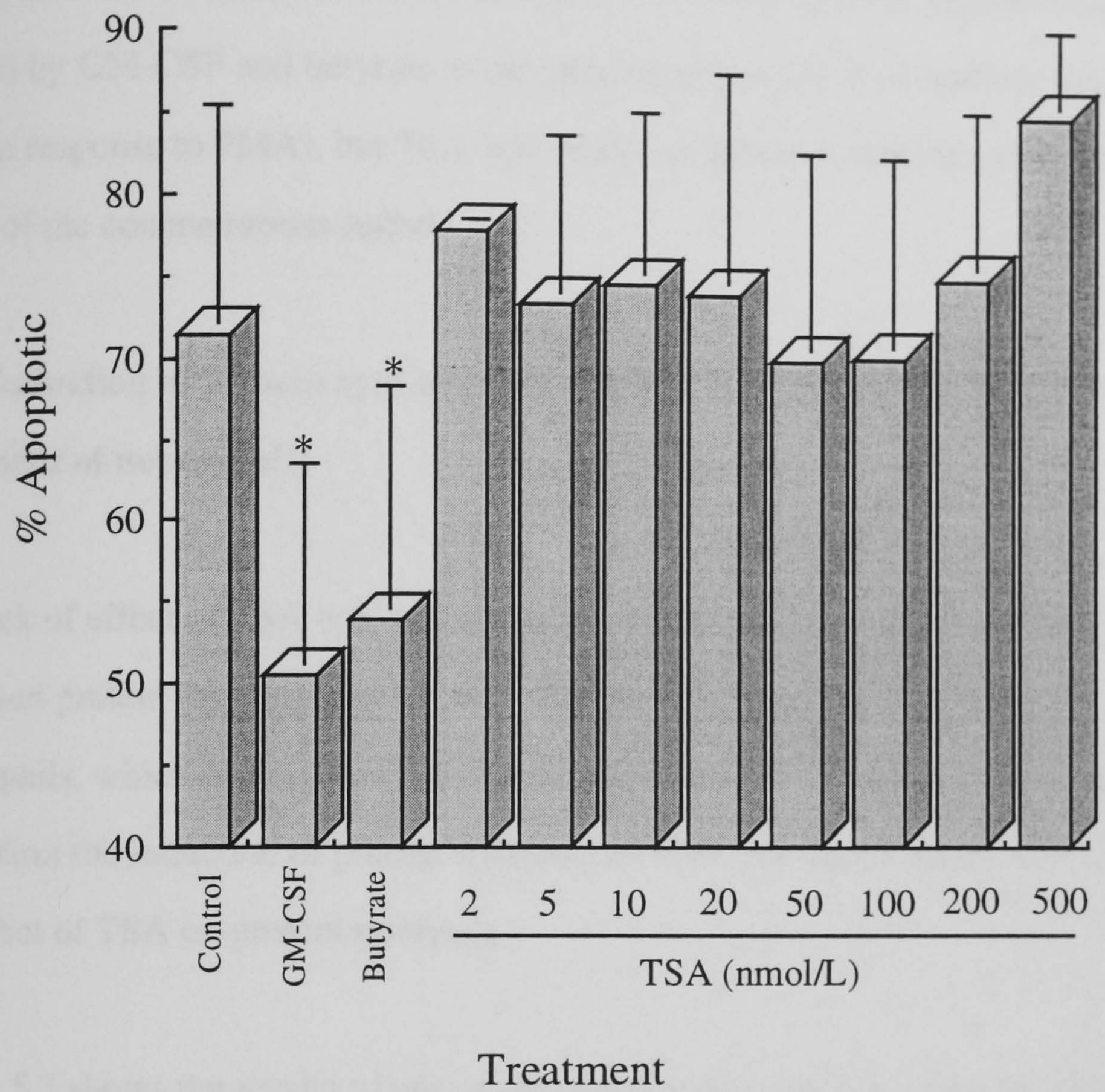
aliquot. To the remaining aliquot a 100-fold excess of cold (unlabelled) acetate (in PBS) was added, and the culture was continued for a further 2 h (chase), before extraction of nuclear proteins. GM-CSF, butyrate or TSA were added to 1 h pulsed samples for a 2 h chase. Nuclear proteins were isolated following the method of Aviram *et al* (1994), with some modifications. Neutrophils were lysed in: 10 mmol/L Tris (pH 7.1); 10 mmol/L $MgCl_2$; 0.25 mol/L sucrose; 1 % (v/v) Triton X-100; 1 mmol/L DTT; 1 mmol/L PMSF, for 10 min on ice. Nuclei were then pelleted at 1,500 g for 5 min, then washed 3 times with: 1 % SDS; 10 mmol/L Tris (pH 7.1); 5 mmol/L EDTA. 10 vol acetone was added to each extract and left overnight at -20^{0}C before collecting protein aggregates by centrifugation at 10,000g for 10 min. Following solubilisation in SDS-PAGE sample buffer, proteins were separated by SDS-PAGE on 16.5 % gels, which were either fixed and stained with coomassie blue for confirmation of equal loading, or fixed, incubated in Amplify, dried and radioactivity detected by exposure to Hyperfilm ECL. Histones on parallel gels were excised from the gels, destained, mixed with 5 mL of scintillant (Cocktail T, BDH), and radioactivity measured by scintillation counting using a Wallac 1219 Rackbeta Liquid Scintillation Counter. [^{3}H]-acetate incorporation into histones is expressed relative to control pulsed samples.

5.3 Results

5.3.1 Apoptosis measurements following culture in the presence of TSA

Trichostatin A was added to neutrophil cultures at concentrations between 2 nmol/L and 500 nmol/L, which encompasses the range of concentrations of TSA shown to be effective in other cell types (50 and 165 nmol/L, Yoshida *et al*. 1990; Schlake *et al*. 1994). Figure 5.1 shows the results of experiments measuring apoptosis of 20 h cultured neutrophils, with GM-CSF and butyrate both significantly delaying

Figure 5.1 Apoptosis measured by morphology following 20 h culture with a range of TSA concentrations



Neutrophils were isolated and cultured as described in Materials and Methods with no additions to the media (control), or supplemented as indicated. Results are means (± SD) of up to 13 experiments; n = 13 (Control, butyrate), n = 12 (GM-CSF, 50 nmol/L TSA), n = 10 (100 nmol/L TSA), n = 7 (10 and 200 nmol/L TSA), n = 4 (5 and 20 nmol/L TSA), n = 2 (500 nmol/L TSA), n = 1 (2 nmol/L TSA). Significant differences from control incubations are indicated by * ($p \leq 0.01$).

apoptosis. Surprisingly, TSA had no effect on neutrophil apoptosis at any of the concentrations used. These results were confirmed by measuring oxidative burst activity after 20 h culture, as shown in Figure 5.2. Apoptosis was significantly delayed by GM-CSF and butyrate as assessed by preservation of function (oxidative burst in response to PMA), but TSA was unable to preserve oxidative burst capacity at any of the concentrations tested.

5.3.2 Induction of protein synthesis following GM-CSF, butyrate and TSA treatment of neutrophils

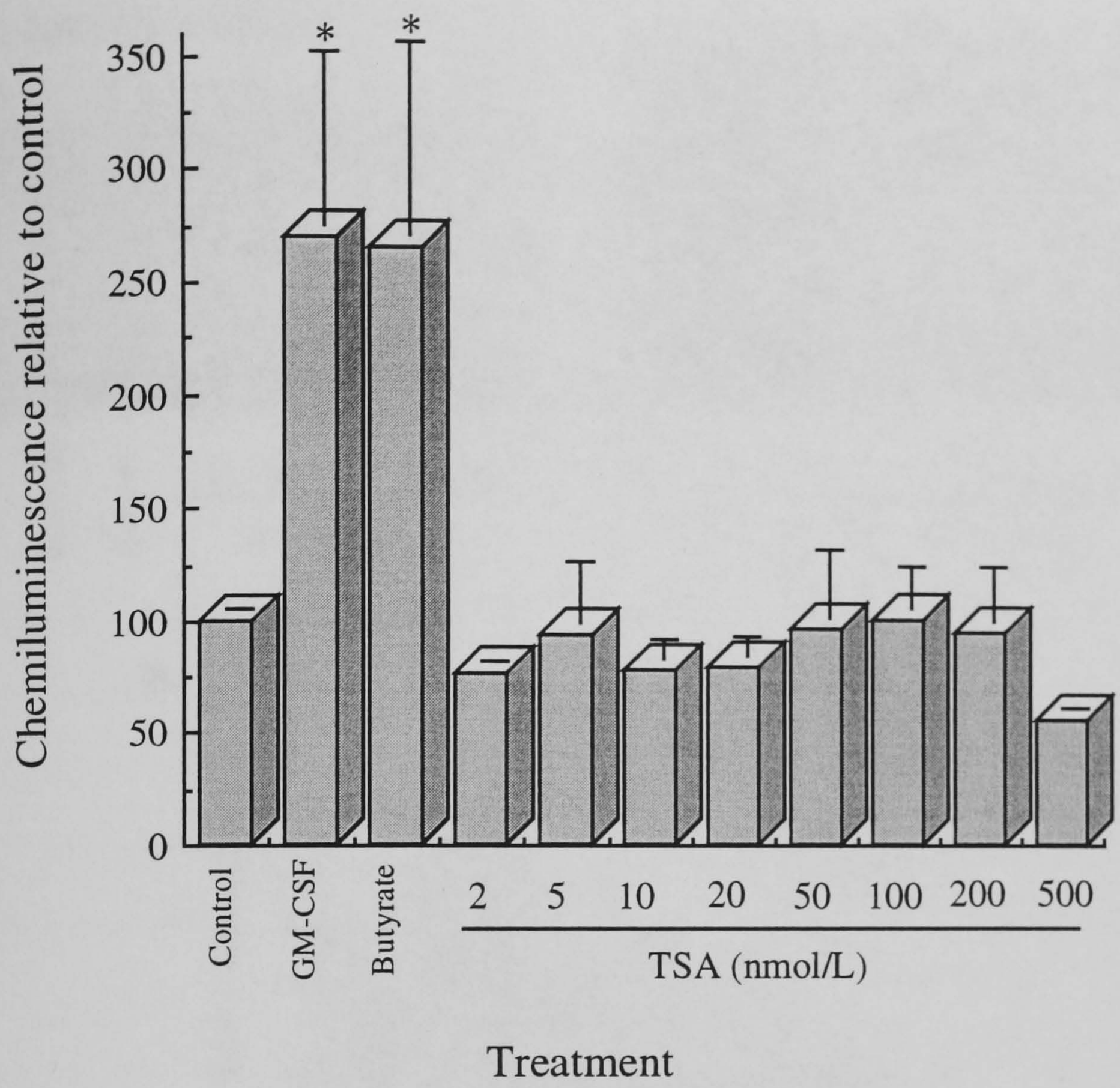
The lack of effect of TSA was not expected, as histone hyperacetylation and increased protein synthesis were predicted to result from TSA treatment of neutrophils, which should then result in delayed apoptosis. It was therefore necessary to confirm the induction of protein synthesis by GM-CSF and butyrate, and to test the effect of TSA on protein synthesis.

Figure 5.3 shows the results of one of two experiments assessing *de novo* protein synthesis following GM-CSF, butyrate or TSA treatment, which gave very similar results. Both GM-CSF and butyrate treated neutrophils showed enhanced protein synthesis after 4 h or 20 h culture, confirming previously published work in this laboratory (Stringer *et al*, 1996). In comparison, TSA resulted in no increase in protein synthesis at any of the concentrations tested (10 - 500 nmol/L), and even resulted in a decrease in protein synthesis at the highest concentration tested.

5.3.3 Histone deacetylase activity and histone acetylation

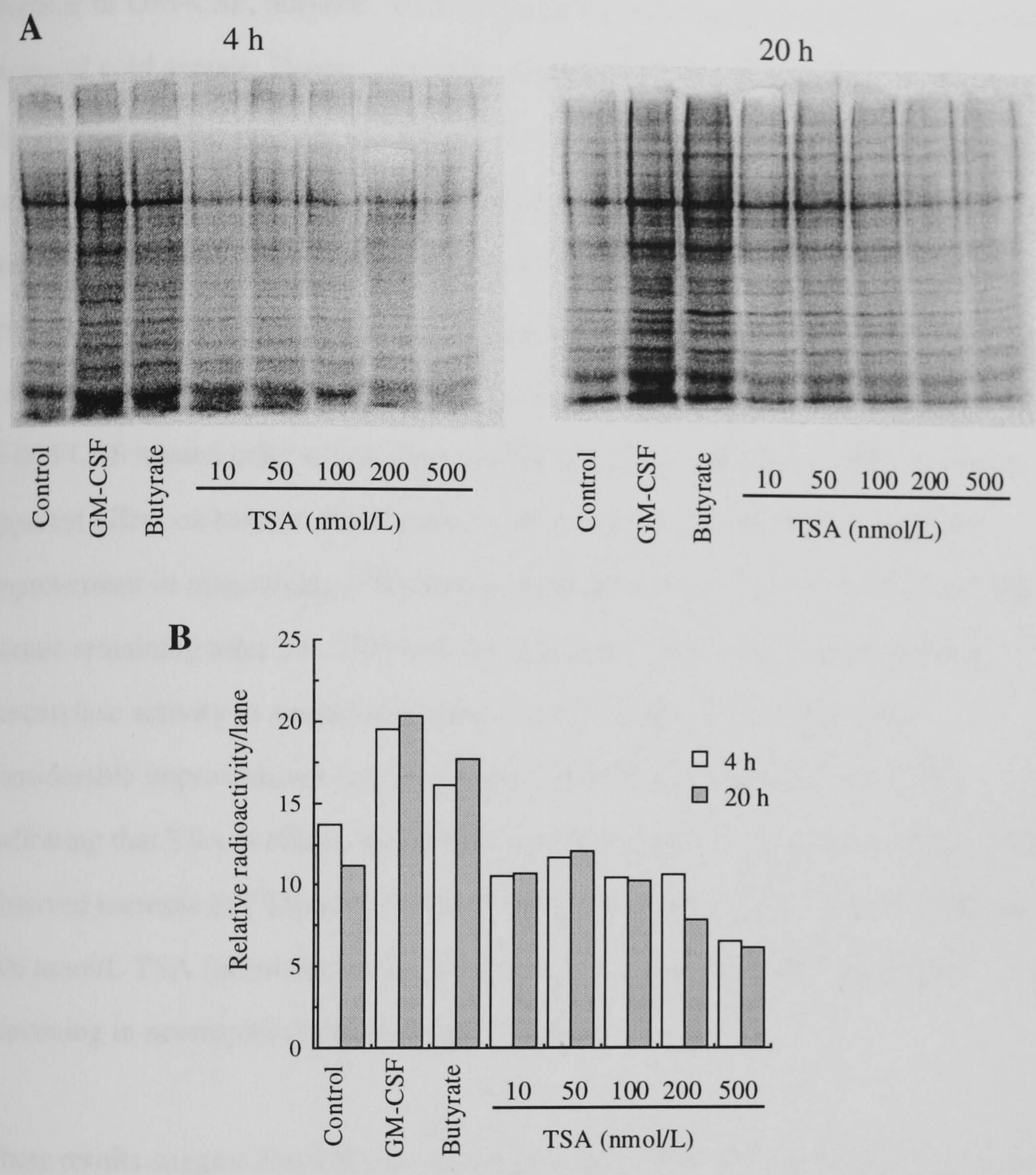
As TSA did not mimic the effect of butyrate in increasing protein synthesis, it was necessary to assess the effect of TSA on histone deacetylase activity in neutrophils. This was achieved by labelling histones with [^{3}H]-acetate for 1 h, then measuring the

Figure 5.2 Apoptosis measured by preservation of oxidative burst activity following 20 h culture with a range of TSA concentrations



Neutrophils were isolated and cultured as described in Materials and Methods with no additions to the media (control), or supplemented as indicated. Results are means (± SD) of up to 12 experiments; n = 12 (Control, butyrate), n = 11 (GM-CSF), n = 9 (100 nmol/L TSA), n = 8 (50 nmol/L TSA), n = 6 (200 nmol/L TSA), n = 5 (10 nmol/L TSA), n = 4 (50 nmol/L TSA), n = 2 (5 nmol/L TSA), n = 1 (2 and 500 nmol/L TSA). Significant differences from control incubations are indicated by * ($p \leq 0.01$).

Figure 5.3 *De novo* protein synthesis after 4 and 20 h culture

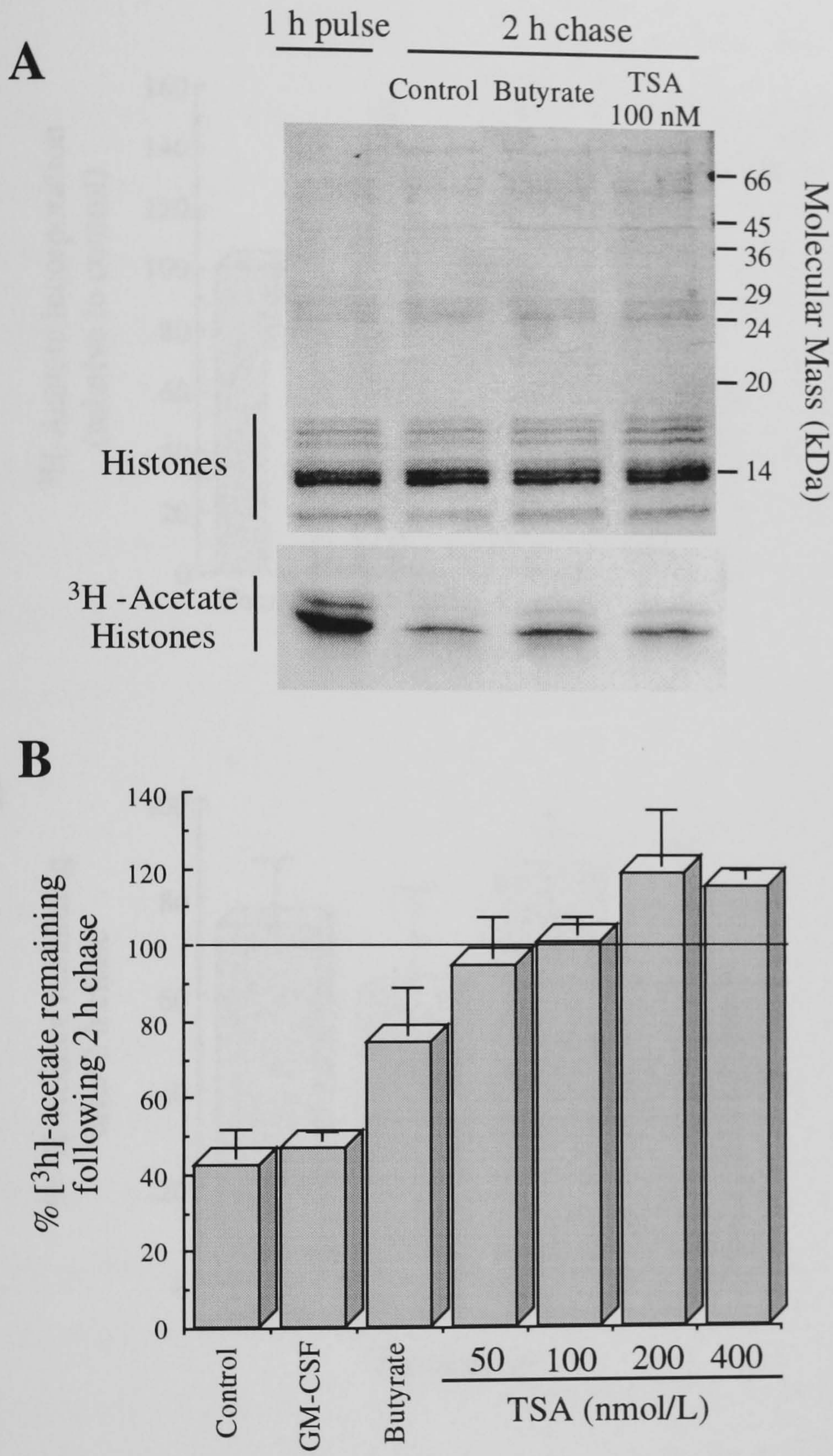


Neutrophils were isolated and cultured as described in Materials and Methods, with no additions to the media (control), or supplemented as indicated. Following culture for 4 or 20 h, equal numbers of cells were solubilised in boiling SDS-PAGE sample buffer, and separated by SDS-PAGE. Radioactivity incorporated into proteins was then detected using a molecular imager, and quantified from each treatment. In B, values from quantification of the gels shown in A are plotted, with the proportion of total radioactivity on the gel shown for each lane. These results are typical of two experiments.

proportion of [^{3}H]-acetate remaining on histones following 2 h culture in the presence of GM-CSF, butyrate, TSA or media alone, in media containing a 100 fold excess of cold acetate. Figure 5.4 shows the results of two experiments measuring histone deacetylase activity. In Figure 5.4a the coomassie stained nuclear proteins from neutrophils are shown, and fluorography shows histones are actively acetylated and then deacetylated as neutrophils were incubated *in vitro*. Figure 5.4b shows the results of quantifying the percentage [^{3}H]-acetate remaining on histones following 2 h chases. Control neutrophils showed 42.1 % (± 7.2) of ^{3}H-acetate remaining, similar to GM-CSF treated cells which showed 46.8 % (± 2.2). Therefore, GM-CSF has no apparent effect on histone deacetylation rates after 2 h. Butyrate gave a marked improvement in maintaining [^{3}H]-acetate on histones, with 74.3 % (± 15.2) of [^{3}H]-acetate remaining after 2 h. This indicates that butyrate is able to inhibit histone deacetylase activity in neutrophils within 2 h of treatment. TSA also gave considerable improvements in maintaining [^{3}H]-acetate on histones over 2 h, indicating that TSA is able to inhibit histone deacetylase activity in neutrophils. The observed increase in [^{3}H]-acetate incorporation on histones seen with 100, 200 and 400 nmol/L TSA following the 2 h chase is likely due to residual [^{3}H]-acetate remaining in neutrophils following the 1 h pulse.

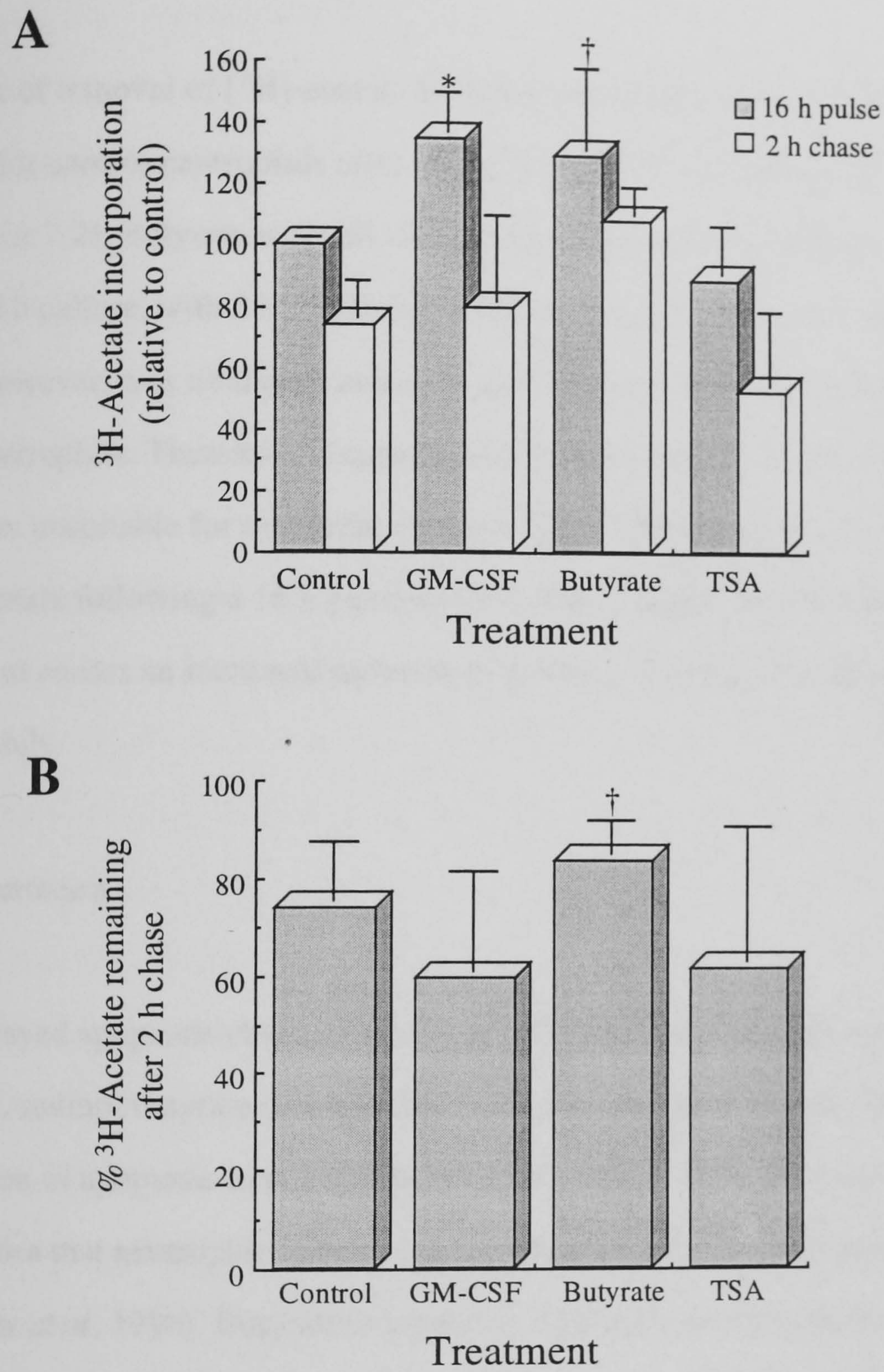
These results suggest that TSA and butyrate both inhibit histone deacetylase, during short-term cultures, but only butyrate is able to increase protein synthesis and delay apoptosis in longer-term cultures. Therefore, the ability of these agents to inhibit histone deacetylase activity in longer-term (18 h) cultures was assessed. Neutrophils were cultured for 16 h in the presence of GM-CSF, butyrate, 100 nmol/L TSA or media alone (control), with [^{3}H]-acetate included in the medium. Unlabelled acetate was then added to a 100-fold excess, and the culture was continued for 2 h. Figure 5.5 shows the results of three experiments measuring histone acetylation after 16 h, and the ability of GM-CSF, butyrate and TSA to maintain [^{3}H]-acetate levels in histones over a further 2 h incubation. Both GM-CSF and butyrate treated

Figure 5.4 Relative histone deacetylation rates in control, GM-CSF, butyrate and TSA treated neutrophils



Neutrophils were isolated a cultured with [^{3}H]-acetate for 1 h as described in Materials and Methods. Cells were then washed and excess unlabelled acetate added, before addition of the agents indicated for a further 2 h incubation. Equal quantities of nuclear proteins were then separated by SDS-PAGE and radioactivity detected by fluography (A). In B, mean (±SD) % radioactivity remaining following 2 h chases, from 2 experiments, measured by scintillation counting of excised histone bands from SDS-PAGE are shown.

Figure 5.5 Incorporation of ^{3}H-Acetate into histones after 16 h culture, and relative deacetylation rates following a further 2 h chase



Neutrophils were isolated and cultured with [^{3}H]-acetate for 16 h with the agents indicated, then chased for 2 h with excess unlabelled acetate as described in Materials and Methods. [^{3}H]-acetate incorporation into equal amounts of histones was the assessed, by scintillation counting. In A, relative levels of [^{3}H]-acetate on histones after 16 h pulse and 2 h chases are shown. In B, the mean percentage [^{3}H]-acetate remaining following the 2 h chase are shown. Results are from 3 experiments, with significant difference from controls indicated by † ($p \leq 0.05$) and * ($p \leq 0.01$).

neutrophils showed significantly higher incorporation of [^{3}H]-acetate into histones after 16 h, but this was not evident for TSA treatments.

The rate of removal of [^{3}H]-acetate from histones as determined by the 2 h chase was slower for control neutrophils after 16 pulse (74.0 % ± 12.1) than for a 1 h pulse (42.1 % ± 7.2). Butyrate was still able to significantly inhibit histone deacetylation after 16 h culture, with 83.6 % (± 6.8) [^{3}H]-acetate remaining after the 2 h chase. TSA, however, was no longer able to inhibit histone deacetylation after 16 h culture with neutrophils. Therefore, it appears that TSA is unstable in this system and therefore unsuitable for overnight cultures. GM-CSF showed an accelerated loss of [^{3}H]-acetate following a 16 h pulse and 2 h chase, suggesting that GM-CSF treatment causes an increased turnover of histones, compared to untreated neutrophils.

5.4 Discussion

The delayed apoptosis observed in human neutrophils following treatment with 0.4 mmol/L sodium butyrate is associated with increased gene expression, as is the inhibition of apoptosis in neutrophils by many agents. Such observations have lead to the idea that neutrophil lifespan can be extended by enhancing gene expression (Stringer *et al*, 1996). Butyrate is known to inhibit histone deacetylase, by a non-competitive mechanism, with a K_i ~ 60 μmol/L (Cousens *et al*, 1979), but is required at concentrations of more than 1 mmol/L to inhibit histone deacetylase of most cells in culture. Therefore, in this Chapter, I set out to confirm the theory that butyrate is able to delay apoptosis due to inhibition of histone deacetylase, despite the fact that the concentration of butyrate used is lower than that known to affect histone deacetylase in other cell types. A specific inhibitor of histone deacetylase, TSA, was therefore used, with a K_i ~ 3.4 nmol/L (Yoshida *et al*, 1990), which I predicted would also inhibit neutrophil apoptosis.

The addition of TSA at a range of concentrations, which would be expected to have a similar potency in inhibiting histone deacetylase in neutrophils as 0.4 mmol/L butyrate, showed no ability to delay neutrophil apoptosis (Figures 5.1 and 5.2). As this result was completely unexpected, I next examined the induction of protein synthesis following butyrate, GM-CSF and TSA treatment of neutrophils. Both GM-CSF and butyrate enhanced gene expression, but TSA was ineffective at any of the concentrations used (Figure 5.3). The lack of enhanced gene expression following TSA treatment may suggest that TSA was unable to inhibit histone deacetylase in neutrophil cultures.

Some cell types are not responsive to TSA, (e.g. BHK cells, Schlake *et al*, 1994), so it was necessary to test the ability of TSA to inhibit histone deacetylase in neutrophil cultures. The turnover of acetyl groups on histones was therefore assessed in neutrophils treated with GM-CSF, butyrate and TSA. These experiments confirmed that butyrate at 0.4 mmol/L is effective in inhibiting histone deacetylase activity, as [^{3}H]-acetate labelled histones showed a slower loss of [^{3}H]-acetate in butyrate treated neutrophils over 2 h (Figure 5.4). TSA was also effective in inhibiting histone deacetylase activity over a range of concentrations during a 2 h culture. This finding indicates that TSA is indeed able to inhibit histone deacetylase in neutrophils, but no increase in gene expression paralleled this inhibition of histone deacetylase activity. The inhibition of histone deacetylase would be predicted to result in enhanced gene expression, due to the increased accessibility of transcription factors to hyperacetylated histones (Grunstein, 1997).

One possible explanation for these disparate results is that TSA is unstable in this culture system with neutrophils, so that whilst it is able to inhibit histone deacetylase activity in short cultures, this activity is lost as culture continues, and no increase in gene expression following this inhibition is observed. There are no published reports of TSA losing activity over time in culture to my knowledge, but personal

communication with Dr Minuro Yoshida (University of Tokyo) confirmed that in some culture systems there is a rapid loss of potency of TSA. Therefore, the ability of TSA to cause higher levels of [^{3}H]-acetate incorporation into histones over 16 h (pulse) culture, and the maintenance of this [^{3}H]-acetate incorporation over a further 2 h (chase) was assessed.

These experiments showed that both GM-CSF and butyrate treated neutrophils showed significantly higher levels of [^{3}H]-acetate incorporated into histones over 16 h, but that TSA was did not lead to histone hyperacetylation (Figure 5.5a). Measurement of the activity of histone deacetylase over a further 2 h showed that in control cultures, the decrease in [^{3}H]-acetate from histones was less marked when labelled for 16 h, then when labelled for 2 h (Figures 5.4 and 5.5). This apparent loss of histone deacetylase activity in 16 h aged neutrophils may be explained by the apoptosis of many of these untreated cells, such that apoptotic cells with [^{3}H]-acetate incorporated into histones show no loss of this [^{3}H]-acetate as the cells are apoptotic and non-functional. GM-CSF treated neutrophils again showed no inhibition of histone deacetylase activity after 16 h (Figure 5.5b). Butyrate treated neutrophils showed a significantly slower loss of [^{3}H]-acetate on histones over 2 h, following 16 h culture with butyrate (Figure 5.5b), indicating that butyrate is sufficiently stable in neutrophil cultures to still inhibit histone deacetylase after overnight cultures. TSA was, however, ineffective at inhibiting histone deacetylase activity after 16 h in culture (Figure 5.5b), indicating that TSA is indeed unstable in overnight neutrophil cultures. There is a recently described molecule, trapoxin, which is a specific and irreversible inhibitor of mammalian histone deacetylases (Kijima *et al*, 1995), which may allow the role of histone deacetylase inhibition in neutrophil apoptosis to be further studied. However, this molecule is not commercially available, and is also in short supply, so I was unable to test this in my cultures.

The link between rescue from apoptosis in neutrophils and enhanced gene expression therefore still holds with the results described in this Chapter. Although TSA was unable to delay neutrophil apoptosis, this lack of effect may be due to the poor stability of TSA in this system. Butyrate was clearly able to inhibit histone deacetylase, and enhance gene expression, strengthening the theory that butyrate acts to delay neutrophil apoptosis via histone hyperacetylation leading to enhanced gene expression. The increased incorporation of [^{3}H]-acetate on histones following 16 h culture with GM-CSF is not due to inhibition of histone deacetylase, but probably due to the increased action of histone acetyltransferases, as there is both increased incorporation of [^{3}H]-acetate on histones, and an increased turnover of acetyl-groups on histones. These results fit well with the current understanding of histone acetylation and control of transcription, such that certain transcription factors (i.e. Gcn5, CBP/c300, TAF 250) have intrinsic histone acetyltransferase activities (Pazin & Kadonaga, 1997).

Butyrate has been show to be associated with delayed apoptosis in one other cell type, namely the proximal colon of guinea pigs (Hass *et al*, 1997). This report shows that colonic cells require the presence of butyrate at the physiologically normal concentration in the colon of ~ 10 mmol/L. Removal of butyrate leads to apoptosis, and is associated with induction of the pro-apoptotic protein Bax. However, most reports show induction of apoptosis by butyrate in other cell types. The protection from apoptosis by butyrate in neutrophils reflects the physiological status of these cells. The neutrophil is pre-programmed to undergo apoptosis and requires external instructions to delay apoptosis, whereas many cell types need stimuli to induce apoptosis. Therefore, enhanced gene expression and removal of transcriptional repression from key genes following butyrate treatment may induce expression of protective genes in neutrophils (and possibly colonic cells), but induces expression of death genes in many other cell types.

CHAPTER 6 BCL-2 FAMILY EXPRESSION IN NEUTROPHILS

6.1 Introduction

The Bcl-2 family of proteins are intimately associated with the control of entry into, and execution of, the apoptotic program (as discussed in sections 1.5.3.1-9). There has been relatively little research into the role of Bcl-2 family members in neutrophil apoptosis, with most researchers finding no expression of anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-X_L, and Mcl-1 expression have been examined) in mature neutrophils (section 1.6.5). The pro-apoptotic protein Bax is expressed at relatively high levels in neutrophils, which it is suggested explains the rapid apoptosis of these cells (Ohta *et al*, 1995).

The Bcl-2 family proteins are reported to act primarily on mitochondrial membranes, but are also located on endoplasmic and nuclear membranes (Kroemer, 1997). There is increasing evidence that anti-apoptotic Bcl-2 family members can prevent entry into apoptosis by inhibiting release of cytochrome c from mitochondria into the cytosol, where cytochrome c is then able to participate in the activation of caspases (section 1.5.3.3). Moreover, the localisation of Bax dimers to mitochondria has been shown to be essential for Bax to exert its cytotoxic effects in both yeast and mammalian cells (Zha *et al*, 1996a). However, Bcl-2 can effectively prevent apoptosis when localisation is restricted to endoplasmic membranes, indicating Bcl-2 family members can exert an anti-apoptotic effect without mitochondrial localisation (Zhu *et al*, 1996). The neutrophil does not use mitochondrial respiration nor oxidative phosphorylation, but relies on glycolysis to meet its energy requirements, and possess few distinguishable mitochondria (Bainton, 1980; Edwards, 1994). The few mitochondrial structures in the cytoplasm of mature neutrophils have been visualised by use of an ultrastructural

cytochemical substrate for elastase-like enzymes and electron microscopy (Clark *et al.* 1980).

The expression of Bax in neutrophils suggests a role for this protein in neutrophil apoptosis, but given the lack of evidence for an anti-apoptotic Bcl-2 family member in neutrophils, and the scarcity of mitochondria, it is uncertain how Bax is involved in neutrophil apoptosis. The ability to extend the lifespan of neutrophils by delaying apoptosis demonstrates that Bax expression does not automatically trigger apoptosis in neutrophils, so it may be predicted that anti-apoptotic Bcl-2 family member is expressed in neutrophils, which can be induced to counteract Bax, and thereby delay apoptosis.

In the course of screening a subtractive cDNA library enriched in GM-CSF regulated clones, a clone encoding Mcl-1 was isolated (Quayle and Edwards, unpublished). The Mcl-1 protein is known to be rapidly inducible in other cell types and has a high rate of turnover due to its PEST motifs targeting it for rapid proteolysis (Kozopas *et al*, 1993; Yang *et al*, 1995a). This protein would therefore potentially fulfil the role of an apoptosis delaying protein in the neutrophil, cells that are subject to acute regulation. In this Chapter, I have therefore studied the expression of the Bcl-2 family proteins Bax, Bcl-2, Bcl-X and Mcl-1, in an attempt to clarify the role of these proteins in neutrophil apoptosis.

6.2 Materials and Methods

6.2.1 Materials

All materials for the isolation and culture of neutrophils are described in section 2.2.1. rhIL-1β was from NIBSC (Potters Bar, UK). Bcl-2 mAb was from Calbiochem-

Novabiochem (UK) Ltd. (Nottingham, UK), Bcl-X antisera from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), Bax and Mcl-1 antisera from Pharmingen (San Diego, CA). Anti-rabbit IgG-HRP secondary Ab, Nitrocellulose membrane, ECL detection reagent and Hyperfilm ECL were all purchased from Amersham (UK). 30% solution of acrylamide/bis-acrylamide was from Severn Biotech Ltd. (Kidderminster. UK). All other specialist materials were purchased from Sigma (Poole, UK), with all reagents used being of the highest purity available.

6.2.2 Isolation of Neutrophils and PBMC

Neutrophils were isolated exactly as described in section 2.2.2, except isolated neutrophils were suspended in media supplemented with 5 % (v/v) FCS. PBMC were isolated exactly as described in section 4.2.3.

6.2.3 Cell culture

Neutrophils were cultured in RPMI 1640 supplemented with 5 % (v/v) FCS and 2 mmol/L L-Glutamine at 5 x 10^6 cells/mL as described in section 2.2.3. Media was supplemented with GM-CSF (100 U/mL), sodium butyrate (0.4 mmol/L), rhIL-1β (500 U/mL) or LPS (10 ng/mL) or control incubations with no further additions to the media.

U937, KLC22, Raji and Jurkat cell lines were maintained and cultured in RPMI 1640 medium supplemented with 10 % (v/v) FCS, 2 mmol/L L-Glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at a cell density maintained between 0.6 and 1.0 x 10^6/mL. Viability was assessed by trypan blue exclusion. and was always greater than 90 %.

In order to measure Mcl-1 induction, U937 cells were cultured in reduced serum conditions containing FCS at 0.3 % (v/v) for 3 days (Kozopas *et al*, 1990). The reduced serum media halts proliferation and causes accumulation of cells in G_0/G_1

phase of the cell cycle, and narrows the time frame of induced differentiation. There was a slight loss of viability in reduced serum media, as previously reported (Kozopas *et al*, 1990), to ~ 80%. Non-viable cells were removed by low speed centrifugation before resuspending serum-starved U937 cells in normal media, with PMA included at 80 nmol/L. Mcl-1 mRNA and protein were induced maximally at 3 h and 6 h (not shown), as reported for ML-1 cells (Kozopas *et al*, 1993; Yang *et al*, 1995).

6.2.4 Morphological assessment of apoptosis

Apoptosis was assessed by morphology after 12 h culture, exactly as described in section 2.2.6.

6.2.5 Western blotting

Following the isolation and culture of Raji, Jurkat, U937, PBMC and neutrophils, whole cell extracts were prepared by boiling cells in SDS-PAGE reducing sample buffer for 5 minutes. Samples were then frozen at -80^{0}C until use. Post-nuclear extracts were prepared exactly as described (Ohta et al, 1995). Briefly, cells were lysed in: 10mmol/L Tris-HCl pH 7.6; 150 mmol/L NaCl; 1% (v/v) Triton-X 100; 5 mmol/L EDTA; 2mmol/L PMSF for 40 min on ice, then nuclei were removed by centrifugation at 15,000g for 10 min. Supernatants were then mixed with 2x SDS-PAGE reducing sample buffer, boiled and frozen as above. An alternative method to prepare nuclear extracts was used to allow crude fractionation of cells into nuclear and non-nuclear fractions. Cells were lysed in: 10 mmol/L Tris-HCl pH 7.6; 10 mmol/L $MgCl_2$; 250 mmol/L sucrose; 1% (v/v) Triton-X 100; 1 mmol/L DTT; 2 mmol/L PMSF; 3 μg/mL of each of aprotinin, chymotrypsin, pepstatin A, leupeptin, antipain for 10 min on ice, then centrifuged at 2,000g for 5 min at 4^{0}C to pellet the nuclei. The resulting supernatants were mixed with 2x SDS-PAGE reducing sample buffer (non-nuclear extracts) and nuclei resuspended in 1x SDS-PAGE reducing sample buffer

(nuclear extracts), boiled and frozen as above. The lysates were mixed with 2x SDS-PAGE reducing sample buffer without the removal of nuclei to prepare lysate samples.

Extracts were separated by SDS-PAGE using the appropriate percentage acrylamide, with 5 x 10^5 cell equivalents loaded per lane for neutrophils, and 2 x 10^5 cell equivalents for Raji, Jurkat, U937 and PBMC. Following electrophoresis, proteins were transferred to nitrocellulose membranes using the BioRad mini protean II transfer apparatus. Membranes were then Ponceau S stained and images captured using a Hamamatsu XC-77CE CCD camera and Image 1.44 VDM software. These Ponceau S stained membranes were used to compare the levels of actin in each lane. Membranes were then washed free of Ponceau S with PBS pH 7.4, and then blocked with: 5% Marvel (dried skimmed milk); TBS pH 8.0; 0.05% (v/v) Tween 20 for 1 h at room temperature. Following two brief washes in TBS pH 8.0; 0.05% (v/v) Tween 20 (wash buffer), membranes were incubated with primary antibodies to either Bcl-2 (1:50), Bcl-X (1:1,000), Bax (1:2,000) or Mcl-1 (1:2,000) diluted in 0.5% Marvel (dried skimmed milk); TBS pH 8.0; 0.05% (v/v) Tween 20 (Ab buffer) overnight at room temperature. Membranes were then washed 2x 30 sec and 3x 5 min in wash buffer before incubation with HRP-linked secondary antibody (1:50,000) diluted in Ab buffer for 1 h at room temperature. Following washes, antisera were detected using Amersham's ECL detection reagents and pre-flashed film. Care was taken not to overexpose film to allow quantitation and comparison of expression of Bcl-2 family proteins between samples using a Hamamatsu XC-77CE CCD camera and Image 1.44 VDM software.

Efficiency of nuclear and non-nuclear fractionation was analysed by visualising histones 2A, 2B and 3 in fixed coomassie stained gels after separation by SDS-PAGE of 5 x 10^5 cell equivalents.

6.2.6 Statistical analysis

All data is expressed and analysed as described in section 2.2.11

6.3 Results

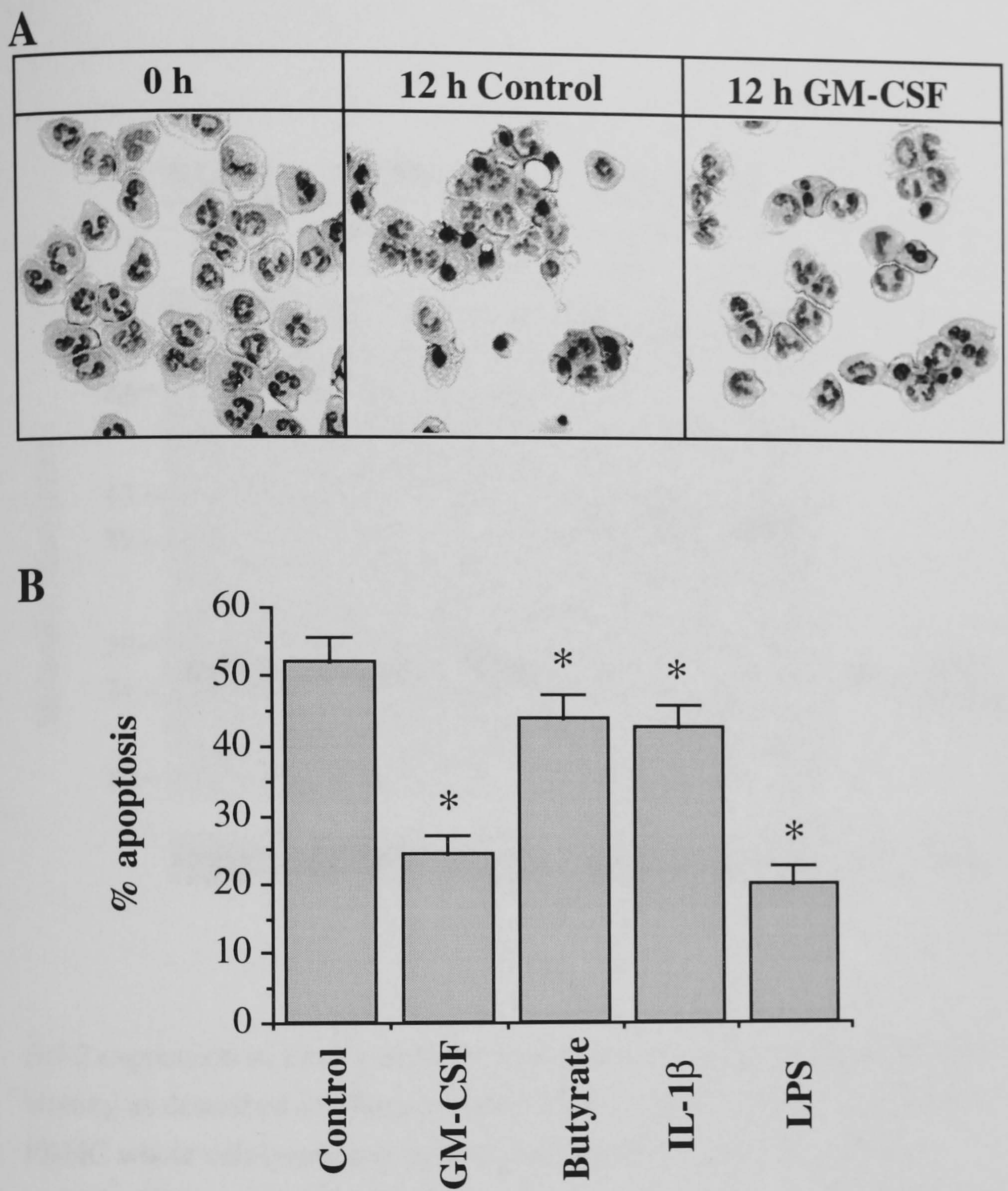
6.3.1 Delay of neutrophil apoptosis by GM-CSF, butyrate, IL-1β and LPS

Freshly isolated neutrophils have a distinct morphology which is easily distinguishable from apoptotic neutrophils which show loss of total cell volume, compaction of chromatin with loss of the multilobed nuclear structure and vacuolation (Figure 6.1a). Control incubations showed 52.0 % (±6.3) apoptosis by morphology after 12 h in culture demonstrating the constitutive apoptosis which cultured neutrophils will undergo. Apoptosis was delayed by all other treatments used (Figure 6.1b) with a difference significant from control using the paired students T-test ($p \leq 0.01$). GM-CSF treatment showed 24.3 % (±5.2), Butyrate showed 44.0 % (±6.6), IL-1β showed 42.9 % (±6.1) and LPS showed 19.0 % (±5.7). These results were obtained from at least three separate experiments.

6.3.2 Expression of Bcl-2, Bcl-X, Bax and Mcl-1 in freshly isolated neutrophils

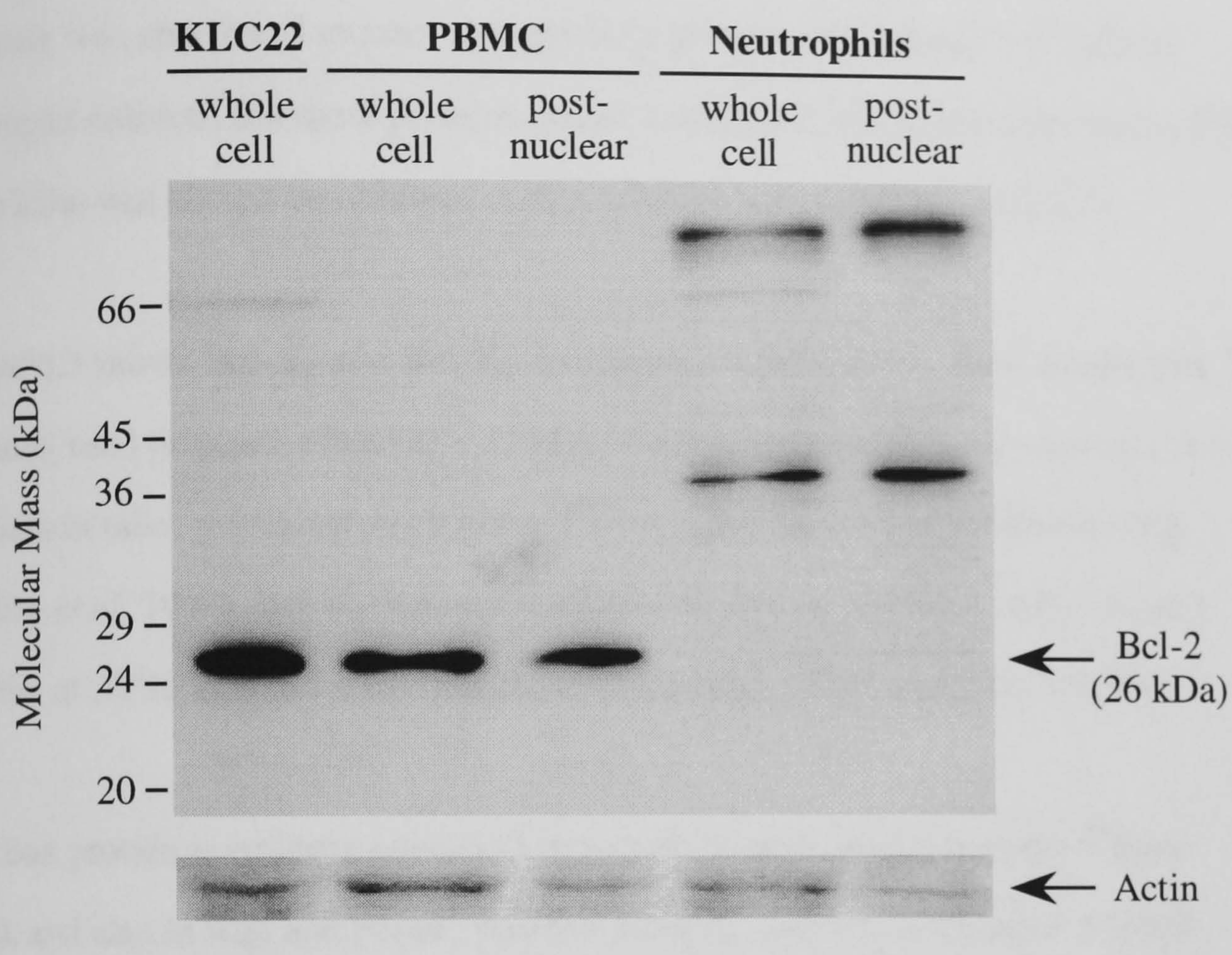
The expression of each of these proteins was examined by western blotting using antibodies specific for each of the proteins to be tested. Figure 6.2 shows that neutrophils essentially express no Bcl-2. PBMC and KLC22 cells used as positive controls confirmed the specificity of the antibody for Bcl-2, which was detected as a single band at ~ 26 kDa. A faint p26 Bcl-2 band was only detected in neutrophil

Figure 6.1 Morphological assessment of neutrophil apoptosis following 12 h culture



Neutrophils were isolated and cultured as described in Materials and Methods. **A** shows cytospins of freshly isolated and 12 h cultured neutrophils. **B** shows % apoptosis after 12 h culture in the conditons indicated. Significant difference from control cultures is indicated by *($p \leq 0.01$), from at least 3 separate experiments.

Figure 6.2 Examination of Bcl-2 expression in neutrophils



Bcl-2 expression in freshly isolated neutrophils was determined by western blotting as described in Materials and Methods. KLC22 whole cell lysate, PBMC whole cell lysate and post-nuclear extracts were run as positive controls. Blots were stained with ponceau S and the stained-actin band is shown to indicate approximately equal loading per lane. The blot shown is representative of two separate experiments.

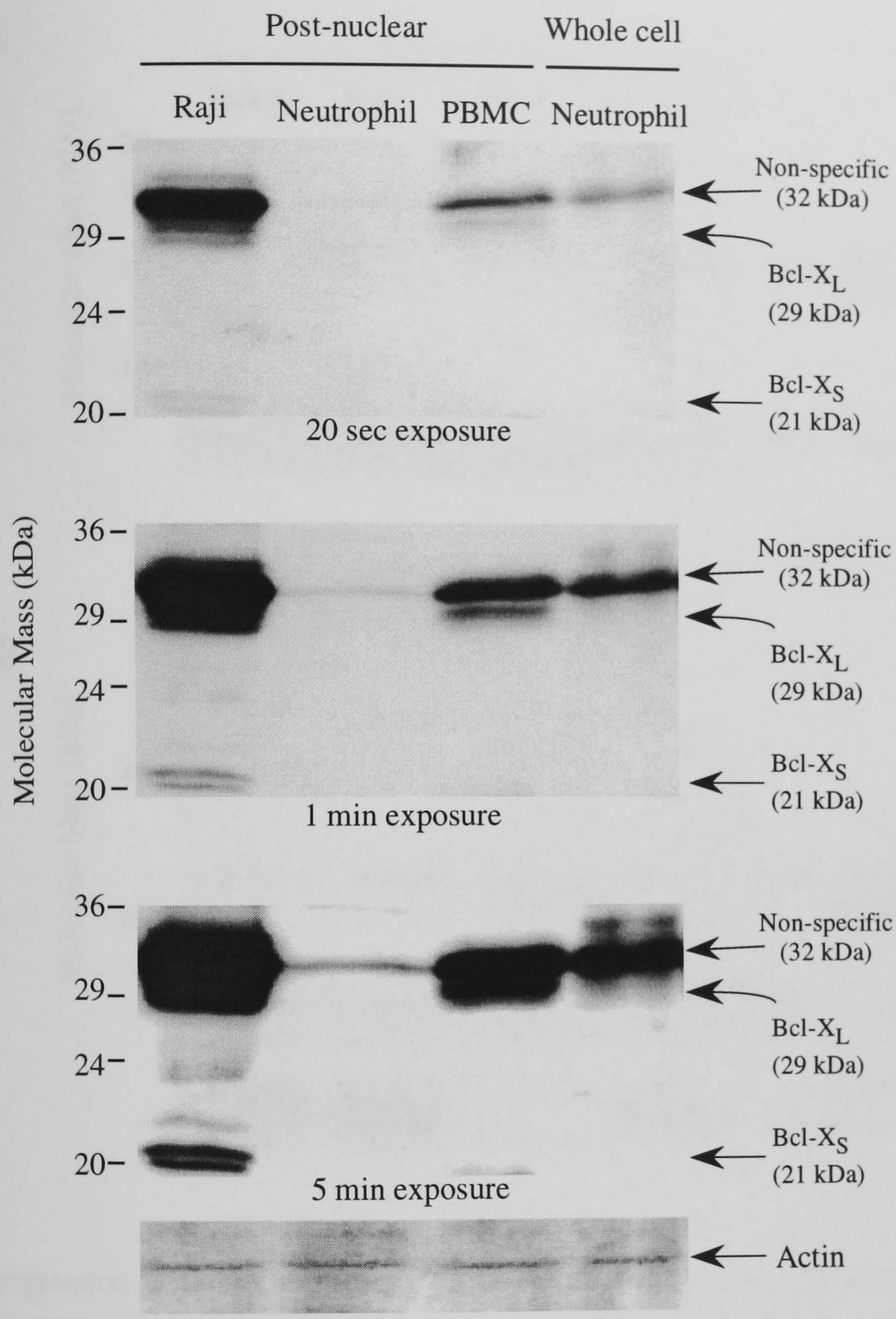
lysates after exposure to film for 10 min (not shown) whilst the film shown in Fig. 6.2 was exposed for only 30 s. This detection of Bcl-2 in neutrophil preparations is at approximately 1/40th the level seen in PBMC, which is the level of PBMC contamination usually found in our neutrophil preparations. The Bcl-2 specific antibody was also found to react strongly with proteins of ~ 80 and ~ 40 kDa in neutrophil extracts, but these proteins do not correspond to the molecular mass of Bcl-2 (26 kDa) that should be obtained in these denaturing, reducing conditions.

Figure 6.3 shows Bcl-X_L and Bcl-X_S are also essentially absent from neutrophils. The antibody used detected a band at ~ 32 kDa which is believed to be non-specific as it is not seen in other published work using different Bcl-X_L specific antibodies (e.g. Findley *et al*, 1997). Bcl-X_L was detected as a doublet at 29 kDa, and Bcl-X_S as a doublet at 20/21 kDa in Jurkat (not shown), Raji and PBMC positive controls.

The Bax protein is strongly expressed in neutrophil post nuclear extracts (Figure 6.4a), and also in Raji and PBMC positive controls, and was detected as a single protein of ~ 21 kDa. Jurkat cell extract was run as a negative control and showed no reactivity with the Bax specific antibody. Crude fractionation of neutrophil extracts (Figure 6.4b) confirms Bax expression in neutrophils extracts depleted of nuclei, and further demonstrate Bax expression is restricted to non-nuclear membranes in neutrophils. The histones 2A, 2B, and 3 are shown from the fractionated samples, and show that fractionation successfully separated nuclear material from the rest of the cell.

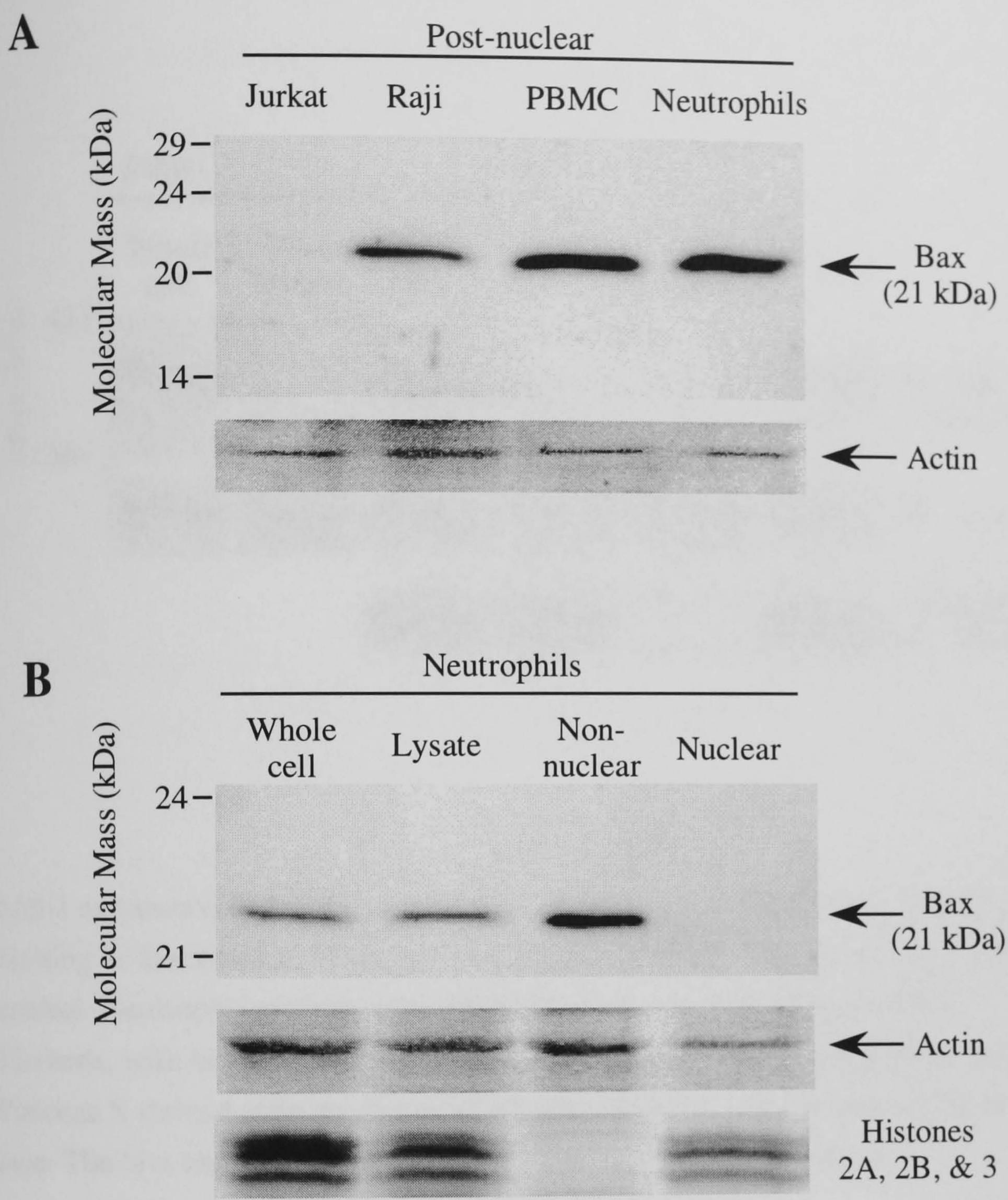
Mcl-1 protein was detected in differentiating U937 cells used as a positive control, which confirmed the specificity of the antibody for Mcl-1, only detecting a doublet at 40/42 kDa (not shown). Mcl-1 was also expressed in PBMC, used as a positive control (Figure 6.5). Post-nuclear neutrophil extracts prepared identically to those used by Ohta *et al* (1995) confirmed no Mcl-1 protein was detected in neutrophil extracts prepared in this manner (Figure 6.5). However, Mcl-1 protein was detected in

Figure 6.3 Examination of Bcl-$X_{L/S}$ expression in neutrophils



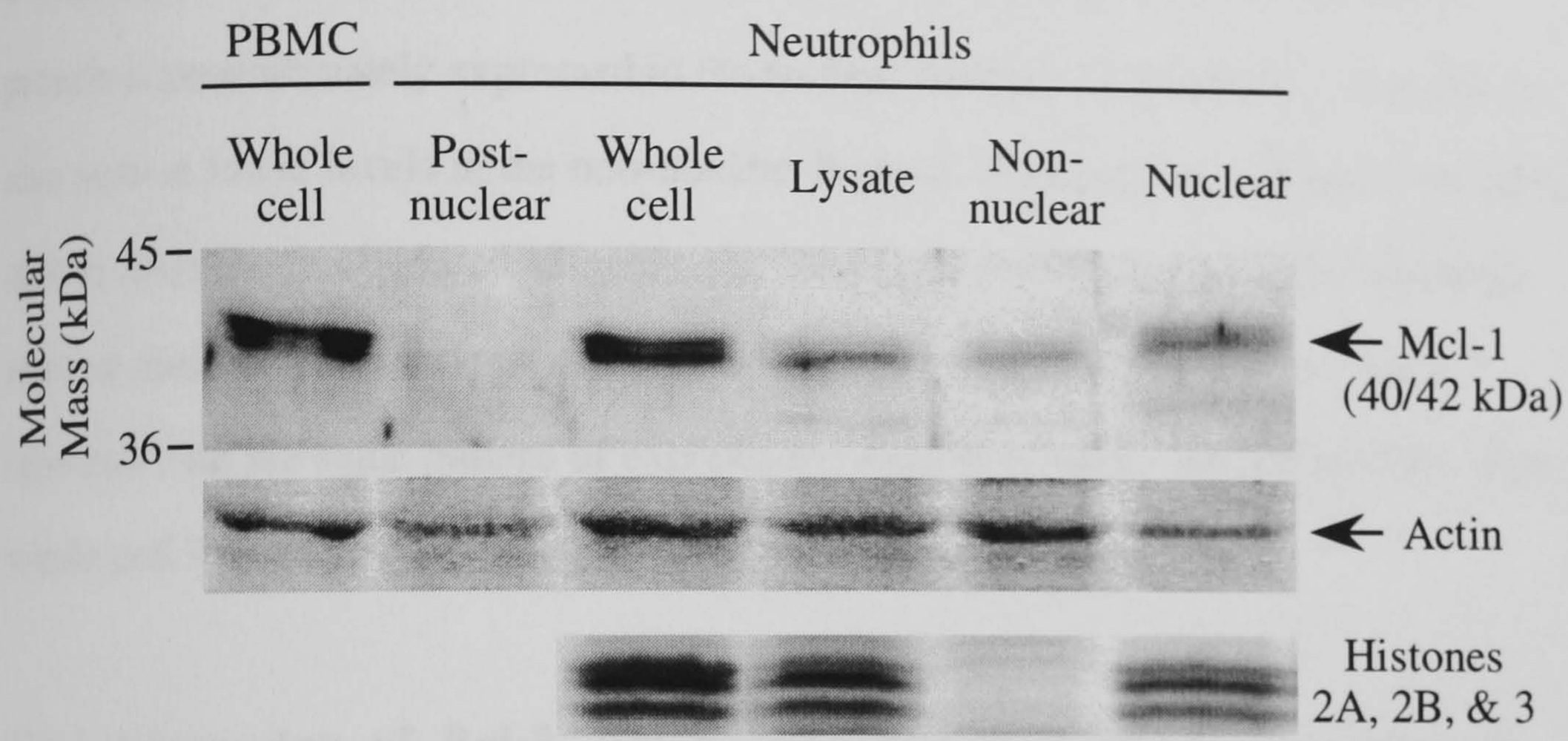
Bcl-$X_{L/S}$ expression in freshly isolated neutrophils was determined by western blotting as described in Materials and Methods. Raji and PBMC post-nuclear extracts were run as positive controls. The antibody used recognised a 32 kDa non-specific band, which was resolved away from the Bcl-X_L doublet. Blots were stained with Ponceau S and the actin stained band is shown to indicate approximately equal loading per lane. The blot shown is representative of two separate experiments, with different exposure times of the same blot shown.

Figure 6.4 Examination of Bax expression in neutrophils



Bax expression in freshly isolated neutrophils was determined by western blotting as described in Materials and Methods. Raji and PBMC post nuclear extracts were run as positive controls, with Jurkat post-nuclear extract as a negative control (**A**). In **B**, neutrophil extracts from whole cell, cell lysate, non-nuclear and nuclear fractions were analysed, with histones from cell fractionation shown. Ponceau S stained actin is shown to indicate approximately equal loading per lane. Each blot is representative of 2 separate experiments.

Figure 6.5 Examination of Mcl-1 expression in neutrophils



Mcl-1 expression in freshly isolated neutrophils was determined by western blotting as described in Materials and Methods. PBMC was run as a positive control. Neutrophil extracts were prepared as described in Materials and Methods, with histones from cell fractionation shown below the relevant lanes. Ponceau S stained actin as shown to indicate approximately equal loading per lane. The blot shown is representative of two separate experiments.

neutrophil whole cell extracts at comparable levels to those found in PBMC, but less abundantly than in differentiating U937 cells (not shown). Preparation of nuclear and non-nuclear neutrophil extracts using an alternative method in which proteolysis is minimised shows that Mcl-1 can be detected in neutrophil lysates and that Mcl-1 protein is predominately expressed in the nuclear fraction, although Mcl-1 protein is also seen at lower levels in the non-nuclear fraction. Histones 2A, 2B, and 3 are again shown from the fractionated samples, showing fractionation successfully separated nuclear material from the rest of the cell. The results shown in Figure 6.5 were repeated with the same pattern of expression found both times. All further blots used whole cell lysates.

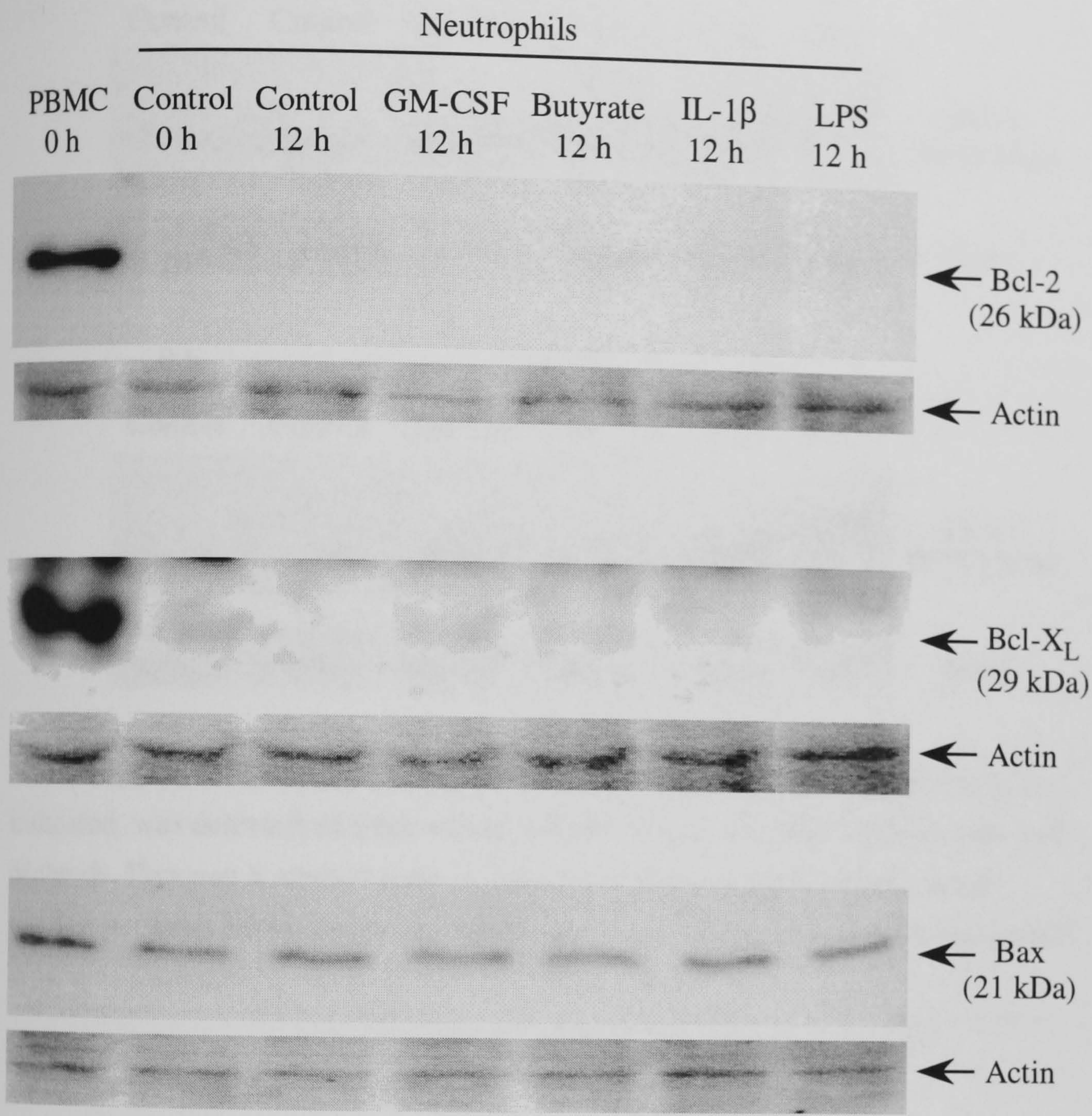
6.3.3 Expression of Bcl-2, Bcl-X_L and Bax in neutrophils treated to delay apoptosis

The expression of Bcl-2, Bcl-X_L and Bax remain unchanged in neutrophils treated to delay apoptosis with GM-CSF, Butyrate, IL-β or LPS, as shown in Figure 6.6. The Bcl-2 and Bcl-X proteins were not induced after 6 h (not shown), 12 h (Figure 6.6) or 24 h (not shown) incubation of neutrophils. The Bax protein remained at essentially constant levels after both 6 h (not shown) and 12 hour incubations and was expressed at levels similar to that found in PBMC. Treatment of neutrophils with the cytokines IL-6, IL-2, Interferon γ (which all delay neutrophil apoptosis), or TNF-α (which accelerates neutrophil apoptosis) also showed no induction of Bcl-2 or Bcl-X, and no change in the levels of the Bax protein (results not shown).

6.3.4 Induction of Mcl-1 protein expression by agents that delay neutrophil apoptosis

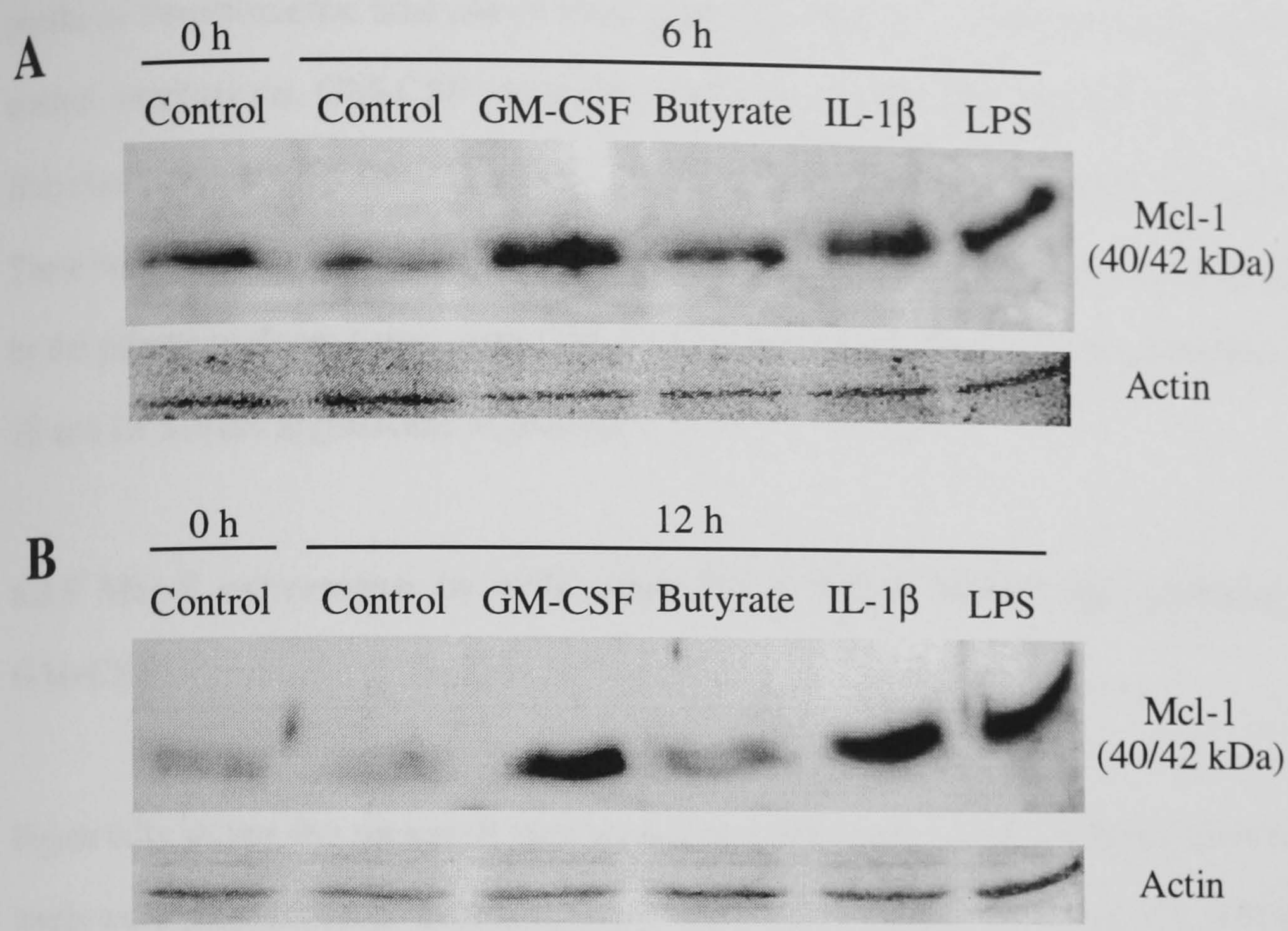
Mcl-1 protein expression is increased relative to untreated neutrophils by the apoptosis delaying agents GM-CSF, Butyrate, IL-1β and LPS. This is shown in Figure 6.7a

Figure 6.6 Expression of Bcl-2, Bcl-X_L, and Bax in neutrophils treated to delay apoptosis for 12 h



Bcl-2, Bcl-X_L, and Bax expression in freshly isolated neutrophils (and PBMC as positive controls), and neutrophils cultured for 12 h with the treatments indicated were determined by western blotting of whole cell extracts as described in Materials and Methods. Ponceau S stained actin is shown to indicate approximately equal loading per lane. Blots shown are representative of 2 separate experiments.

Figure 6.7 Mcl-1 expression in neutrophils treated to delay apoptosis



Mcl-1 expression in freshly isolated, 6 h and 12 h cultured neutrophils, treated as indicated, was determined from whole cell extracts as described in Materials and Mehtods. Ponceau S stained actin is shown to indicate approximately equal loading per lane. Blots shown are representative of at least 3 separate experiments.

Table 6.1 Relative Mcl-1 levels in 12 h cultured neutrophils

	Control	**GM-CSF**	**Butyrate**	**LPS**	**IL-1β**
Relative Mcl-1 expression (12 h)	1.00 ---	2.87* ±0.69	1.46† ±0.23	2.41† ±1.29	2.70† ±0.94
n =	6	6	3	3	4

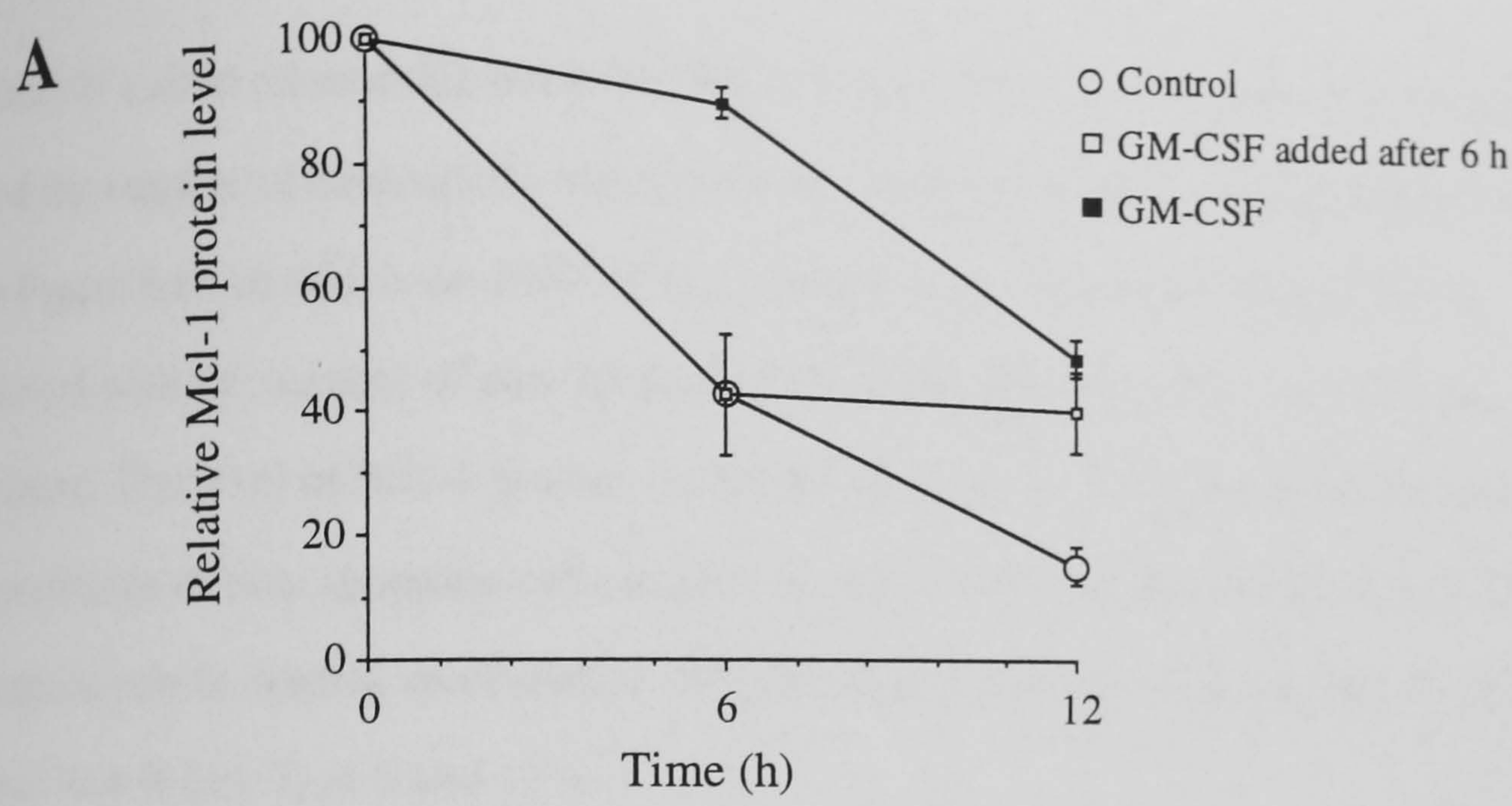
Relative levels of Mcl-1 protein were calculated by densitometric analysis of western blots prepared as described in Figure 6.7, following 12 h culture in the conditions indicated. Significant difference from control cultured neutrophils is indicated by † ($p \leq 0.05$), or * ($p \leq 0.01$).

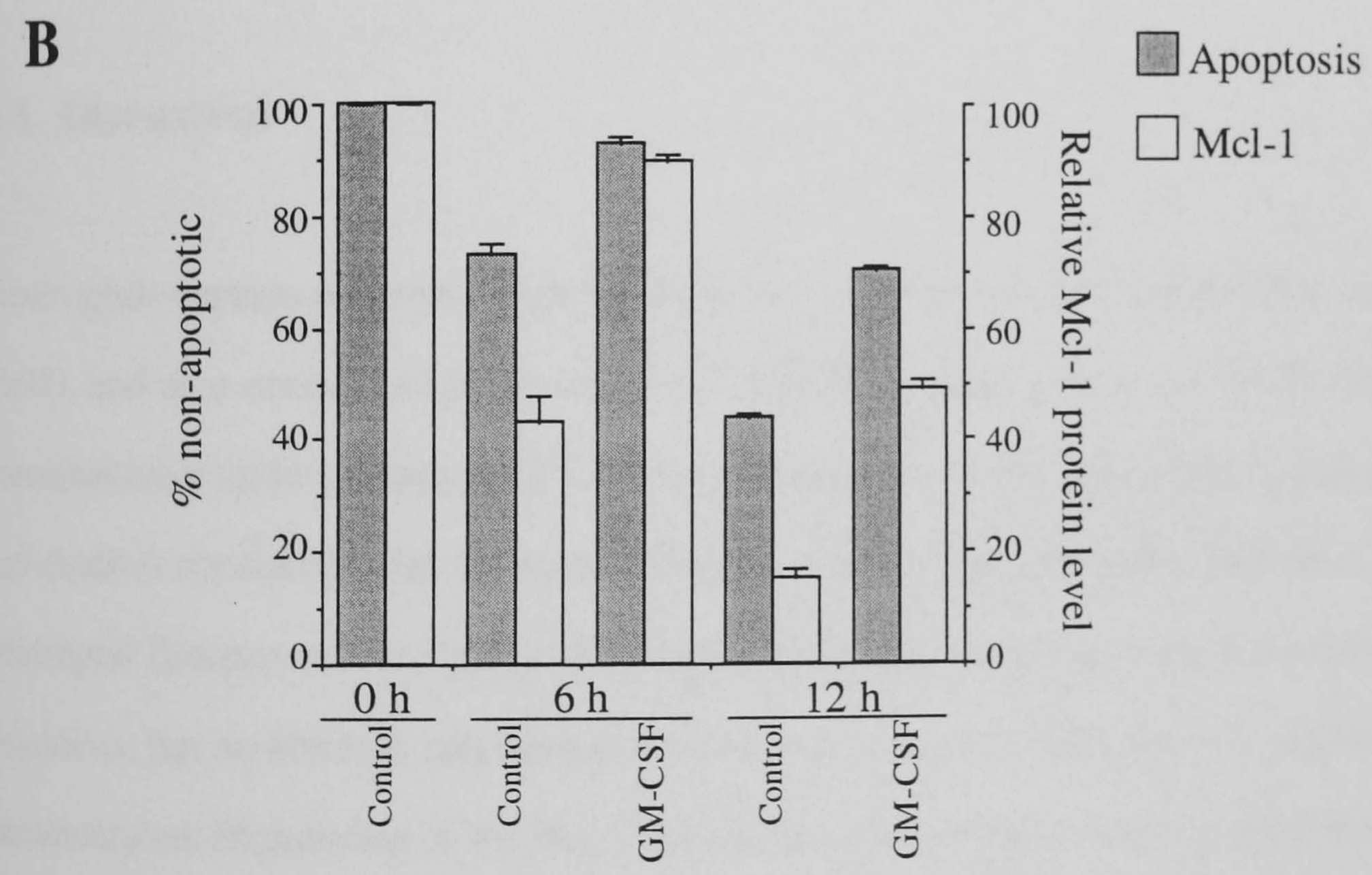
after 6 h incubations and Figure 6.7b after 12 h incubations. Table 6.1 shows the results of densitometric analysis of blots prepared after 12 h cultures. Compared to control incubations, GM-CSF treatment gave 2.87 (±0.69) fold higher Mcl-1 protein. Butyrate 1.46 (±0.23) fold, IL-1β 2.41 (±1.29) fold and LPS 2.70 (±0.94) fold. These values were significantly different from control levels of Mcl-1 protein assessed by the paired students t-test, with GM-CSF significant at $p \leq 0.01$, while Butyrate, IL-1β and LPS were significant to $p \leq 0.05$.

6.3.5 Mcl-1 expression in cells aged for 6 hours before the addition of GM-CSF

Figure 6.8a shows the means of two separate experiments, in which Mcl-1 protein levels were determined in freshly isolated cells, cells cultured for 6 and 12 h with and without GM-CSF, and cells cultured for 6 h before the addition of GM-CSF. Control incubations showed a steady decline in levels of Mcl-1 protein to 42.9 % (±9.8) and 15.4 % (±2.9) at 6 and 12 h, respectively of the time zero value. GM-CSF greatly decreased the loss of Mcl-1 protein levels with 89.3 % (±2.1) and 49.1% (±3.3) remaining after 6 and 12 h in culture. The addition of GM-CSF to 6 h aged cells also greatly diminished the loss of Mcl-1, with Mcl-1 protein levels only falling a further 1.5 % to 40.4 % (±6.5) after a total of 12 h culture. This addition of GM-CSF also decreased the number of cells becoming apoptotic for the final 6 h of culture. Without GM-CSF, apoptosis increased from 26.7 % (±4.1) at 6 h to 56.2 % (±1.9) after 12 h. The addition of GM-CSF for the final 6 h resulted in apoptosis increasing to only 30.5 % (±7.2) (results not shown).

Figure 6.8 Maintenance of Mcl-1 expression by GM-CSF and correlation with delayed apoptosis





A, Mcl-1 expression was determined by western blotting and densitometric analysis as described in Materials and Methods after 0 h, 6 h and 12 h in culture in medium alone(open circles), with GM-CSF (closed boxes), or with GM-CSF added to control incubations after 6 h (open boxes). Results shown are means (±SD) of two separate experiments. **B**, shows the relationship between Mcl-1 expression (open bars), and non-apoptotic neutrophils (shaded bars), from two separate experiments. Apoptosis was assessed by morphology as described in Materials and Methods.

6.3.6 The proportion of non-apoptotic neutrophils in a population correlates with the abundance of Mcl-1 protein.

There is a clear relationship between Mcl-1 protein levels in a population of neutrophils and the number of neutrophils which have not become apoptotic. This is demonstrated in Figure 6.8b in which the level of Mcl-1 expression (shown in Figure 6.8a) is plotted with the number of non-apoptotic neutrophils present after 6 and 12 h in culture. The level of Mcl-1 protein shown are the same as in Figure 6.8a, with the percentage of non-apoptotic cells at 73.3 % (±4.5) and 43.8 % (±1.9) at 6 and 12 h, respectively in control incubations, with GM-CSF treatment showing 92.8 % (±2.5) and 70.4 % (±1.7) at 6 and 12 h.

6.4 Discussion

Neutrophils express relatively high levels of the proapoptotic protein Bax (Ohta *et al.* 1995), and also strongly express caspase-3 (CPP-32) (Krajewska *et al.* 1997) and constituitively undergo apoptosis. The expression of these two molecules involved in cell death is consistent with the short lifespan of the mature neutrophil. However, the neutrophil lifespan can be significantly increased during exposure to agents such as cytokines, but no obvious mechanism for this rescue from death has previously been demonstrated. Expression of the Bcl-2 family member proteins in neutrophils have not been conclusively defined, due in part to the fact that members of this family are increasing as more are discovered, but mainly due to the different methodologies used by researchers to detect these proteins. Bcl-2 has been reported to be expressed in neutrophils and needed for rescue from apoptosis (Keihntopf *et al*, 1995; Keihntopf *et al.* 1996; Hseih *et al*, 1997). Other reports suggest no detectable Bcl-2 in mature neutrophils (Afford *et al*, 1992; Hannah *et al*, 1994; Iwai *et al.* 1994; Lagasse & Weissmann, 1994; Hannah *et al*, 1995; Krajewski *et al*, 1995; Liles & Klebanoff,

1995; Wei *et al*, 1996; Watson *et al*, 1997c). In this paper we show by western blot that neutrophils essentially express no Bcl-2, even if rescued from apoptosis by a variety of agents.

The conflicting reports on Bcl-2 expression in neutrophils may be explained by the methodologies used. Flow cytometric or immunohistochemical analysis of Bcl-2 expression in neutrophils may give false results due to non-specific binding of the antibodies to other neutrophilic proteins. Indeed, in my western blots using a monoclonal antibody raised against residues 44-52 of Bcl-2 we found strong binding of the primary antibody in neutrophil extracts to proteins of approximately 40 kDa and 80 kDa (Figure 6.2), which were not seen in PBMC or KLC 22 cells. These proteins have not been identified but will clearly give false positives in FACS assays or following immunohistochemical staining. Such proteins may represent non-specific binding of the antibodies or may indeed represent novel Bcl-2 family members expressed in neutrophils. Further work needs to be carried out to identify these proteins.

Bcl-X is also shown in this chapter to be absent from mature neutrophils, in agreement with work of other groups (Krajewski *et al*, 1994; Ohta *et al*, 1995; Liles & Klebanoff, 1995; Sanz *et al*, 1997), although Krajewski *et al* (1994) do find a small minority of granulocytes which have slight Bcl-X_L immunoreactivity. We have also shown that protection from apoptosis does not result in induction of Bcl-X to levels detectable by western blot.

Previous work in our laboratory identified a partial cDNA of Mcl-1 in a cDNA library enriched in GM-CSF regulated clones (Quayle & Edwards, unpublished). This strongly suggested Mcl-1 protein would be expressed in neutrophils, and that it could be upregulated by GM-CSF. However the work of Ohta *et al* (1995) showed no expression of the Mcl-1 protein in post-nuclear extracts from mature neutrophils. I

repeated their work and, following their protocol, also found no expression of Mcl-1 in neutrophil post-nuclear extracts. However, I found abundant Mcl-1 in extracts solubilized directly into boiling sample buffer, indicating Mcl-1 was lost during the post-nuclear extract preparation. This is, to my knowledge, the first demonstration of the presence of any anti-apoptotic Bcl-2 family protein in mature human neutrophils by western blotting, where the reactivity of an antibody to its specific ligand is confirmed by the molecular mass of the ligand.

An alternative method to separate nuclei from the rest of the neutrophil structures and cytoplasm that minimises protein degradation showed that Mcl-1 was expressed primarily in the neutrophil nuclear fraction, but was also expressed in the non-nuclear fraction at lower levels. Even with this alternative method of crude cell fractionation we saw considerable degradation of Mcl-1 protein (Figure 6.5 neutrophil whole cell and lysate samples). This degradation was not seen with PBMC lysates (not shown), and may be explained by the release of the many proteases found in neutrophil granules rapidly degrading the labile Mcl-1 protein.

Therefore, Mcl-1 appears to be predominately located on neutrophil nuclear membranes, whose fraction may also contain some endoplasmic reticulum, as this is contiguous and closely associated with the nuclear membrane (Clark *et al*, 1980). The Mcl-1 found in the non-nuclear fraction is likely located on endoplasmic reticulum not removed with the nuclear fraction, and on the few mitochondria in neutrophils. Indeed, Mcl-1 is localised on mitochondrial membranes, nuclear membranes and also on light membranes (containing golgi and endoplasmic reticulum) in ML-1 cells (Yang *et al*, 1995). It seems likely then that in neutrophils, Mcl-1 is only sparsely found in the non-nuclear fraction due to the small numbers of mitochondria.

Another group has also reported a lack of Mcl-1 protein in neutrophils by immunohistochemical staining (Krajewski *et al*, 1995). However, this discrepancy

with our western blot results may be explained by the origin of the neutrophils used. Krajewski *et al* (1995) used bone marrow neutrophils and report moderate Mcl-1 protein levels in metamyelocytes, occasional Mcl-1 expression in band cells, and rare Mcl-1 expression in mature bone marrow neutrophils. In my experiments I have used peripheral blood neutrophils, which have migrated from the bone marrow. It is possible that upon release from bone marrow Mcl-1 expression is induced in neutrophils. Indeed, work by the same group shows no Bax detected in mature bone marrow granulocytes (Krajewski *et al*, 1994), although Bax is expressed in peripheral blood neutrophils (Ohta *et al*, 1994).

The expression of Bax in neutrophils is confirmed in this Chapter, with crude fractionation indicating that Bax is not found in the nucleus of freshly isolated neutrophils. We also show that the level of Bax protein remains essentially constant in neutrophils treated to delay apoptosis with a variety of agents. These results suggest Bax is not co-localised with Mcl-1 in freshly isolated neutrophils, and may be exclusively found on the few mitochondria in the neutrophil cytosol.

The presence of both Bax and Mcl-1 in mature neutrophils suggests a possible explanation for the constitutive apoptosis of mature neutrophils and the ability to extend neutrophil lifespan. As Mcl-1 is a labile protein its cellular levels would be expected to fall quickly if expression of Mcl-1 was stopped or diminished, allowing the endogenously expressed Bax to dominate and lead to apoptosis. Induction of Mcl-1 expression would maintain the levels of Mcl-1 protein in the neutrophil, thereby preventing Bax exerting its apoptotic effects. This simple model requires the constant production of Mcl-1 protein to replace previously synthesized Mcl-1 that would be expected to be constantly and rapidly degraded. Further, this system would allow for neutrophil death and survival to be quite tightly regulated and rapidly modulated, as the lability of the Mcl-1 protein would allow Bax levels to predominate following suspension of Mcl-1 expression.

This model for the control of entry into apoptosis in mature neutrophils is strengthened by our findings that Mcl-1 levels are significantly higher in cultured neutrophils that have been protected from entry into apoptosis by a variety of agents (Figure 6.7 and Table 6.1). The level of Mcl-1 expression can also be maintained by the addition of GM-CSF to neutrophils that have already been aged for 6 h without GM-CSF (Figure 6.8) whilst also protecting these aged cells from further entry into apoptosis. This suggests that induction of Mcl-1 expression before the endogenous level of Mcl-1 has fallen below the level necessary to counteract Bax, is sufficient to protect the cells from entry into apoptosis. It is also possible that other anti-apoptotic Bcl-2 family members that have not been investigated to date (i.e. Bcl-w, A1, unknown Bcl-2 family proteins) are expressed in neutrophils which may also counter the effects of Bax.

The level of Mcl-1 expression in differently treated neutrophil populations also correlates well with the proportion of the population that have not become apoptotic. This is evident by comparing the relative levels of Mcl-1 in cultured cells (Table 6.1), with the percentage of cells which are apoptotic following each treatment for 12 h (Figure 6.1b). The more effective the treatment is at delaying apoptosis, the higher the level of Mcl-1 protein found in the aged cells. This correlation is further shown in Figure 6.8b, where both Mcl-1 protein levels and apoptosis were determined in parallel from the same cultures after both 6 and 12 h of culture. It is clear that Mcl-1 protein levels decline more rapidly in cells not protected from apoptosis. These results also show that the decline in Mcl-1 protein levels from each neutrophil population is more rapid than the rate of entry into apoptosis, as assessed by morphology. Therefore, it is proposed that Mcl-1 protein loss from neutrophils leads to the initiation of the apoptotic program. This theory should be further tested in future studies by assessing the abundance of Mcl-1 protein in individual neutrophils whilst simultaneously measuring apoptosis in these cells. Specifically disrupting Mcl-1

expression with antisense oligonucleotides, and determining the effect on cell survival, will allow the role of Mcl-1 in neutrophil apoptosis to be confirmed.

Recent work has shown that Bcl-2 and Bcl-X_L are able to prevent caspase activation (and therefore the initiation of apoptosis) by preventing the release of cytochrome c from mitochondria (Yang *et al*, 1997; Kluck *et al*, 1997). The localisation of Bax dimers to mitochondria has also been shown to be essential for Bax to exert its cytotoxic effects in both yeast and mammalian cells (Zha *et al*, 1996). In the nematode *Caenorhabditis elegans* Ced-9 (a Bcl-2 homologue) binds to Ced-4 (of which functional homologues exist in mammals, Lui *et al*, 1996, Zou *et al*, 1997) pulling Ced-4 out of the cytosol. This prevents Ced-4 activating Ced-3 (a caspase) thereby preventing apoptosis. These events have not been demonstrated as yet in mammalian cells, but Bcl-X_L has been shown to pull Ced-4 out of the cytosol (Wu *et al*, 1997). Overexpression of pro-apoptotic Bcl-2 family members prevents Ced-4 binding by Ced-9 (or Bcl-X_L) (Reed 1997), which would allow Ced-4 to activate the caspases found in the cytosol.

Similar processes may control entry into neutrophil apoptosis, with Bax located on the few mitochondria present in the neutrophil cytosol, and Mcl-1 located on other membranes, and also possibly on the mitochondria. The decline in Mcl-1 protein levels may then allow Bax dimers to release cytochrome c from neutrophil mitochondria, and also result in release of an as yet unidentified Ced-4 homologue from Mcl-1 on membranes, and into the cytosol, allowing activation of the caspases.

In this final Chapter I have shown that induction of Mcl-1 expression is associated with delayed apoptosis, by all the anti-apoptotic agents tested. This provides the first evidence for Mcl-1 expression in the neutrophil, and the first possible explanation for the observation that agents which increase neutrophil gene expression invariably delay neutrophil apoptosis.

CHAPTER 7 GENERAL DISCUSSION AND CONCLUSIONS

This thesis has investigated the mechanisms by which diverse agents delay neutrophil apoptosis. In particular, the role of active gene expression and new protein synthesis have been investigated. The main areas of investigation were:

- Relationship between active gene expression and apoptosis;
- Maintenance of surface CD16 expression during delayed apoptosis;
- Determination of effects of bacterial superantigens on neutrophil apoptosis;
- Histone acetylation during delayed apoptosis;
- Bcl-2 family expression and role in neutrophil apoptosis.

The major findings of the work presented in this thesis can be summarised as follows:

- Not all protein synthesis activators delay neutrophil apoptosis: DNA demethylating agents which increase neutrophil gene expression do not delay neutrophil apoptosis. Protein synthesis stimulated by sustained histone hyperacetylation is sufficient to delay neutrophil apoptosis. Therefore the key gene(s) required to delay neutrophil apoptosis cannot be activated by DNA demethylation, whereas histone hyperacetylation is able to induce expression of these gene(s).
- CD16 surface expression falls in apoptotic neutrophils due to shedding of the surface receptor, without replenishment by mobilisation of internal stores. Maintenance of surface CD16 by apoptosis delaying agents is achieved by maintaining the ability to mobilise internal stores in response to loss of surface CD16.
- Superantigens are able to modulate neutrophil functions by a direct, but unknown, interaction with neutrophils, but have no direct effect on the rate of neutrophil apoptosis. However, the activation of cytokine production from

other immune cells by superantigens is sufficient to delay neutrophil apoptosis, even with less than 3 % contamination by non-neutrophilic white blood cells.

- Delayed apoptosis mediated by enhanced gene expression due to histone hyperacetylation requires prolonged, not transient, hyperacetylation of histones. GM-CSF accelerates turnover of acetyl groups on histones.
- Bcl-2, Bcl-X_L and Bcl-X_S proteins are not expressed in peripheral blood neutrophils, nor in neutrophils treated to delay apoptosis. Bax is constitutively expressed in peripheral blood neutrophils, and protein levels are not modulated by manipulation of neutrophil apoptosis.
- Mcl-1 protein is expressed in peripheral blood neutrophils. Mcl-1 protein level declines during neutrophil culture, correlating with entry into constitutive apoptosis. Agents which delay neutrophil apoptosis maintain Mcl-1 protein levels, with maintenance of Mcl-1 protein expression correlating with delayed apoptosis.
- Bax protein is located on non-nuclear membranes, whilst Mcl-1 is primarily located on nuclear membranes, with remaining Mcl-1 located on non-nuclear membranes.

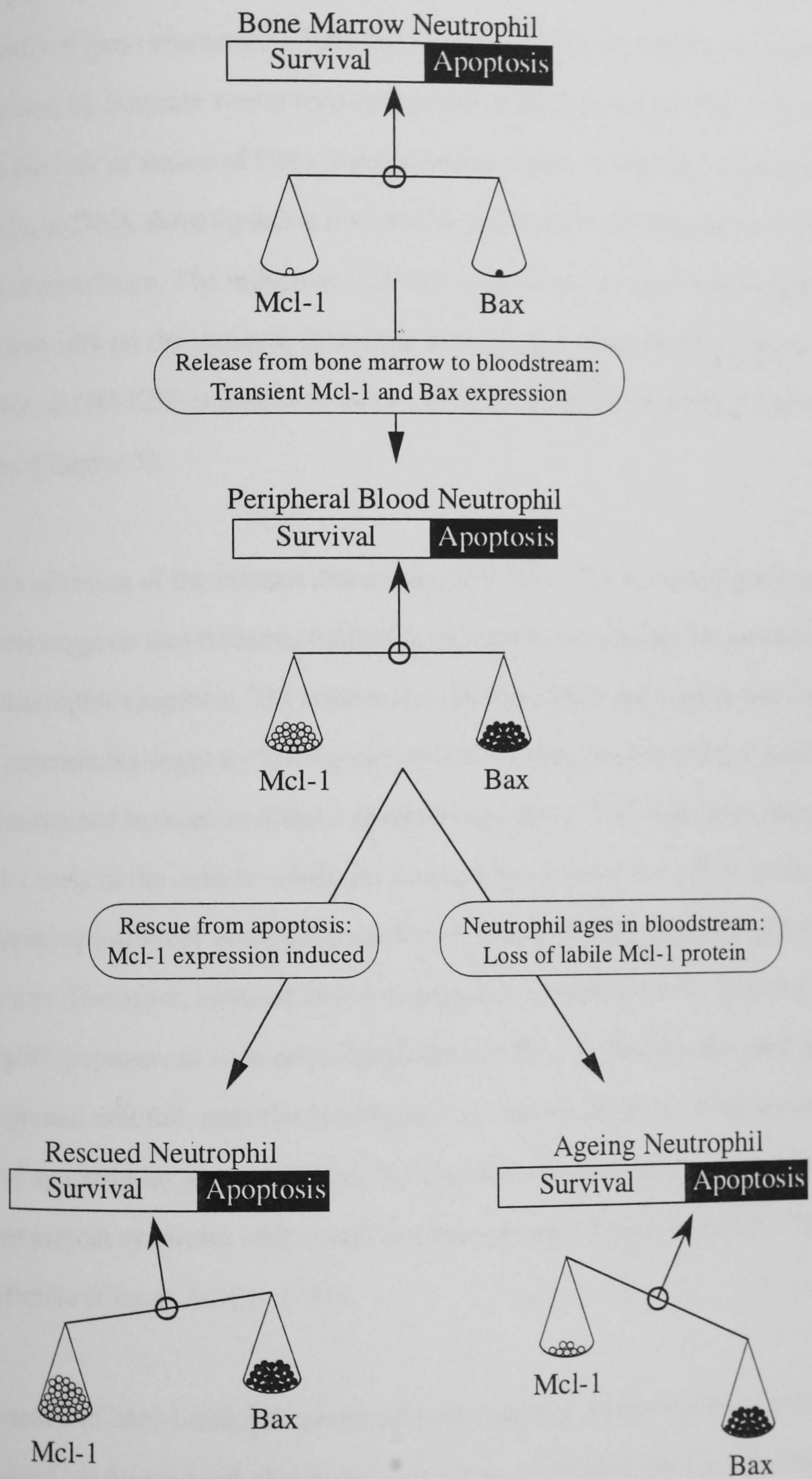
The work presented in this thesis demonstrates the importance of maintained gene expression in neutrophil survival. Evidence is also presented which indicates the involvement of specific gene(s) in delaying neutrophil apoptosis, as enhanced gene expression by DNA demethylating agents is not sufficient to delay apoptosis, though all other agents tested which delay neutrophil apoptosis are associated with increased protein synthesis. The novel finding that Mcl-1 is expressed in neutrophils, and that the expression of this protein correlates with delayed apoptosis suggests that the expression of this protein is involved in controlling entry into neutrophil apoptosis. The correlation between enhanced gene expression and delayed apoptosis may

therefore be explained by induction of Mcl-1 (and perhaps other unidentified gene products), which act to prevent entry into apoptosis.

The lack of Mcl-1 and Bax expression in the majority of mature bone marrow neutrophils (Krajewski *et al*, 1995; Krajewski *et al*, 1994), and the expression of both of these proteins in peripheral blood neutrophils suggests that both genes may be expressed upon release from the bone marrow. The Mcl-1 protein has PEST sequences accompanied by pairs of arginine residues, which are associated with rapid protein turnover (Kozopas *et al*, 1993), with Mcl-1 protein half-life reported to be less than 3 h (Yang *et al*, 1995). The Bax protein does not have these signals for rapid degradation, so without further expression of either protein Bax would soon predominate, as the more labile Mcl-1 is degraded, and thereby initiate apoptosis. Rescue from apoptosis would be achieved by inducing Mcl-1 gene expression, replenishing Mcl-1 protein levels, and preventing initiation of apoptosis (Figure 7.1). In this way both constitutive apoptosis, and rescue from apoptosis in the neutrophil could be controlled by Mcl-1 expression. This should be addressed in further studies, by specifically blocking Mcl-1 protein synthesis with antisense oligonucleotide technology.

This model for the control of neutrophil apoptosis requires transient expression of both Bax and Mcl-1 upon release from the bone marrow, with expression of both these genes quickly repressed. Bax expression seems to not be further modulated by either delaying or accelerating apoptosis (Chapter 6), suggesting Bax expression is no longer possible in the neutrophil, or that expression of Bax proceeds at a constant level which maintains a steady level of Bax protein. Mcl-1 gene expression appears to be repressed in peripheral blood neutrophils (Chapter 6), but expression can be induced by agents which delay apoptosis.

Figure 7.1 Model for control of neutrophil apoptosis by Mcl-1 and Bax



The ability of sodium butyrate to maintain Mcl-1 protein levels in cultured neutrophils suggests that Mcl-1 expression may be repressed by recruitment of histone deacetylases to the Mcl-1 gene promoter, as this has been shown to be a mechanism of gene repression (Pazin &Kadonaga, 1997). Inhibition of histone deacetylases by butyrate would then release this gene from repression. This may also explain the lack of action of DNA demethylating agents in delaying neutrophil apoptosis, as DNA demethylation may not be sufficient to lift repression of genes by histone deacetylases. The induction of Mcl-1 expression by other apoptosis delaying agents may rely on the removal of histone deacetylases from the Mcl-1 gene promoter, as GM-CSF is shown to cause increased turnover of acetyl groups on histones (Chapter 5).

The lack of action of the histone deacetylase inhibitor TSA in delaying neutrophil apoptosis suggests that transient inhibition of histone deacetylases is not sufficient to delay neutrophil apoptosis. The transient inhibition of histone deacetylases by TSA in my experiments suggests that the removal of repression of the Mcl-1 gene needs to be maintained in order to delay subsequent apoptosis. The short term expression of Mcl-1 early in the culture would not be expected to have any effect on the subsequent apoptosis of neutrophils, as Mcl-1 would be degraded soon after production. Therefore, constant Mcl-1 expression is predicted to be required to delay neutrophil apoptosis, as soon after repression of Mcl-1 expression the total levels of Mcl-1 protein will fall, until the neutrophil is no longer protected from apoptosis. Delayed apoptosis by glucocorticoids has also been recently demonstrated to require constant protein synthesis, with transient enhancement of gene expression being far less effective (Cox & Austin, 1997).

The location of Mcl-1 and Bax proteins in the neutrophil have not been precisely determined, so future work should be undertaken to confirm the localisation of these proteins to specific intracellular membranes. The expression of Mcl-1 in both non-

nuclear and nuclear membranes, whilst Bax is expressed solely on non-nuclear membranes suggests that Mcl-1 may act by both co-localising with Bax, whilst also acting on other membranes where Bax is not located. Therefore, Mcl-1 may act to prevent Bax mediated apoptosis, and also prevent apoptosis induction by a separate mechanism. This may involve Mcl-1 protein holding an as yet unidentified neutrophilic Ced-4 homologue away from the cytosol, preventing the putative Ced-4 homologue mediated activation of cytosolic caspases.

Mcl-1 has been shown to bind to FADD, via the N-terminal region of Mcl-1 which is not found in other Bcl-2 family proteins, and block Fas induced apoptosis (Wei *et al*, 1996). This presents the intriguing possibility that, in neutrophils, Mcl-1 may bind to FADD (the adaptor protein that allows Fas and TNF receptors to activate the caspases as shown in Figure 1.7). Fas induces apoptosis rapidly in neutrophils (Liles *et al*, 1996), with the co-expression of both the Fas receptor and ligand on neutrophils suggested to be an important, but not exclusive, mechanism involved in the control of spontaneous neutrophil apoptosis (Liles & Klebanoff, 1995; Liles *et al*, 1996). Addition of Fas neutralising agents to neutrophil cultures retards the spontaneous apoptosis of neutrophils (Liles *et al*, 1996; Hsieh *et al*, 1997). Therefore, Mcl-1 expression may both prevent Bax mediated apoptosis, and also prevent Fas mediated apoptosis by preventing FADD joining the Fas and TNF receptors on the cytoplasmic membrane. As Mcl-1 levels fall, FADD would be released from the Mcl-1 protein, allowing FADD to join the Fas and TNF receptors, and allow activation of the caspases.

The induction of apoptosis via TNF receptor signalling to the caspases can be prevented in neutrophils by glucocorticoids (Kato *et al*, 1995), LPS (Hachiya *et al*, 1995) and platelet activating factor priming (Murray *et al*, 1997). Similarly, G-CSF, GM-CSF, IFN-γ, dexamethasone (Liles *et al*, 1996) and LPS treatments or CD11b cross-linking (Watson *et al*, 1997a) will inhibit Fas induced apoptosis in the

neutrophil. This modulation of the action of Fas and TNF may be mediated by increased Mcl-1 expression, with maintained levels of Mcl-1 protein able to hold FADD away from these receptors.

Other factors are believed to regulate neutrophil apoptosis, notably oxidative stress (section 1.6.2). Neutrophils cultured in hypoxic conditions show a markedly delayed entry into apoptosis (Hannah *et al*, 1995). Oxidative stress in the neutrophil is prevented, in part, by the tripeptide GSH, which converts H_2O_2 into water. Depletion of GSH results in accelerated neutrophil apoptosis, so oxidative stress within the neutrophil can induce apoptosis, via a mechanism which requires protein tyrosine phosphorylation (Watson *et al*, 1996d). This is unlikely to explain the constitutive apoptosis of neutrophils in the bloodstream, as they should not become exposed to any oxidative stress. Inflammatory neutrophils do experience oxidative stress, as these neutrophils produce ROS, so this stress may contribute to apoptosis, as demonstrated by the observation that following *E. coli* phagocytosis, neutrophils rapidly become apoptotic, but this is inhibited by artificially increasing GSH levels (Watson *et al*, 1996a).

Induction of apoptosis by Fas-mediated caspase activation is inhibited by LPS, or CD11b cross-linking, which is associated with a rise in intracellular GSH (Watson *et al,* 1997a). Preventing the rise in GSH abolishes the protection from Fas induced apoptosis conferred by LPS, with artificial restoration of GSH levels in LPS treated neutrophils restoring much of this protection. The requirement of the rise in GSH levels, for protection from Fas induced apoptosis, following CD11b cross-linking was not addressed in this work. As GSH is able to protect proteins from oxidative damage, it is possible that GSH mediated protection from Fas induced apoptosis is a result of maintaining the function of proteins that would otherwise be oxidatively damaged. This idea is strengthened by the fact that augmented GSH levels have no effect on spontaneous neutrophil apoptosis (Watson *et al*, 1996b; Watson *et al*,

1997a), where oxidative stress is not experienced. The rise in GSH following LPS treatment or CD11b cross-linking is possibly a physiological response of neutrophils to recruitment to an inflamed site, in order to protect the neutrophil from the ROS it will produce. LPS is able to trigger this ROS production, so if GSH is artificially depleted in LPS treated neutrophils, the neutrophil is likely to experience oxidative damage. The lack of protection from Fas induced apoptosis may merely be due to the loss of function of protective proteins due to oxidative damage.

These authors also show that the protection from Fas induced apoptosis following LPS treatment or CD11b cross-linking is lost within 4 h of removal of LPS or CD11b cross-linking antibodies (Watson *et al*, 1997a). This shows the protection is short lived, so may be mediated by a molecule with rapid turnover. Therefore, it is possible that Mcl-1 may be the molecule that allows protection from Fas induced apoptosis, as Mcl-1 is able to bind to FADD, and prevent Fas induced apoptosis (Wei *et al*, 1996), and is rapidly degraded. Furthermore, the oxidative stress that occurs when GSH is depleted may prevent Mcl-1 binding to FADD, thereby releasing FADD, and allowing Fas to induce apoptosis. This theory should be addressed in future studies.

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