Investigations into the Involvement of Calcium Ions and Glutathione in Apoptosis

Declaration

The research presented in this thesis is my own work, utilizing information collected in my own laboratory. I have not drawn on any other degree work or any other degree

A thesis submitted for the degree of

Doctor of Philosophy

Australian National University

Joanne Beaver

January 1996
Declaration

The research presented in this thesis is my own work unless otherwise stated. This work was carried out in the Division of Immunology and Cell Biology, John Curtin School of Medical Research, Australian National University. The material presented in this thesis has not been submitted for any other degree.

Joanne Beaver
January, 1996
Acknowledgments

I could not have completed this task without the support and encouragement of my treasured husband, Michael Bird, and two wonderful sons, Alex and Sam. I thank them wholeheartedly for their forbearance and for their never ending love. I have eternal gratitude to our golden retrievers, Buttercup and Sunlight, who constantly offered me cuddles and smiling faces and so contributed greatly to the maintenance of my sanity!

Multitudes of thanks to my “Great and Illustrious Supervisor”, Dr Paul Waring, who provided me with excellent intellectual support and friendship. He welcomed me into his laboratory and allowed me to find my way with just the right amount of interference! Allan Sjaarda deserves a huge vote of thanks, he knew where chemicals and instruments lived and was always quick with an offer of help or a joke.

My thanks to Geoff Osborne and Sabine Grueninger for their expert assistance with the flow cytometers and their great sense of humour. I spent so many hours in the FACS lab, and they both always made me feel part of the “FACS-Users Family”! Thanks to Ms Cathy Gillespie and her diligent team who fixed and prepared my samples for the electron microscope, and taught me, and then entrusted me with the microscope so I could take the electron micrographs. Thank you to Dr Gary Buffington for his advice about the HPLC assay for GSH, and for the use of his instrument and column. Thank you also to the lovely people in Photography for their production of the photographic plates in this thesis.

There were so many other individuals who contributed to my success including Dr Arno Müllbacher, Dr Joe Altin, Ms Jan Bateman, Ms Cathy Woodhams and Mrs Esmee Weil.

I remember so much laughter during my PhD preparation, so much in the laboratory and with my friends, especially with Kirsten Flynn and Tahira Khan, fellow doctoral students - two of the world’s treasures.
Thank you to Professor Allan Cripps, Dean of the Faculty of Applied Science at the University of Canberra. When he offered me a full-time lectureship in Biochemistry in January 1995, Professor Cripps demonstrated his faith in my tenacity and determination to finish writing my thesis and submit in good time whilst teaching an unfamiliar program.

Finally, many thanks to my examiners, who have taken time to read and comment on my thesis.
Abstract

Apoptosis is a form of cell death in which the cell plays an active role. It is common in both physiological and pathological situations and has been observed in organisms throughout the evolutionary spectrum, both plant and animal. Apoptotic cells are characterised by several morphological and biochemical features including condensed chromatin, oligonucleosomally fragmented DNA, increased transglutaminase activity, and the division of the cell into many smaller fragments called apoptotic bodies.

In this project, the role of calcium ions and glutathione in apoptosis was investigated. The cell type used was primarily thymocytes. P815 cells were used for some experiments in which an actively cycling cell with a low rate of spontaneous apoptosis was required. Apoptosis was induced mainly by three disparate agents: dexamethasone, a synthetic glucocorticosteroid analog; gliotoxin, a toxic fungal metabolite; and thapsigargin, an inhibitor of the endoplasmic reticulum Ca\(^{2+}\)-ATPase. Other areas covered in this project included an investigation of other effects of thapsigargin within the cell, and a brief study of changes in cell surface sugar expression during apoptosis.

The role of calcium ions in the apoptotic process was, for some time, considered to be an important universal occurrence. Calcium was proposed to act by direct activation of a nuclear Ca\(^{2+}\)-dependent endonuclease. Evidence is presented here for the role of calcium ions in apoptosis being more complex than previously thought. Intracellular calcium ion concentrations were measured with the fluorescent calcium-chelating dye fluo-3. Concentrations of gliotoxin and dexamethasone, both of which induced apoptosis over 6 hours in mouse thymocytes, were found to have no effect on the intracellular calcium ion concentration ([Ca\(^{2+}\)]). Thapsigargin treatment, on the other hand, stimulated a rapid and prolonged increase in the [Ca\(^{2+}\)], but induced apoptosis over a much longer time (18 hours).
This longer time scale of induction indicated thapsigargin did not induce apoptosis by direct activation of a nuclear endonuclease. However, thapsigargin did severely disrupt the cell's calcium ion homeostasis. This was indicated by the appearance of swollen mitochondria with disrupted internal structure similar to that observed in mitochondrial phase transition. Thapsigargin-treated cells contained mitochondria with reduced membrane potential (measured with Rhodamine 123 staining) and had a reduced intracellular ATP concentration. It is proposed here that apoptosis induced by thapsigargin occurs as a result of a loss of mitochondrial function caused by calcium-induced phase transition.

Glutathione (GSH) is an important intracellular antioxidant. Many other researchers have implicated GSH in the inhibition of prooxidant-induced apoptosis. In this work, evidence is presented that GSH may have a more general role in the onset of apoptosis. The intracellular concentration of GSH ([GSH]) was found to decrease before the onset of DNA fragmentation. This was the case however apoptosis was induced. It was also found that GSH treatment of the cells could inhibit dexamethasone-induced apoptosis.

Oxidised glutathione (GSGG) treatment was found to provoke apoptosis. GSGG is known to react with proteins to form mixed disulphides (glutathiolated proteins), and this can alter protein activity. An increase in the abundance of six glutathiolated proteins was found as a result of thapsigargin-induced apoptosis in P815 cells.

These results led to the formation of the hypothesis that GSH/GSGG metabolism has a role in the onset of apoptosis, possibly through the formation of glutathiolated proteins with altered activity. These proteins may have an active role in apoptosis or a role in the propagation of the apoptotic signal.

Other aspects of thapsigargin’s activity were investigated, including its poor activity as a protein synthesis inhibitor and its ability to block the cell cycle before S phase.
The expression of N-acetylglucosamine (glc-NAc) on the outer surface of the plasma membrane makes up the final part of this project. Glc-NAc expression was found to decrease slightly early in the apoptotic process and then to increase to a level highly elevated above normal in cells well progressed through apoptosis.
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<table>
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<th>Description</th>
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<tr>
<td>[ATP]</td>
<td>concentration of ATP</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid acetoxy-methyl ester; intracellular calcium ion chelator</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSO</td>
<td>L-Buthionine-(SR)-sulfoximine</td>
</tr>
<tr>
<td>[Ca$^{2+}$]</td>
<td>calcium concentration</td>
</tr>
<tr>
<td>[Ca$^{2+}$]_i</td>
<td>cytosolic calcium ion concentration</td>
</tr>
<tr>
<td>[Ca$^{2+}$]_m</td>
<td>mitochondrial matrix calcium ion concentration</td>
</tr>
<tr>
<td>CPA</td>
<td>cyclopiazonic acid</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic lymphocytes</td>
</tr>
<tr>
<td>Dex</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>DiOC$_6$(3)</td>
<td>3,3′-dihexyloxacarbocyanine iodide; a lipophilic cationic fluorescent dye for the measurement of mitochondrial membrane potential</td>
</tr>
<tr>
<td>“early”</td>
<td>cells appearing in Region2 of a plot of EB fluorescence versus FSC after staining with ethidium bromide; these are the cells that have most recently entered the apoptotic process</td>
</tr>
<tr>
<td>EB</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMEM</td>
<td>modified Eagle’s minimum essential medium with Earle’s salts; without methionine or cysteine</td>
</tr>
<tr>
<td>F</td>
<td>fluorescence of sample in bulk cell assay of Ca$^{2+}$ using fluo-3</td>
</tr>
<tr>
<td>F15</td>
<td>Eagle’s minimum essential medium</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>Fl-1</td>
<td>fluorescence collected in the fluorescence channel number 1 for detecting fluorescence from fluo-3 bound to Ca$^{2+}$ and from WGA-FITC bound to glc-NAc on the cell plasma membrane</td>
</tr>
</tbody>
</table>
Fl-2 fluorescence collected in the fluorescence channel number 2; used for detecting fluorescence from ethidium bromide stained cells and from propidium iodide bound to DNA.

Fl-32 fluorescence collected in the fluorescence channel number 3 from the UV laser; used for the collection of fluorescence associated with OPT-protein binding.

Fl-4 fluorescence collected in the fluorescence channel number 4; used for the collection of fluorescence associated with OPT-glutathione binding.

Fluo-3-AM tetramethoxyester of 9-[4-bis-(carboxymethyl)amino-3-[2-(2-bis(carboxymethyl)amino-5-methylphenoxy)ethoxy]-phenyl-2,7-dichloro-6-hydroxy-3H-xanthin-3-one-acetoxy methyl ester.

Fmax maximum fluorescence of sample in bulk cell assay of Ca^{2+} using fluo-3, obtained after the addition of digitonin to permeabilise the cells' plasma membrane.

Fmin minimum fluorescence of sample in bulk cell assay of Ca^{2+} using fluo-3, defined as the background fluorescence before the addition of agonist.

FSC forward scatter.

G_0/G_1 phases of the cell cycle during which the cell is resting in a non-growing, non-dividing state (G_0 phase) and when the cell is preparing for DNA synthesis (G_1 phase).

GSH reduced glutathione.

[GSH]_i intracellular reduced glutathione concentration.

GSSG oxidised glutathione.

[GSSG]_i intracellular oxidised glutathione concentration.

GT gliotoxin.

Ho342 Hoechst 33342 (bis-benzimidazole), fluorescent DNA intercalating dye.

JC-1 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide; a lipophilic cationic fluorescent dye for the measurement of mitochondrial membrane potential.

"late" cells appearing in Region3 of a plot of EB fluorescence versus FSC after staining with ethidium bromide; these...
are the cells that have most recently entered the apoptotic process

<table>
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<tr>
<td>mBBr</td>
<td>monobromobimane</td>
</tr>
<tr>
<td>MTX</td>
<td>maitotoxin</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmorpholine</td>
</tr>
<tr>
<td>NK cells</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>OPT</td>
<td>α-phthalaldehyde</td>
</tr>
<tr>
<td>P815</td>
<td>mastocytoma cell line</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>Procysteine®</td>
<td>L-2-oxothiazolidine-4-carboxylate</td>
</tr>
<tr>
<td>R123</td>
<td>rhodamine 123; a lipophilic cationic fluorescent dye for the measurement of mitochondrial membrane potential</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>S/G₂/M</td>
<td>phases of the cell cycle during which DNA is synthesised (S phase), after DNA synthesis during which the chromosomes are assembled (G₂ phase) and when the duplicated chromosomes are separated into two daughter cells (M phase)</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>side scatter</td>
</tr>
<tr>
<td>tBHQ</td>
<td>2,5-di-(t-butyl)-1,4-hydroquinone</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>Tg</td>
<td>thapsigargin</td>
</tr>
<tr>
<td>uM</td>
<td>µM; used in figures when the graphical or analysis package utilised to create the figure did not allow the proper use of µM.</td>
</tr>
<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
</tr>
<tr>
<td>WGA-FITC</td>
<td>wheat germ agglutinin conjugated to fluorescein</td>
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Publications and Conference Proceedings
Arising from This Work

Publications


Beaver JP, Waring P (1996) Thapsigargin induces mitochondrial dysfunction and apoptosis in the mastocytoma P815 cell line and in mouse thymocytes. Submitted for publication


Conference proceedings


Beaver JP, Waring P (1994) Thapsigargin causes mitochondrial ultrastructural damage which may play a role in the induction of apoptosis in thymocytes. Australian Society for Biochemistry and Molecular Biology Annual Conference, Gold Coast, Australia. Talk


1. Introduction

1.1 Cell Death

1.1.1 General

Apoptosis and necrosis are types of cell death that differ from each other both morphologically and biochemically. Unlike necrosis, apoptosis is a process under the control of the dying cell.

Apoptosis was first described in 1972 (Kerr et al., 1972). In this process, the final biochemical and morphological outcomes, regardless of cause or mode of induction, have been described as very similar (Kerr et al., 1980). This suggests that the process follows a common final pathway with an ancient evolutionary origin (Carrison et al., 1990). It is an entirely different process from necrosis (Wyke, 1981; Beach et al., 1982) which could be thought of as accidental death in which the cell plays no active part.

The severity of an insult to a cell or tissue is related to the degree of hypoxia, toxic assault or heat shock have been shown to damage to apoptosis. Severe hypoxia, larger concentrations of toxic substances, or temperatures have been demonstrated to affect the cell's viability (Lennon et al., 1992; Lennon et al., 1991).

1.1.2 Characteristics

1.1.2.1 Morphological characteristics

1.1.2.1.1 Necrosis

Necrosis is a form of cell death in which the cell is the death victim rather than a participant. It can occur as a result of a variety of types of cell damage such as complement attack, lytic viral infection or apoptosis is a type of cell death due to chemical assault (Dawid & Wyke, 1990). Whatever the cause, the outcome is an increase in the permeability of the plasma membrane. This occurs either through modification in its structure or as a result of the alteration of the activity of the plasma membrane (Dawid et al. 1990). The cell is no longer able to maintain ion homeostasis, high levels such as...
1.1 Cell Death

1.1.1 General

Apoptosis and necrosis are types of cell death that differ from each other both morphologically and biochemically. Unlike necrosis, apoptosis is a process under the control of the dying cell.

Apoptosis was first described in 1972 (Kerr et al., 1972). In this process, the final biochemical and morphological outcomes, regardless of cell type or of the mode of induction, have been described as very similar (Wyllie et al., 1980). This suggests that the process follows a common final pathway with an ancient evolutionary origin (Cornillon et al., 1994). It is an entirely different process from necrosis (Wyllie, 1981; Searle et al., 1982) which could be thought of as accidental death in which the cell plays no active part.

The severity of an insult determines the form of death it induces. Mild degrees of hypoxia, toxic assault or heat shock have been shown to lead to apoptosis. Severe hypoxia, larger concentrations of toxin and higher temperatures have been demonstrated to lead to necrosis (Searle et al., 1982; Lennon et al., 1991).

1.1.2 Characteristics

1.1.2.1 Morphological characteristics

1.1.2.1.1 Necrosis

Necrosis is a form of cell death in which the cell is the victim rather than a participant. It can occur as a result of a variety of types of cellular damage, such as complement attack, lytic viral infection or exposure to a variety of chemical assaults (Duvall & Wyllie, 1986). Whatever the stimuli, the outcome is an increase in the permeability of the plasma membrane. This occurs either through modification in its structure or as a result of the alteration of the activity of the plasma membrane ion pumps. As a result, the cell is no longer able to maintain ion homeostasis and ions such as
Ca\textsuperscript{2+} and Mg\textsuperscript{2+} move down concentration gradients with an associated redistribution of water.

Initially the endoplasmic reticulum dilates, the mitochondria become denser as the inner membrane shrinks away from the outer mitochondrial membrane, and the nuclear chromatin flocculates. These early changes are reversible (Carafoli et al., 1971; Duvall & Wyllie, 1986), but are rapidly followed by irreversible changes including high amplitude mitochondrial swelling and finally the lysis of the cell (Campbell, 1983). The permanent cell changes are proposed to be a result of the activation of Ca\textsuperscript{2+}-dependent phospholipases leading to a widespread and catastrophic disruption of cellular membranes (Trump et al., 1981). In this final irreversible phase, lysosomal enzymes are released and contribute to the final dissolution of the cell (Hawkins et al., 1972). The degradation of DNA to a point where it would form a smear after agarose gel electrophoresis takes several days (reviewed in Campbell, 1983). The morphological changes associated with necrosis may not be detectable until several hours after irreversible chemical changes have occurred and mitochondrial functioning has been lost (reviewed in Campbell, 1983).

The ultimate outcome of necrosis is the rupture of the plasma membrane and the release of intracellular compounds that have chemotactic properties (Cohen 1991). These compounds provoke an inflammatory response, which leads to damage to neighbouring cells.

1.1.2.1.2 Apoptosis

Apoptosis differs from necrosis biochemically and morphologically, but most importantly because apoptosis is a process under the control of the cell. The process is characterised by the condensation of chromatin, reduction in cell volume, and finally, the formation of apoptotic bodies that are phagocytosed by macrophages or by adjacent tissue cells.

After a lag period, where no morphological changes are obvious, the cells round up, sever junctions with their neighbours, and lose microvilli (Wyllie
et al., 1980). Normal mitochondrial structure is generally maintained, but the endoplasmic reticulum membranes alter in appearance and form vesicles that fuse with the plasma membrane, contributing to a bubbling appearance in electron micrographs (Wyllie et al., 1980). The nuclear chromatin condenses and then forms into crescent shapes that abut the nuclear membrane (Wyllie et al., 1980). Associated with these changes is a dramatic reduction in cell volume. In rat thymocytes, the volume has been recorded as dropping by one third (Thomas & Bell, 1981; Wyllie & Morris, 1982). All these changes are followed by the rapid breakup of the cell into a cluster of membrane bound segments (apoptotic bodies). These apoptotic bodies are rapidly phagocytosed by neighbouring cells or macrophages, where they are broken down by normal lysosomal degradation (Wyllie et al., 1980). The apoptotic bodies are usually phagocytosed before the plasma membrane becomes permeable to vital dyes (Cohen, 1991). The apoptotic process is very rapid; the in vivo cells may die and be phagocytosed within 4 hours (Bursch et al., 1990; Wyllie et al., 1980).

1.1.2.2 Biochemical characteristics of apoptosis

1.1.2.2.1 DNA fragmentation

Double stranded DNA fragmentation by a nuclear endonuclease activity is a common feature of apoptotic cell death. The DNA is cut into pieces of oligonucleosomal lengths (Skalka et al., 1976; Wyllie et al., 1980, 1984). This occurs after the chromatin is condensed and can be visualised with electron microscopy abutting the nuclear membrane (Cohen et al., 1992). Prior to internucleosomal fragmentation, the DNA is cut into larger fragments of 50 and 200kbp (Brown et al., 1993; Cohen et al., 1993, 1994; Sun et al., 1994b). The large fragments are consistent with cleavage of the DNA within its chromosomal structure, such as in the supercoiled loops and rosettes (Filipski et al., 1990; Solov'yyan et al., 1991a, b) and requires either magnesium ions (Sun & Cohen, 1994) or calcium ions (Zhivotovsky et al., 1994). The ion requirement of these processes probably depends on the cell type. The condensation and
fragmentation of the DNA are nearly universal outcomes of the apoptotic process and precede cellular uptake of vital dyes by about 5 hours (Cohen, 1991). This contrasts with necrotic cell death, where the digestion of the DNA into small fragments can occur as late as several days after the cell has lost its membrane integrity and died (reviewed in Campbell, 1983).

1.1.2.2 Endonuclease identity

The search for the identity of the endonuclease responsible for the internucleosomal DNA fragmentation has produced conflicting results. The “ladder” pattern of DNA fragments observed after agarose gel electrophoresis is very similar to the pattern created by an endogenous endonuclease found in normal cells, especially hepatocytes (Hewish & Burgoyne, 1973; Burgoyne & Mobbs, 1975). A Ca\(^{2+}\)-dependent endonuclease has been implicated in the fragmentation of DNA during apoptosis observed in prostate tissue after androgen withdrawal (Kyprianou et al., 1988). In thymocytes a neutral endonuclease (Nakamura et al., 1981) has been characterised. This endonuclease was shown to be activated by Ca\(^{2+}\) and Mg\(^{2+}\) and inhibited by Zn\(^{2+}\), conditions which match those of the early experiments where the DNA in isolated nuclei became fragmented into oligonucleosomal lengths (Cohen & Duke, 1984).

Several other candidate Ca\(^{2+}\)-dependent endonucleases have been identified. These include deoxyribonuclease 1 (Peitsch, 1993), a high molecular weight enzyme isolated from thymocytes (Arends et al., 1990), a 27kD protein from human spleen (Ribeiro & Carson, 1993), a proteolysis activated chromatin bound endo-exonuclease (Fraser, 1994), and an 18kD protein from rat thymocytes (Gaido & Cidlowski, 1991) later identified as cyclophilin (Montague et al., 1994). However a Ca\(^{2+}\)-dependent endonuclease has not been detected in the nuclei of some cell types that undergo apoptosis-associated DNA fragmentation, such as the S49 thymoma cell line (Ucker, 1987; Ucker et al. 1992).
Early reports suggested there was always an increase in \([\mathrm{Ca}^{2+}]\), associated with the onset of apoptosis (Kaiser & Edelman, 1978b, Cohen & Duke, 1984, McConkey et al., 1989c). However, more recent reports show that rises in \([\mathrm{Ca}^{2+}]\) (Iseki et al., 1991; Iwata et al., 1993; Beaver & Waring, 1995) can inhibit apoptosis, while others report chromatin degradation to be \(\mathrm{Ca}^{2+}\)-independent (Bansal et al., 1990; Alnemri & Litwack, 1990). These inconsistencies have stimulated the search for other putative endonucleases that are active in the apoptosis-associated DNA fragmentation but are not activated by calcium ions. One such endonuclease has been described as being activated by acidification rather than \(\mathrm{Ca}^{2+}\) (Barry & Eastman, 1992) and identified as deoxyribonuclease II (Barry & Eastman, 1993).

Morphological changes characteristic of apoptosis have been described as appearing without concomitant DNA fragmentation in both developmental cell death (Zakeri et al., 1993) and in cell systems containing mature vertebrate cells (Sun et al., 1994a; Tomei et al., 1993; Falcieri et al., 1993; Oberhammer et al., 1992; Cohen et al., 1992). This can be seen as evidence that the internucleosomal fragmentation of the DNA is not the absolute outcome of DNA fragmentation but one of several processes which together result in the death of the cell. Also DNA fragmentation has been reported as occurring without the concomitant condensation normally accepted as being the hallmark of apoptosis (Duke et al., 1994)

### 1.1.2.3 Protein synthesis

Apoptosis is an active process that appears to require active protein synthesis to proceed when induced in thymocytes by dexamethasone. The evidence for the role of protein synthesis has come from experiments with thymocytes in which inhibitors of macromolecular synthesis were shown to inhibit the onset of apoptosis (Wyllie et al., 1984; Yamada & Ohyama, 1988; Christ et al., 1993; Evans & Dive, 1993). However, recent evidence suggests that the effect of translation inhibitors on thymocyte
apoptosis may be non-specific, and may delay the onset of apoptosis rather than prevent it (Chow et al., 1995).

Evidence for the role of protein synthesis gathered from experiments using thymocytes may not be readily generalised to other cell types. Thymocytes are poised ready for death by negative selection whereas in other cell types apoptotic death is the result of senescence or cellular damage. The biochemical mechanisms within cells in these two very different states could be expected to differ. When the role of protein synthesis in other cell types has been investigated, macromolecular synthesis inhibitors have been found to either induce apoptosis in their own right, or to potentiate apoptosis induced by another means, or even have no effect on the onset of apoptosis. Cycloheximide, a protein synthesis inhibitor, has been shown to induce apoptosis in a variety of cell types including T-blasts (both rodent and human) (Waring, 1990; Martin, 1993b), the human promyelocytic leukaemia cell line HL-60 (Martin et al., 1990) and cultured splenic B lymphocytes (Illera et al., 1993). Ricin is also a potent inhibitor of protein synthesis and has been demonstrated to induce apoptosis in T blasts (Waring, 1990), macrophages (Waring, 1990; Khan & Waring, 1993) and epithelial cells (Griffiths et al., 1987). The potentiation of apoptosis by protein or RNA synthesis has been demonstrated in Burkitt's lymphoma cells after hyperthermia treatment (Takano et al., 1991), in human mammary adenocarcinoma after TNF-α treatment (Bellomo et al., 1992a), in mature spleen T cells treated with glucocorticoids (Perandones et al., 1993), in glial cells induced by staurosporine (Koh et al., 1995), and in many other cell types (reviewed in Martin, 1993a). Cycloheximide treatment has been demonstrated not to inhibit dexamethasone-induced apoptosis in human medullary thymocytes and in T cell chronic lymphocytic leukaemia cells (Nieto et al., 1992; Baxter et al., 1989) or in murine thymocytes treated with valinomycin (Deckers et al., 1993).
1.1.2.2.4 Transglutaminase activity

Another biochemical characteristic of apoptosis is the activation of a Ca\(^{2+}\)-dependent tissue transglutaminase activity, which forms ε(γ-glutamyl)lysine crosslinks and γ-glutamyl-bis-spermidine crosslinks between some protein substrates (Fesus \textit{et al.}, 1989). The ε(γ-glutamyl)-lysine isopeptide has been shown to be released by cells that have phagocytosed apoptotic bodies. It could be isolated from cell medium in which both apoptosis and phagocytosis were occurring (Fesus \textit{et al.}, 1991). It has also been found in mouse, rat and human plasma where its concentration increased following the induction of large scale apoptosis in the thymus or liver (Fesus \textit{et al.}, 1991).

As a result of the crosslinking of proteins associated with the plasma membrane, the dying cell is surrounded by a highly stable envelope that prevents the leakage of macromolecules into the extracellular space (Fesus \textit{et al.}, 1989). This allows apoptotic cell death to be a very "clean" process in which inflammation is not provoked, peripheral damage to surrounding cells is kept to a minimum and the tissue architecture maintained.

1.1.2.2.5 Protease activity

In recent years the role of proteases in the apoptotic process has been under increasing investigation. Two superfamilies of proteins have been implicated in apoptosis, the serine proteases and the cysteine proteases.

The serine proteases are a superfamily of evolutionarily related enzymes with a reactive serine in the active site. Members of this family have for some time been recognised as having important roles in blood homeostasis, including blood clotting and leucocyte activation (reviewed in Altieri, 1995). Recently members of this family have been recognised as having a vital role in the target cell apoptotic death stimulated by CTL (cytotoxic lymphocytes) and NK cells (natural killer cells). Granzymes A and B both have been demonstrated to be contained in the secretory granules of CTL and NK cells and to play a role in the onset of DNA
fragmentation associated with CTL and NK cell activity (Hayes et al., 1989; Shi et al., 1992; Shiver et al., 1992; Heusel et al., 1994).

The interleukin-1β-converting enzyme (ICE) family of cysteine proteases has been implicated in the onset of apoptosis after a range of triggering stimulations and in a variety of cell types. The studies conducted on the genes regulating cell death in the nematode Caenorhabditis elegans showed that the ced-3 gene product was a homolog of ICE (Yuan et al., 1993). Subsequently several other members of this family of proteases have been identified and demonstrated to be involved in apoptosis, including Nedd-1/Ich-1, CPPP32/Yama, Tx/Ich-2 and Mch-2 (reviewed in Martin & Green, 1995).

The involvement of these proteases has been demonstrated by a number of experimental approaches. These have included overexpression of the protein in cells and observation of increased cell death (Miura et al., 1993), inhibition of apoptosis upon the expression of antisense protease mRNA (Kumar, 1995), the use of specific cysteine protease inhibitors to block cell death (Sarin et al., 1993; Enari et al., 1995; Los et al., 1995) and the identification of cellular proteins which become degraded during the onset of apoptosis (Lazebnik et al., 1994; Casciola-Rosen et al., 1994; Neamati et al., 1995).

Members of the ICE family of proteases have been shown capable of activating other members of the family through proteolysis of inactive pro-forms. Tx/Ich-2 can process both pro-TX and pro-ICE (Faucheu et al., 1995) and Ice can process both pro-ICE and pro-YAMA/CPPP23 (Thornberry et al., 1992; Tewari et al., 1995). Such activities can lead to proteolysis cascades which can amplify the initial apoptotic signal (Martin & Green, 1995). Furthermore their action of other cellular proteins may mediate the activation of some apoptotic processes, such as nuclease activity, or the removal of inhibitor subunits, similar to the activation of NFκB by the removal of IκB (Martin & Green, 1995).
1.1.2.2.6 Cell surface changes

Apoptotic cells are rapidly removed from tissue. In the thymus, where up to one third of lymphocytes may die each day (Cohen, 1991), the tissue architecture is maintained and the apoptotic cells removed so rapidly that they are difficult to locate in histology sections. Thymic lymphocytes with productive gene rearrangement, but low levels of surface CD3 and TCR, were demonstrated to be rapidly phagocytosed by macrophages after apoptosis (Inaba et al., 1988). As no inflammatory response is provoked by apoptotic cell death, it is interesting to ask how these apoptotic cells were recognised by the phagocytic cells that removed them.

Phagocytosis by local cells and macrophages is most likely to be stimulated by cell surface changes on the apoptotic cells. Morris et al. (1984) proposed that the surface glycosylation of cells was changed during apoptosis. They suggested that the terminal sialic acid residues may be lost exposing N-acetyl glucosamine (glc-NAc), N-acetyl galactosamine, and galactose. This was supported by an early experiment, where the preferential binding of mouse peritoneal macrophages to apoptotic rather than normal cells (Duvall et al., 1985) could be blocked by the addition of glc-NAc and its dimer N,N'-diacetylchitobiose. These experiments suggested that the apoptotic cells express more glc-NAc on their plasma membrane glycosyl groups than do normal cells. Further support for the involvement of glycosylation changes came from experiments with cultures of neonatal rat liver cells. It was demonstrated the asialoglycoprotein receptor was implicated in the ingestion of cells undergoing apoptosis by healthy neighbouring hepatocytes (Dini et al., 1992).

These changes in surface glycosylation could be the result of removal of terminal sialic acid residues, or could alternatively be the result of the expression of immature glycoproteins and glycolipids. During the process of apoptosis the cell’s membrane structures are rearranged and the total
surface area must be increased to enable the cell to form into many apoptotic bodies. In this process it is likely that internal membranes become incorporated into the plasma membrane. One means by which immature glycoproteins could be expressed on the cell surface is through the incorporation of Golgi complex and endoplasmic reticulum membranes into the plasma membrane. This is supported by the observation of profound changes in cell surface structure during apoptosis (Morris et al., 1984) and the observation of increased amounts of phosphotidylserine exposed in the outer leaflet of apoptotic cells (Fadok et al., 1992).

The appearance of phosphotidylserine in the outer membrane leaflet of apoptotic cells has also been proposed to act as a stimulant of phagocytosis. Evidence for this came from experiments in which liposomes containing phosphotidylserine were shown to inhibit the phagocytosis of apoptotic thymocytes, whereas liposomes containing other anionic phospholipids had no inhibitory effect (Fadok et al., 1992).

Human neutrophils have a short half-life and die by apoptosis both in vivo and in vitro. Apoptotic neutrophils which still excluded vital dyes were shown to be phagocytosed by macrophages, whereas the viable nonapoptotic neutrophils were not (Savill et al., 1989b). This phagocytosis was not inhibited by the addition of glc-NAc or other sugars active in the system described by Duvall et al., 1985. Recognition of these apoptotic cells has been suggested to occur by means of a "charge-sensitive" system that is more sensitive to the net charge on the cell surface than to a particular molecular structure (Savill et al., 1989a).

Two further mechanisms by which macrophages may recognise apoptotic cells have been proposed. The first involves macrophage secreted thrombospondin (Savill et al., 1992), which could bind to a thrombospondin binding moiety on the surface of the apoptotic cell and to the thrombospondin receptor on the macrophage surface. The second involves the vitronectin receptor (integrin αvβ-3) (Haslett et al., 1994) on the plasma membrane of the macrophage.
1.1.2.7 Gene products and the program

Many gene products have been implicated in the control of apoptosis. These include p53, Bcl-2, Fas, myc and the *C. elegans* gene products CED-3, CED-4 and CED-9 (reviewed in brief in Vaux *et al.*, 1994). Discussion of this interesting topic is outside the scope of this present work.

1.2 Importance of Apoptosis

1.2.1 General

Apoptotic cell death has been observed in an enormous variety of situations, with both physiological and pathological stimuli. These stimuli can occur in many parts of the cell, including the nucleus, cytosol and plasma membrane. For example, the trigger for a cell to undergo apoptosis can be in the nucleus, such as damage to DNA as a result of radiation or chemical damage, on the plasma membrane when an appropriate ligand binds the Fas receptor and provokes apoptosis, or in the cytosol when for example glucocorticoid hormone complexes with the glucocorticoid receptor and migrates to the nucleus to exert its activity. Whilst the outcomes of the apoptotic program are remarkably similar, the speed with which a particular cell type responds to the initiating stimuli can vary widely (Silverstone *et al.*, 1994). The rate of the apoptotic process can depend on how the stimulus impinges on the cell's metabolism or regulatory processes. In general, apoptosis appears to be involved in shaping during embryonic development, the modulation of immune responses, the removal of senescent cells, maintenance of tissue size and shape, and, the removal of cells that have been damaged by radiation or toxins.

1.2.2 Physiological processes

1.2.2.1 General

Apoptosis has been recognised as an important physiological means by which cell numbers are regulated *in vivo* (Wyllie, 1987; Raff, 1992). It is likely to be as important as mitosis in the regulation of the growth and the

1.2.2.2 Embryogenesis and morphogenesis

The process of focal cell death during the morphogenesis of normal embryos has been clearly defined (Hinchliffe & Thorogood, 1974; Glucksmann, 1951; Saunders, 1966). It is now clear that the cells die by apoptosis (Wyllie et al., 1980; Hinchliffe, 1981). Many examples of apoptosis during organised tissue changes in embryonic development have been described. These include the disappearance of interdigital cells during the formation of digits from the solid limb paddle (Saunders, 1966; Duvall & Wyllie, 1986), the elimination of cervical motor neurones from the abortive visceral system during the development of the chick spinal chord (O'Connor & Wyttgenbach, 1974) and the development of the chick wing bud (Hurle & Hinchliffe, 1978).

The morphological characteristics of apoptotic cell death have also been observed in the changes associated with metamorphosis, including the resorption of tadpole tail (Kerr et al., 1974). Apoptosis has also been recognised as the primary mode of removal of senescent cells such as in the gastrointestinal epithelium (Hall et al., 1994) and neutrophils (Savill et al., 1989b).

Apoptosis has been described in endocrine-dependent tissue atrophy. These processes include the thinning of the adrenal cortex of the rat after birth (Wyllie et al., 1973), prostate atrophy after androgen removal (Kyprianou et al., 1988), the control of normal tissue turnover in breast tissue during the menstrual cycle (Anderson et al., 1982), endometrium sloughing during menstrual cycle (Martin et al., 1973; Sandow et al., 1979) and glucocorticosteroid-induced lymphoid tissue atrophy (Wyllie et al., 1980).
1.2.2.3 Cell-mediated immune response

Cytotoxic T lymphocytes (CTL) have been demonstrated to mediate target cell killing by two major pathways. One is through the release of a series of cytotoxic enzymes such as serine esterases (granzymes) and perforin from the cytotoxic lymphocyte. The second mechanism involves a cell-cell interaction and the stimulation of surface receptors. Both these processes have been reported to precipitate apoptosis in the target cell (Young & Cohn, 1987; Tschopp & Nabholz, 1990; Ucker, 1987; Liu et al., 1989). In these processes the DNA is cleaved into the nucleosomal lengths characteristic of apoptosis but, whereas the onset of apoptosis induced by glucocorticosteroids or toxins generally has a half life of 5 to 6 hours, the DNA fragmentation in CTL-mediated killing is detectable within a few minutes (Russell & Dobos, 1980). The ultrastructural changes observed in the target cell during CTL-mediated killing are also typical of apoptosis (Russell et al., 1982; Don et al., 1977). Natural killer cells have also been reported to induce apoptosis in their target cells (Bishop & Whiting, 1983; Roder et al., 1978).

1.2.2.4 Regulation the immune responses

The cell-mediated immune response must be able to differentiate between self and non-self to function to the benefit of the organism. Since the T cell receptor (TCR) functions to recognise antigens within the host organism, these lymphocytes have the potential to initiate a dangerous autoimmune response. The process of negative selection in the thymus during thymocyte development minimises the risk of self-reactive mature T cells being released. During negative selection self-reactive cells are triggered to undergo apoptosis (Kappler et al., 1987; Jenkinson et al., 1989; Smith et al., 1989; Shi et al., 1991). At least 80% of the cells die in situ during thymic negative selection (Joel et al., 1977). The induction of apoptosis by negative selection has been shown to occur at the level of double positive (CD4⁺CD8⁺) (MacDonald et al., 1988) or single positive CD4⁺ (MacDonald & Lees, 1990) or CD8⁺ thymocytes with low or high expression of the TCR/CD3 complex (Debatin, 1994; Inaba, 1988). It has also been
demonstrated to involve engagement of the Fas receptor (Yonehara et al., 1994). Certain autoimmunity-prone mouse strains carry a defect in the protein Fas (CD95) caused by the lpr mutation. In these mice, both Fas-induced apoptotic cell death and negative selection are impaired (Kroemer & Martinez-A, 1994).

Thymocytes also die during thymic atrophy associated with severe physical stress (Selye, 1936), bacterial infections (Abo et al., 1991) and malignancies (Fu et al., 1989). In all these situations the benefit of thymic atrophy is likely to be the prevention of non-self antigens being expressed on nurse cells in the thymus. This reduces the risk of mature T cells tolerant to foreign antigen being released into the peripheral circulation.

Clonal elimination of peripheral T cells may also represent a mechanism by which tolerance to self-antigens is prevented. D'Adamio et al. (1993) reported in vitro and in vivo that, following TCR stimulation, a large fraction of the peripheral T cell population underwent deletion before the proliferation of the remaining cells. This death of T cells has often been referred to as activation induced cell death (AICD). The Fas receptor and its ligand have been implicated in its initiation (Russell et al., 1993).

B-cell selection in germinal centres and bone marrow has also been characterised as involving apoptosis (Murakami et al., 1992; Nemazee & Bürki, 1989). Rodriguez-Tarduchy et al. (1990) demonstrated that a cell line with a pre-B phenotype dependent on IL-3 and died by apoptosis if the IL-3 was withdrawn. A lack of appropriate growth factor has been proposed as a mechanism by which many cell types are provoked to undergo apoptosis in vivo (Raff, 1992).

AICD may play an important role in the regulation of immune responses, possibly by contributing to the establishment of peripheral tolerance (Kabelitz et al., 1993; Hiruma et al., 1992).
1.2.2.5 Tumour induction and treatment

Mutations in genes that yield proteins with a role in the regulation of apoptosis have been shown to be important factors in the onset of tumours. There is now an extensive literature on the role of p53 and related proteins in the stimulation of apoptosis after DNA damage (Zhan et al., 1993). It is also clear that tumours arise and undergo malignant progression in cells with no functional p53 (Lavigueur et al., 1989; Kemp et al., 1993). Bcl-2 is another protein that has an important, but as yet undefined, role in the regulation of apoptosis (reviewed in Reed, 1994). In contrast to p53, normal Bcl-2 activity inhibits the onset of apoptosis (Reed, 1994) and in many cell types the overexpression of Bcl-2 has prevented apoptotic death induced by various agents. It has been proposed to act through an antioxidant pathway (Hockenbery et al., 1993). It has also been proposed to have an action associated with regulation of the calcium ion pool in the endoplasmic reticulum (Lam et al., 1994).

Many tumours over-express Bcl-2 (Reed, 1994). The first to be recognised was Burkitt’s lymphoma. In this B cell tumour the Bcl-2 gene is translocated to a site near the immunoglobulin heavy chain promoter, leading to over-expression of Bcl-2 and a monoclonal expansion of a B cell clone (Cleary & Sklar, 1985; Tsujimoto et al., 1986).

Agents that inhibit apoptosis are likely to act as tumour promoters. Nicotine has been shown to inhibit apoptosis induced by TNF and by chemotherapeutic drugs in a variety of cell lines (Wright et al., 1993) implicating it as a potential tumour-promoting agent in tobacco-related carcinogenesis.

Apoptosis has been observed in solid tumours (Kerr & Searle, 1972; Fukuda et al., 1993). This cell death has been attributed to several possible factors including a residual attempt at autoregulation within the expanding tumour population, and mild cellular injuries such as hypoxia or nutritional deficiency. Many treatments designed to destroy tumours have been shown to kill the cells by apoptosis. These include chemotherapies
Introduction


1.2.3 Toxicological process

Cell function can be compromised in its ability to maintain cellular homeostasis or as a result of damage to the structure of its DNA. The removal of a cell by apoptosis after it has been compromised provides protection to the host organism from the proliferation of damaged cells. Where cells are severely damaged and immediately unable to maintain homeostatic controls, the cells die in an uncontrolled fashion by necrosis and this is followed by local inflammation. However when the cell has, by some means, registered critical damage but still has control over its metabolism and its cell cycle, the apoptotic pathway can be initiated. The cell's response will be largely determined by the severity and type of insult inflicted upon it.

A range of stimuli and cytotoxic treatments can induce apoptosis. The response to these damaging stimuli differs, depending on the dose and cell type (Lennon et al., 1991; Bonfoco et al., 1995). Chemical treatments have been shown to induce apoptosis. These treatments include drugs (Dive & Hickman, 1991), fungal and plant toxins (Waring et al., 1988b; Waring, 1990; Zhivotovsky et al., 1994), and environmental toxins such as dioxin (McConkey et al., 1988) and tributyltin (Chow & Orrenius, 1994). Low dose \( \gamma \)-irradiation has been demonstrated to induce apoptosis in small resting lymphocytes from thymus or spleen (Sellins & Cohen, 1987). Viral infections have been shown to provoke an apoptotic response (Rojko et al., 1992). These include infections with the HIV virus (Ameisen & Capron, 1991; Meyaard et al., 1992; Gougeon & Montagnier, 1993) and the Sendai virus and Herpes virus Type 1 (Portolani et al., 1995). Oxidative stress has been demonstrated to provoke apoptosis (Hockenbery et al., 1993) and damage by ionising radiation to be enhanced by oxygen (Dewey, 1960). As mentioned above, many cancer treatments act by inducing apoptosis in the target cells, such as cisplatin,
cyclophosphamide and γ-radiation. Unfortunately this is usually associated with the induction of death in many other healthy rapidly proliferating tissues.

1.3 Calcium

1.3.1 General

1.3.1.1 Buffering calcium ion fluxes

The concentration of calcium ions in the cytosol of a normal unstimulated cell is in the order of 100 - 200nM (Cobbold & Rink, 1987). This can be compared with millimolar concentrations of calcium ions in the serum and extracellular fluids (Rasmussen & Barrett, 1984). Cells use several membrane-located pumps and channels to maintain this large concentration differential.

Calcium entry into the cytosol occurs through calcium channels in the plasma membrane and in the endoplasmic reticulum. These channels rely on the substantial calcium ion concentration gradient between the cytosol, and both the extracellular medium and the lumen of the endoplasmic reticulum. The calcium ion concentration is often as great as 10,000-fold lower in the cytosol (reviewed in Carafoli, 1987). Calcium ions are also able to refill depleted endoplasmic reticulum stores directly from outside the cell (Putney, 1986, 1990). Activation of store-depletion activated channels has been demonstrated in white blood cells, in both rat thymocytes and human T cells (Mason et al., 1991a, b; Gouy et al., 1990).

To remove excess calcium ions from the cytosol, the cell employs calcium exporting pumps in the endoplasmic reticulum, mitochondrial and plasma membranes. Both the endoplasmic reticulum and plasma membranes contain high affinity low capacity Ca\(^{2+}\)-ATPases (Carafoli, 1987). These pumps hydrolyse ATP to provide the energy required to pump calcium ions out of the cytosol against the large concentration gradient. The plasma membranes of many cells also contain a calcium-sodium exchanger but T lymphocytes do not express this activity (Donnadieu et
This calcium-sodium exchanger is a low affinity but high capacity pump that exchanges one calcium ion for three sodium ions. It makes use of the large sodium ion gradient into the cell to provide energy for calcium pumping (reviewed in Carafoli, 1987). Mitochondria also have some ability to take up calcium ions (Gunter & Pfeiffer, 1990). For a long time they were thought to be an important sink for $\text{Ca}^{2+}$ (Carafoli, 1987) however now it is recognised that mitochondria do not play a major role in the buffering of $[\text{Ca}^{2+}]$ fluxes. This aspect of mitochondrial function is discussed in more detail later in section 1.3.3 Mitochondria and calcium.

1.3.1.2 Calcium ions and cellular damage

Calcium ions have been long implicated in cell death. The involvement of calcium ions in the necrotic death of hepatic tissues after chemical treatment has been studied in detail (Schanne et al., 1979; Thor et al., 1984; Landon et al., 1986; Itoh et al., 1988). Neurotoxic agents have also been shown to induce large increases in intracellular $[\text{Ca}^{2+}]$, before the onset of necrotic death (Komulainen & Bondy, 1987), as has oxidative injury (Geeraerts et al., 1991; Kaneko et al., 1994). Some of the effects of raised $[\text{Ca}^{2+}]$, that have been examined include the extensive blebbing of necrotic cells associated with the saturation of $\text{Ca}^{2+}$-binding sites on cytoskeletal proteins (Jewel et al., 1982), loss of mitochondrial function (Moore et al., 1985), the destruction of intracellular proteins through the action of $\text{Ca}^{2+}$-activated proteases (Farber, 1982; Nicotera et al., 1986), DNA damage (Sidhartha et al., 1993), disruption of protein kinase control (Blackshear et al., 1988), and the degradation of membrane structures and release of arachidonate following the activation of $\text{Ca}^{2+}$-dependent phospholipases (Chien et al., 1979; Farber, 1982).

1.3.2 Calcium ions in apoptosis

The role of calcium ions in apoptosis has been a controversial topic for some years. In 1977, Kaiser and Edelman presented the first evidence for the involvement of calcium ions in the onset of apoptosis in thymocytes after glucocorticoid treatment. They supported their initial evidence with
experiments using calcium ionophores to mimic the effect of glucocorticoid treatment in thymocytes (Kaiser & Edelman, 1978a, b). In 1980, Wyllie showed DNA degradation was an early event in the apoptotic process and suggested the enzyme responsible could be the Ca$^{2+}$/Mg$^{2+}$-dependent endonuclease previously described by Hewish and Burgoyne in 1973. Experiments associated with attempts to identify the enzyme responsible for DNA degradation produced further evidence for a role of calcium ions in the apoptotic process. Cohen and Duke (1984) demonstrated internucleosomal DNA fragmentation within 90 minutes, when isolated nuclei were incubated in a calcium rich medium. Wyllie et al. (1984) observed that calcium ionophore treatment could induce endonuclease activation in thymocytes and many of the morphological changes typical of apoptosis (Wyllie et al., 1984). Both groups concluded calcium ions were integral to the onset of apoptosis. However the ability of cycloheximide to inhibit the ionophore-induced DNA fragmentation suggests that the calcium concentration does not lead to a simple activation of the endonuclease. The next published observation of a rise in [Ca$^{2+}$]$_i$ associated with glucocorticoid-induced apoptosis in thymocytes came in 1989 (McConkey et al., 1989b, c). This induction was able to be inhibited by either cycloheximide or actinomycin D. A similar rise in [Ca$^{2+}$]$_i$ was observed in rat thymocytes treated with 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD) (McConkey et al., 1988).

After contact with a cytotoxic T lymphocyte (CTL) the target cells had been observed to experience sudden and dramatic increases in [Ca$^{2+}$]$_i$ (Poenie et al., 1987). This was probably mediated through the action of cytotoxic granules released by the CTL. DNA fragmentation in target cells after CTL activity had also been observed and along with ultrastructural changes was suggestive of apoptosis (Russell et al., 1982; Don et al., 1977). However the death process in the target cell appeared to take only minutes (Russell & Dobos, 1980) rather than the several hours expected with glucocorticoid treatment of thymocytes. This led to the proposal of a causative relationship between the onset of DNA fragmentation in the
target cell and the observed increase in calcium concentration (Allbritton et al., 1988). A rise in [Ca\(^{2+}\)], was also observed in the targets of natural killer cells (McConkey et al., 1989a).

Since these results from thymocytes, CTL and NK target cells were published, there has followed a flurry of research into the role of calcium ions in apoptotic cell death. This has been associated with an increasing understanding in the field that DNA degradation was likely to be one of many biochemical outcomes of apoptosis, rather than the central defining event. Results since 1989 have been confusing, with some results showing involvement of calcium ions in apoptosis, and others showing no involvement. A sample of the published results is presented in Table1-1.

Many of the early experiments measured intracellular calcium fluxes during the induction of apoptosis. Another approach to the problem of calcium ion involvement has been to measure apoptosis induced by an already defined induction agent, such as dexamethasone, whilst simultaneously modulating the internal or external calcium ion concentration with another agent, such as an ionophore. If calcium ions are involved in the early apoptotic processes, the raising of the [Ca\(^{2+}\)], along side the action of another inducer should potentiate the process and the decreasing of [Ca\(^{2+}\)], should inhibit or postpone apoptosis. These methods have also met with mixed results.

Dowd et al. (1992) used calbindin, a protein capable of the high affinity complexing of five or six calcium ions, to decrease [Ca\(^{2+}\)]. They were able to demonstrate that the over-expression of calbindin in the WEHI 7.2 myeloid cell line was able to decrease the apoptotic effects of dexamethasone, forskolin, and A23187. This result gave support to the theory that calcium ions are integrally involved in the apoptotic process. In another example that supports this hypothesis, anti-CD3 induction of apoptosis in a T-cell hybridoma was inhibited by a reduction in the extracellular [Ca\(^{2+}\)] and was not inhibited by an increase in the intracellular [Ca\(^{2+}\)] induced with ionomycin treatment, (Iseki et al., 1991). On the other
hand using the same cell type, these workers found dexamethasone-induced apoptosis was inhibited by application of ionomycin. These results present an apparent paradox that may be resolved by suggesting the involvement of calcium ions is stimulus specific. That is, depending how and where the apoptotic stimulus feeds into the final apoptotic pathway, a calcium dependent step may or may not be involved. A similar result in an entirely different system provides further evidence for the complexity of the situation. When a lymphoma cell line with an immature B cell phenotype and some Group I Burkitt's lymphoma cell lines were treated with calcium ionophore, apoptosis was provoked which could be inhibited with phorbol esters (Bonnefoyberard et al., 1994). As phorbol esters stimulate the activity of PKC and a rise in [Ca^{2+}], also activates PKC an immediate paradox is obvious. How can two agents that both activate PKC be able to act in opposition? Furthermore this group found that when the cells were treated with cyclosporin A, an inhibitor of the phosphoprotein phosphatase calcineurin, calcium-induced apoptosis was inhibited but not that induced by the PKC-inhibitor chelerytherine or by serum deprivation. Clearly in these cell lines, calcium is not a universal participant in the apoptotic process.

Other workers have limited the extracellular calcium concentration during the onset of apoptosis and drawn conclusions about the role of calcium ions from the results. Zhivotovsky et al. (1994) could inhibit the formation of large molecular weight DNA fragments in glucocorticoid treated human thymocytes by using a Ca^{2+}-free medium, or by pretreatment of the cells with the intracellular calcium ion chelator bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM). From these results they concluded the formation of high molecular weight fragments was mediated by Ca^{2+}. On the other hand, calcium chelators both extracellular, EGTA, and intracellular, BAPTA-AM, have been demonstrated to induce apoptosis in three apoptosis sensitive myeloid cell lines (Kluck et al., 1994).
When different cell types are investigated, further contradictory results have been found. In neutrophils, apoptosis has an important role in the control of the number of granulocyte cells present in a tissue during inflammation (Haslett et al., 1994). An elevation of $[\text{Ca}^{2+}]$, provoked by inflammatory mediators has been demonstrated to maintain the longevity of neutrophils in vivo. In in vitro experiments calcium ionophores (A23187 and ionomycin) retarded the onset of apoptosis by 20 hours (Whyte et al., 1993). Conversely, $\text{Ca}^{2+}$-chelation using BAPTA-AM and calmodulin inhibition with W-7 promoted apoptosis in these cells.

Table 1-1 Conflicting results have been published about the role of $\text{Ca}^{2+}$ in the onset of apoptosis

**Part A** Positive involvement for calcium ions

<table>
<thead>
<tr>
<th>cell type</th>
<th>induction mechanism</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cell leukaemia</td>
<td>$\text{Ca}^{2+}$ ionophore, A23187</td>
<td>McConkey et al., 1991</td>
</tr>
<tr>
<td>rat thymocytes (4-5 weeks old)</td>
<td>tributyltin</td>
<td>Aw et al., 1990</td>
</tr>
<tr>
<td>rat thymocytes</td>
<td>thapsigargin</td>
<td>Jiang et al., 1994</td>
</tr>
<tr>
<td>cultured mouse hepatocytes</td>
<td>acetaminophen</td>
<td>Shen et al., 1991</td>
</tr>
<tr>
<td>human B cell line</td>
<td>anti-Fas mAb</td>
<td>Oshimi &amp; Miyazaki, 1995</td>
</tr>
<tr>
<td>mammary adenocarcinoma cell line</td>
<td>TNF$\alpha$</td>
<td>Bellomo et al., 1992a</td>
</tr>
<tr>
<td>human B cell line (Ramos cells)</td>
<td>$\text{Ca}^{2+}$ ionophore, A23187</td>
<td>Ning &amp; Murphy, 1993</td>
</tr>
</tbody>
</table>
**Part B**  Decrease in calcium ions observed during apoptosis

<table>
<thead>
<tr>
<th>cell type</th>
<th>induction mechanism</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>anti-CD3 stimulation</td>
<td>Cohen <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>IL-3-dependent myeloid cell line (mouse)</td>
<td>IL-3 withdrawal</td>
<td>Magnelli <em>et al.</em>, 1993</td>
</tr>
</tbody>
</table>

**Part C**  No involvement for calcium ions

<table>
<thead>
<tr>
<th>cell type</th>
<th>induction mechanism</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL-60 cells</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;, ethanol, UV irradiation</td>
<td>Lennon <em>et al.</em>, 1992</td>
</tr>
<tr>
<td>IL-3-dependent haematopoietic cell line (32D)</td>
<td>IL-3 withdrawal</td>
<td>Baffy <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>mouse thymocytes</td>
<td>etoposide, teniposide</td>
<td>Ye <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>B cells</td>
<td>TCDD</td>
<td>Karras &amp; Holsapple, 1994</td>
</tr>
<tr>
<td>mouse tumour haematopoietic cell lines</td>
<td>ionophores</td>
<td>Duke <em>et al.</em>, 1994</td>
</tr>
<tr>
<td>human lymphoid cell line (CEM-C7)</td>
<td>novobiocin</td>
<td>Alnemri &amp; Litwack, 1990</td>
</tr>
<tr>
<td>human lymphoid cell line (CEM-C7)</td>
<td>dexamethasone</td>
<td>Bansal <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>cultured central nervous system neurons</td>
<td>β-amyloid peptide</td>
<td>Loo <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>thymocytes</td>
<td>TPEN (Zn&lt;sup&gt;2+&lt;/sup&gt;-chelator)</td>
<td>Jiang <em>et al.</em>, 1995</td>
</tr>
<tr>
<td>Jurkat T lymphocytes</td>
<td>Fas/APO-1</td>
<td>Weis <em>et al.</em>, 1995</td>
</tr>
</tbody>
</table>
In other systems, an increase in $[\text{Ca}^{2+}]$ induced by either ionophores or thapsigargin has been demonstrated to inhibit apoptosis. Calcium ionophores (A23187 and ionomycin) have been demonstrated to inhibit apoptosis induced by growth factor withdrawal in IL-3-dependent bone marrow cells (Rodriguez-Tarduchy et al., 1992) and, as described above, ionophores have been used to inhibit dexamethasone-induced apoptosis (Iseki et al., 1991).

The results published up to now do not offer unanimity of opinion on the question of calcium's role in apoptosis. However, it would appear that the original hypothesis, that $\text{Ca}^{2+}$ acts directly in the nucleus to activate a resident endonuclease, was simplistic. In cell types where rises in $[\text{Ca}^{2+}]$ are observed to be associated with apoptosis, this rise could be the result of cell signalling processes, with the resultant activation of $\text{Ca}^{2+}$-dependent enzymes (kinases and phosphatases, for example) or of alterations in membrane arrangements in the cell which must inevitably accompany the dissection of the cell into apoptotic bodies. In some cases it could even be an artefact of the method used to detect the calcium flux.

Many of the published studies used bulk cell fluorimetry to detect intracellular calcium ion concentration changes (Kaiser & Edelman, 1978a; McConkey et al., 1989b, c). This method is fraught with difficulties as the total fluorescence of the cells and the medium are measured together. Only a small percentage of the cells needs to release their fluorescent dye into the medium that is $\text{Ca}^{2+}$-rich, or alternatively a small number of cells undergo a very large increase in $[\text{Ca}^{2+}]$, for there to be a large increase in the fluorescence signal. The flow cytometric method allows both of these possibilities to be overcome. In this method only the fluorescence of individual cells is measured and leakage of dye into the medium from a few cells will not contribute to the result. Furthermore, the proportion of responding cells can be quantified.

Other experimenters have used chelating compounds to complex calcium inside the cell (Kluck et al., 1994) or in the extracellular medium. These
compounds have the potential to complex such large quantities of calcium that cellular calcium homeostasis mechanisms and cellular functions that rely on calcium ion signalling, would be compromised. When chelators are used there is also the risk that other ions essential to cellular functioning will be complexed. Experiments with calcium chelators have been used to demonstrate both that complexing intracellular calcium contributed to the inhibition of apoptosis (Zhivotovsky et al., 1994) and its induction (Whyte et al., 1993). There is an alternative explanation for the apparent inhibition of apoptosis by chelating agents. These agents may have prevented DNA fragmentation by causing necrosis (Waring & Sjaarda, 1995). Cells that have died by necrosis can be difficult to distinguish from normal cells if only DNA fragmentation is measured (see 3.2 Detection of apoptosis).

The use of ionophores to substantiate the argument that an increase in $[\text{Ca}^{2+}]_i$ is involved in apoptosis is also open to argument. The apoptosis induced by ionophores could be the cellular response to an unacceptably severe assault on its homeostatic mechanisms. It is not clear from these experiments that calcium ions are involved in control of the apoptotic pathway per se.

In summary, it is difficult to conclude that calcium ions definitely do, or do not, have an integral role in the onset of apoptosis in all cell types. They certainly do not seem to have a universal role. It is certain to be the case that different cell types express their final pathway to apoptotic death differently. This may include some pathways provoked by some stimuli involving calcium and others that do not, even in the same cell type.

1.3.3 Mitochondria and calcium

1.3.3.1 General

The outer mitochondrial membrane is permeable to $\text{Ca}^{2+}$ ions but the inner membrane has both calcium ion export and import mechanisms. The export of calcium ions from the mitochondrial matrix to the cytosol is effected by a pair of exchangers which result in electroneutral transport of calcium ions. In the first member of the pair, one $\text{Ca}^{2+}$ is exported in
exchange for two Na\(^+\), and these imported Na\(^+\) are then re-exported by the second exchanger with the concomitant entry of hydrogen ions (Puskin et al., 1976; Denton & McCormack, 1985; Gunter & Pfeiffer, 1990, McCormack & Denton, 1993). This export mechanism is inhibited physiologically by raised extra-mitochondrial Mg\(^{2+}\) and Ca\(^{2+}\) concentrations. Heart muscle mitochondria were investigated before and after stimulation of the cell. The [Ca\(^{2+}\)]\(_{\text{m}}\) was less than cytosolic [Ca\(^{2+}\)] in unstimulated cells but higher in stimulated cells (McCormack & Denton, 1993). This was probably as a result of the inhibition of the Ca\(^{2+}\)/Na\(^+\) exchanger by raised cytosolic Mg\(^{2+}\) and Ca\(^{2+}\) concentrations.

The inward flow of calcium ions occurs through an electrophoretic uniporter that transports calcium ions down the mitochondrial charge gradient. This uniporter can be inhibited by Ruthenium red, Mg\(^{2+}\) and La\(^{3+}\) and inhibited by the polyamine spermine (Rizzuto et al., 1994). This uniporter has about a ten-fold greater capacity than the export mechanism. Therefore in conditions when the extramitochondrial [Ca\(^{2+}\)] is substantially raised, there is the potential for the mitochondria to accumulate calcium ions (Nicholls & Akerman, 1982) and contribute to intracellular buffering of cytosolic [Ca\(^{2+}\)] (Fiskum & Lehninger, 1982). However, this is likely to be reserved for conditions of severe cytosolic calcium overload (McCormack et al., 1990) with cytosolic concentrations of greater than 1\(\mu\text{M}\) (Pietrobon et al., 1990).

The pumps of the endoplasmic reticulum and plasma membrane have been implicated as the major sites of control of cytosolic [Ca\(^{2+}\)]. The mitochondrial calcium pumps are primarily concerned with the regulation of the intramitochondrial [Ca\(^{2+}\)], and not with the maintenance of cytosolic calcium ion concentrations (McCormack et al., 1990, McCormack & Denton, 1994). The [Ca\(^{2+}\)]\(_{\text{m}}\) under physiological conditions has been shown to be low (Somlyo et al., 1985) and the matrix calcium concentration has been demonstrated to have an important role in the modulation of the activity of many mitochondrial enzymes, especially
those involved in respiration (Denton et al., 1978; Hansford, 1991; McCormack et al., 1990; McCormack & Denton, 1994). When cells are stimulated with an increase in [Ca\(^{2+}\)], an increase in oxygen gas uptake is observed and the ATP/ADP ratio increases (Hansford, 1991; McCormack et al., 1990; McCormack & Denton, 1994).

As stated above, when the cell experiences prolonged rises in [Ca\(^{2+}\)], which are beyond the capacity of the endoplasmic reticulum and plasma membrane pumps, the mitochondria have a substantial capacity to accumulate calcium ions (Altin & Bygrave, 1986). This accumulation can lead to mitochondrial damage, including the deposition of hydroxyapatite crystals in the mitochondrial matrix as the calcium ions complex with phosphate ions (Carafoli, 1987). A non-specific pore in the inner mitochondrial membrane opens when the [Ca\(^{2+}\)]\(_{m}\) increases to greater than 20µM. This leads to changes described in the literature as phase transition effects (Compton et al., 1988a).

1.3.3.2 Phase transition effects

The effects of extreme increases in the [Ca\(^{2+}\)]\(_{m}\) include an increase in matrix volume (Halestrap, 1989) with marked ultrastructural changes (Duncan & Shamsadeen, 1991). The ultrastructural changes may be the result of the activation of a Ca\(^{2+}\)-dependent mitochondrial phospholipase A\(_2\) (Duncan, 1988, 1991). Uncoupling of the electron transport chain has been found to be usually associated with the development of ultrastructural damage (Corton et al., 1994) which resulting in a drop in the ATP/ADP ratio as mentioned above.

The phase transition effect, also known as permeability transition, was first described as occurring in isolated mitochondria in the presence of high concentrations of calcium and phosphate ions (Chappell & Crofts, 1961; Carafoli, 1987; Savage et al., 1991). During phase transition, calcium ions accumulated in the matrix (Gunter & Pfeiffer, 1990) and resulted in the loss of small molecules from the matrix including the cellular antioxidant glutathione (Savage et al., 1991). It has also been described in
reperfusion injury (Compton et al., 1988a; Broekemeier et al., 1989; Halestrap & Davidson, 1990) and has been causally related to a loss of oxidative phosphorylation (Compton et al., 1988a). The high amplitude swelling that accompanies the phase transition effect is thought to occur because the matrix proteins are slow to equilibrate through the pore opening. This leads to a colloid-osmotic pressure imbalance which drives the entrance of water molecules. This results in dilution of the matrix and massive swelling (Gunter & Pfeiffer, 1990) until the matrix and cytosolic protein concentrations become the same. The time course for this swelling is of the order of 800ms in isolated mitochondria (Gunter & Pfeiffer, 1990). They suggested three possible roles for the phase transition effect in vivo. Firstly, it could contribute to the removal from the mitochondrial matrix of calcium ions sequestered after a period of high cytosol $[\text{Ca}^{2+}]$. Secondly, it could allow a slow release of matrix proteins and therefore contribute to protein turnover. The third suggested role is to allow the diversion of energy gained from substrate oxidation away from ATP synthesis towards heat production. Another proposed role for the occasional opening of the pore associated with phase transition is the release of by-products of metabolism for which no specific transport pathways exist (Griffiths and Halstrup, 1991).

In isolated mitochondria, cyclosporin A (CsA) has been found to act as a high potency inhibitor of phase transition (Broekemeier et al., 1989, 1992; Igbavboa et al., 1989, Griffiths & Halestrap, 1991). The reversal after CsA treatment occurs on a time scale of seconds and appears to involve a synchronised closing of all the pores in individual mitochondria (Compton & Costi, 1988). The inhibition offered by CsA is transient (between 10 and 20 minutes), although its effect can be extended with the additional use of an inhibitor of phospholipase $A_2$ (Broekemeier & Pfeiffer, 1989). The role of phospholipase $A_2$ is likely to involve the regulation of the opening of the transition pore (Broekemeier & Pfeiffer, 1989).
1.3.3.3 Mitochondrial heterogeneity

Mitochondria have been found to vary enormously in morphology and density within a cell and it is possible that each mitochondrion responds to its local environment (Chen & Smiley, 1993). Rizzuto et al. (1994) used mitochondrially-expressed aequorin to measure calcium ion transients in mitochondria within intact cells. They observed a heterogeneity of mitochondrial response, with approximately 30% of the mitochondria being highly responsive to IP$_3$-stimulated calcium release. The response of mitochondria to phase transition is also heterogeneous, with subpopulations of differing stability in vitro (Gunter & Pfeiffer, 1990). If this property holds inside the cell, it would be likely that, even in conditions that favoured phase transition, a proportion of mitochondria would remain in the impermeable state with full functioning for an extended period of time (Gunter & Pfeiffer, 1990). However, eventually the calcium ions that have leaked from the mitochondria, which have undergone phase transition, would be redistributed to the still intact mitochondria. These, in turn, would then be precipitated into phase transition (Gunter & Pfeiffer, 1990).

1.3.3.4 Mitochondria in Apoptosis

During the apoptotic process, mitochondrial structure has been characterised as becoming crowded in the cell, as cell volume decreases but as retaining their integrity (Wyllie et al., 1980). The functioning of the mitochondria has been measured using fluorescent dyes that make use of the transmembrane potential in functioning mitochondria. The transmembrane potential is a key parameter for the measurement of mitochondrial functioning and energy production (Chen, 1988) because without this electrochemical gradient ATP is unable to be synthesised (Engelbrecht & Junge, 1990). These dyes include Rhodamine 123 (R123) (Darzynkiewicz et al., 1981, 1992; Papadimitriou et al., 1994), DiOC$_6$(3) and JC-1 (Reers et al., 1991; Smiley et al., 1991; Chen & Smiley, 1993).
Most reports have provided evidence that mitochondrial structure and functioning, including the maintenance of the transmembrane potential, remains intact in apoptotic cells but not in necrotic cells (Darzynkiewicz et al., 1992). This has been demonstrated in a range of cell types and after a variety of treatments (Wyllie 1981; Del Bino et al., 1991; Darzynkiewicz et al., 1992; Weis et al., 1995) and in a detailed study of dexamethasone-treated thymocytes (Cossarizza et al., 1994). However, recent reports have suggested the mitochondrial transmembrane potential decreased before the onset of apoptosis in dexamethasone-treated thymocytes, splenocytes and rat embryo cell lines transvected with SV40 (Vayssière et al., 1994, Zamzami et al., 1995a, b; Petit et al., 1995). The main difference between the earlier and more recent reports appears to be technical. The Darzynkiewicz and Cossarizza papers reported thoroughly washing of the cells before flow cytometry to remove background staining due to the potential associated with the plasma membrane. Whereas, the Vayssière, Zamzami and Petit papers did not wash the cells prior to analysis. Their measurements could therefore be include a component due to the retention of the dye in the cytosol.

This confusion about mitochondrial function during apoptosis can be resolved by a comparison of the ATP concentration in the treated and normal cells. If the mitochondrial function does remain intact well into the apoptotic process, as the earlier reports suggested, the ATP concentration should not decrease until after DNA oligonucleosomal fragmentation. On the other hand, if a loss of mitochondrial functioning is universally an early event in the process, the ATP concentration should fall before the onset of DNA fragmentation.

1.4 Glutathione in apoptosis

1.4.1 Glutathione

Glutathione (GSH) is the major non-protein thiol in cells (Kosower & Kosower, 1987), and is present in most cells in millimolar concentrations (Meister & Anderson, 1983). GSH is a tripeptide (γ-glutamylcysteinyl-
glycine) but interestingly is not synthesised by the protein synthesis machinery of the cell (Halliwell & Gutteridge, 1989). GSH is occurs in cells in the reduced and oxidised forms. It is present in the cytosol, mitochondria (Reed, 1990) and nucleus (Ketterer & Meyer, 1989; Bellomo et al., 1992b). It is also present as mixed disulphides with proteins, Coenzyme A or cysteine (Kosower & Kosower, 1975; Halliwell & Gutteridge, 1989).

GSH is recognised as having three major roles related to protecting the cell from toxic substances and oxidative stress (Halliwell & Gutteridge, 1989). Firstly, it can reduce peroxides in a reaction mediated by the enzyme GSH peroxidase with concomitant release of oxidised glutathione (GSSG). Secondly, it has an important role in detoxification mediated by the enzyme glutathione-S-transferase. Thirdly it helps keep protein cysteines in a reduced state, preventing crosslinking (reviewed in Halliwell & Gutteridge, 1989).

The oxidised glutathione (GSSG) produced by GSH peroxidase is a dimer of glutathione molecules joined by a disulphide bridge. The rate of GSSG formation has been proposed to represent an index of the oxidative stress a cell is experiencing (Lauterberg et al., 1984). The oxidised GSSG is recycled to form two molecules of GSH by the action of GSH reductase, with the investment of NADPH (Flohé & Günzler, 1975). When the rate of GSSG production is greater than the rate of its reduction to GSH, the GSSG can be extruded from the cell (Bellomo et al., 1987). If it is not reduced or extruded from the cell the GSSG can interact with intracellular protein thiol groups to form mixed disulphides (Bellomo et al., 1987). This is discussed in more detail in the later section 1.4.3 S-thiolation of protein by glutathione.

GSH also has roles in normal physiological processes such as amino acid transport into the cell, cytoskeletal assembly and the synthesis of peptidoleukotrienes (Meister, 1983; Cotgreave et al., 1990). GSH also has a role in the synthesis of deoxyribonucleotides, as an intermediate in the
transfer of reducing equivalents from NADPH to ribonucleotide reductase using the glutaredoxin system (Holmgren, 1990). This reaction is inhibited by high [GSSG] (Holmgren, 1979); therefore only a high ratio of GSH/GSSG will promote the synthesis of deoxyribonucleotides (Holmgren, 1990) and allow DNA synthesis.

Treatment of cells with the precursors of GSH synthesis can augment the intracellular GSH levels. The initial rate-limiting step for GSH synthesis involves the reaction between cysteine and glutamate catalysed by γ-glutamylcysteine synthase. The availability of cysteine is the major determinant of the rate of this reaction (Meister & Anderson, 1983). The uptake of cysteine into cells is dependent on the activity of the γ-glutamyl transpeptidase enzyme, which has different activity depending on the tissue investigated (Griffith & Meister, 1979). Lymphocytes have a γ-glutamyl transpeptidase activity greater than 10 fold higher than the activity in the liver (Martensson et al., 1989; Orlowski & Wilk, 1975).

N-acetyl-L-cysteine (NAC) has been reported to cause an increase in the intracellular [GSH] by acting as a precursor for the production of cysteine (Burgunder et al., 1989) and for cystine (Issels et al., 1988). N-acetyl-L-cysteine (NAC) has been used to supplement GSH levels rather than cysteine, because of the recognised in vivo toxicity of cysteine even when administered in moderate amounts (Anderson & Meister, 1987). This toxicity is likely to be mediated through the production of H₂O₂ generated as a result of the spontaneous oxidation of cysteine to cystine (Anderson & Meister, 1987). The NAC is readily transported into cells and deacylated in vivo at unknown cellular sites (Anderson & Meister, 1987).

Addition of GSH to the extracellular medium can lead to an increase in the [GSH]. GSH itself is not directly transported into cells but is broken down outside the cell with the release of cysteine, cysteinylglycine and γ-glutamylcysteine. These are readily transported into the cell where the GSH can be reformed (Meister & Anderson, 1983; Anderson & Meister, 1983, 1987).
Another compound that has been used to supplement GSH concentrations is L-2-oxothiazolidine-4-carboxylate (Procysteine®) which is decarboxylated and oxidised inside cells to form cysteine by the enzyme 5-oxoprolinase (Williamson & Meister, 1981, 1982). Procysteine® has the advantage of being apparently non-toxic and readily transported into cells (Boettcher & Meister, 1985) but unfortunately difficult to obtain commercially. L-Buthionine-(SR)-sulfoximine (BSO) is a potent inhibitor of γ-glutamylcysteine synthase (Meister, 1988) and therefore of GSH synthesis. The ability of BSO to inhibit Procysteine® stimulated GSH synthesis provides further evidence that Procysteine® is able to deliver cysteine inside the cell (Williamson et al., 1982).

Intracellular [GSH] can be lowered with the use of BSO or diamide. BSO inhibits GSH synthesis (Meister, 1988) and is therefore is suited to long term culture. Diamide rapidly reacts the GSH (Kosower & Kosower, 1969, 1987) with a stoichiometry of a one to one and does not cause the formation GSSG. It enters the cells within seconds and reacts at a high rate at physiological pH and in most cases the diamide treatment did not cause irreversible damage if washed out of the cell culture medium within 10 minutes (Kosower & Kosower, 1969, 1987).

1.4.2 S-thiolation of proteins by glutathione

Protein cysteine sulphydryl groups are usually maintained in the reduced form (Reed, 1990). However, when the cell is under doing oxidative stress, intracellular GSH is oxidised. Concomitant with this oxidation, proteins can form mixed disulphides with glutathione (Bellomo et al., 1987). The amount of mixed disulphides produced and the kinetics of their formation is each dependent on both the intracellular [GSH]/[GSSG] ratio and the activity of the GSSG recycling enzyme glutathione reductase (Bellomo et al., 1987). Normally the intracellular GSSG concentration is kept extremely low, at less than 1% of the total cellular glutathione pool (Cotgreave et al., 1988).
Reduced GSH is the single most important reactant in the removal of the GSH moiety from the protein mixed disulphides. As the concentration of GSH drops, during the maintenance of the cellular redox defences, GSSG is produced resulting in mixed disulphides. The decrease in the [GSH] further compromises the cell's ability to remove the mixed disulphides from proteins (Bellomo et al., 1987). The formation of GSH-protein disulphides has been reported in perfused liver tissue treated with the redox active dipyrdilium compound, paraquat (Brigelius et al., 1982), with hydroperoxides (Brigelius et al., 1983), in cultured macrophages treated with various oxidants (Tsukahara et al., 1987), during respiratory burst in human neutrophils (Chai et al., 1994), and in human endothelial cells after treatment with oxidant chemicals (Schuppe et al., 1992).

It has been suggested that the presence of protein bound-GSH may provide an "emergency" pool of GSH that could be mobilised according to the needs of the cell (Modig, 1968; Kosower & Kosower, 1978). Other studies have suggested that the formation of these mixed disulphides represents a physiological mechanism for the modulation of enzyme activity (Gilbert, 1982; Brigelius, 1985). Mixed disulphides between GSSG and proteins have been shown to affect the activities of a number of enzymes and ion transporters. In a review of the role of thiol-disulphide exchange in cellular metabolism, Cotgreave et al., (1990) listed 23 examples of enzymes that have been demonstrated to have their activities modulated in vitro as a result of S-thiolation with glutathione. These enzymes have a diverse range of cellular functions from carbohydrate metabolism to protein kinase and Ca$^{2+}$-ATPase activities. Other examples include the activation of glycogen phosphorylase a (Heinle, 1982), the inhibition of adenylate cyclase (Baba et al., 1978), phosphofructokinase (Gilbert, 1982; Walters & Gilbert, 1986), 3-hydroxy-3-methylglutaryl-CoA reductase (Cappel & Gilbert, 1988) and pituitary growth hormone-releasing factor receptor binding (Lefrancois et al., 1995). Intracellular GSH and GSSG levels have also been shown to regulate transcription factor induction and gene expression (Bergelson et al., 1994, Eposito et
Along with the possible roles of protein-S-glutathiolation as a source of GSH, and in the modulation of enzyme activity, Brigelius (1985) suggested GS-protein mixed disulphide formation may protect delicate cellular structures against oxidative damage. Li et al. (1994) demonstrated the S-glutathiolation of carbonic anhydrase III in rat hepatocytes subjected to chemical-induced oxidative stress. They proposed this protected the enzyme against irreversible oxidation. However, when the cell is depleted of reduced GSH, its capacity to remove the mixed disulphides is lost and the original activity of the thiolated proteins cannot be readily recovered (Kosower & Kosower, 1978).

GSH and the S-thiolation of proteins have also been implicated in intracellular signalling. Aspects of the effects of insulin signalling can be mimicked by sulphydryl reagents (Czech, 1980) with the receptor signalling process having been shown to involve an alteration to the thiol status of insulin-receptor complex (Varandani & Nafz, 1986, Wilden & Pessin, 1987). GSSG has been postulated to be the ultimate second messenger to insulin, and to modulate the activity of critical enzymes in glucose metabolism through the formation of protein-glutathione disulphide bonds (Cotgreave et al., 1990). These results support the proposition that S-glutathiolation of proteins may have an important role in intracellular signalling.

1.4.3 Cell death and glutathione

The damage caused within cells by acute oxidative stress has been well described. This damage is mediated through the formation of free radicals capable of inactivating macromolecules. Such damage affects DNA, proteins, lipids and carbohydrates (Jones, 1985). It has been shown that necrotic cell death induced by oxidative stress is preceded by a drop in the intracellular GSH concentration (Nicotera & Orrenius, 1986).
Pretreatment of cells with compounds that deplete GSH potentiate necrotic cell death (Di Monte, 1984). Moreover, GSH has been shown to give protection to cells against necrosis-inducing prooxidant treatments (Jochmann et al., 1994; Cossarizza et al., 1995).

GSH metabolism has also been recognised as having a role in prooxidant-induced apoptosis. Prooxidant treatments induce either apoptosis or necrosis in a dose dependent manner. These include H$_2$O$_2$, cyano-hydroxybutene and 2,3-dimethoxy-1,4-naphthoquinone, (Lennon et al., 1991; Wallig et al., 1992; Dypbukt et al., 1994), with low concentrations able to stimulate apoptosis while higher concentrations induce necrosis. In T cells with deficient glutathione peroxidase activity, and therefore a reduced ability to deal with reactive oxygen species, lipid peroxides are able to induce apoptosis (Sandstrom et al., 1994b). In embryology, oxidative stress-induced apoptosis has been suggested as a means by which tissue modelling occurs (Pierce et al., 1991; Parchment, 1991; Ratan et al., 1994a). The physiological roles of both TNF$_\alpha$ and nitric oxide (NO) have been linked to their abilities to induce apoptosis as a result of the intracellular generation of free radicals (Buttke & Sandstrom, 1994; Albina et al., 1993; Larrick & Wright, 1990).

Other treatments, which are not recognised as prooxidant stresses, have also been shown to be sensitive to antioxidant inhibition. These treatments include serum withdrawal from dependent cell lines, activation-induced cell death (AICD) and dexamethasone treatment, (Sandstrom et al., 1993, 1994a; Stefanelli et al., 1995; Matsuda et al., 1991; Chang et al., 1992; Hirose, 1993; Ramakrishnan & Catravas, 1992). This has been a fertile area of research and Table 1-2 provides examples of apoptosis inhibited by antioxidants or treatments that supplement the intracellular GSH concentration.
Table 1-2 Apoptosis can be induced by prooxidants and inhibited with agents which supplement [GSH]

<table>
<thead>
<tr>
<th>cell type</th>
<th>induction mechanism</th>
<th>inhibitor</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>chronically HIV-infected human T cell line (8EA)</td>
<td>serum withdrawal or H$_2$O$_2$</td>
<td>catalase, Vitamin E, 2-mercaptoethanol,</td>
<td>Sandstrom et al., 1993</td>
</tr>
<tr>
<td>HIV-infected human T cell line (U937)</td>
<td>TNF</td>
<td>N-acetylcysteine (NAC)</td>
<td>Malorni et al., 1993, 1994</td>
</tr>
<tr>
<td>T cell hybridomas</td>
<td>activation-induced cell death</td>
<td>NAC</td>
<td>Sandstrom et al., 1994a</td>
</tr>
<tr>
<td>rat thymocytes</td>
<td>methylprednisolone, etoposide</td>
<td>dihydrolipoic acid, lipoamide</td>
<td>Bustamante et al., 1995</td>
</tr>
<tr>
<td>peripheral blood mononuclear cells</td>
<td>2-deoxy-d-ribose</td>
<td>NAC</td>
<td>Barbieri et al., 1994</td>
</tr>
<tr>
<td>thymocytes</td>
<td>H$_2$O$_2$</td>
<td>Trolox (water soluble vitamin E analog)</td>
<td>Forrest et al., 1994</td>
</tr>
<tr>
<td>pig epithelial cells</td>
<td>lipopolysaccharide and heat shock</td>
<td>dithiothreitol, NAC</td>
<td>Abello et al., 1994</td>
</tr>
<tr>
<td>neuron &amp; oligodendrocyte</td>
<td>nerve growth factor withdrawal, TNF$_\alpha$</td>
<td>NAC</td>
<td>Mayer &amp; Noble, 1994</td>
</tr>
</tbody>
</table>

When thymocytes and related cell lines were considered the same picture emerged. Apoptosis induced by many diverse agents involved an oxidation event (Hockenbery et al., 1993) and that this oxidation event, and therefore apoptosis, could be inhibited by antioxidant treatment (Ramakrishnan & Catravas, 1992). Hockenbery et al. (1993) proposed that the target of the implicated reactive oxygen species may be redox
regulated transcription factors such as NF-κB, Fos/Jun and other helix-loop-helix factors. This is a reasonable proposition for those cell types in which macromolecular synthesis is a concomitant step in the apoptotic process. However, not all cell types require the synthesis of new proteins as discussed above in section 1.1.2.2.3 Protein synthesis. In those cells there must be another target. Raff et al. (1993) suggested, that in many cells, the proteins required for the "death program" are likely to be constitutively expressed and subject to inhibition by a control protein, also constitutively expressed. They proposed that the apoptotic process could occur only when the function of the inhibitory control protein was prevented. Perhaps this control protein is subject to redox control in a similar fashion to the redox sensitive transcription factors, or has its activity altered through a process involving S-glutathiolation.

Recent observations have reported a fall in the cellular GSH concentration before the appearance of DNA fragmentation. This suggests GSH does have a role in apoptosis signalling (Wu et al., 1994; Slater et al., 1995; Beaver & Waring, 1995). Further evidence for the role of GSH in apoptotic signalling has come from experiments in which BSO was used to deplete GSH, which did not provoke apoptosis in itself. This treatment did allow alkylating agents to induce necrosis, rather than the apoptosis observed without GSH depletion (Fernandes & Cotter, 1994; Sato et al., 1995). Another interesting related observation was that macromolecular synthesis inhibitors prevented apoptosis by increasing the supply of intracellular cysteine available for the synthesis of GSH (Ratan et al., 1994b) by diverting it from protein synthesis. Any treatment that increases intracellular GSH would lead to a decrease in the S-glutathiolation of proteins. Taken together, these results suggest that the fall in [GSH], may not be the trigger for apoptosis in itself. Rather, the more important trigger might be the rise in [GSSG], upon GSH oxidation and the concomitant increase in protein S-glutathiolation.
1.4.4 Glutathione and the mitochondria

Mitochondria contain a pool of GSH equivalent to approximately 13% of the total cellular GSH in liver tissue (Wahländer et al., 1979). The mitochondrial store of GSH is essential to maintenance of mitochondrial function because the mitochondrial electron transport chain is a source of reactive oxygen species (Chance et al., 1979; Cadenas, 1989). Many studies have demonstrated a direct relationship between the loss of mitochondrial GSH and a decrease in cellular viability, especially under conditions that involve a disruption of calcium homeostasis (Olafsdottir et al., 1988; Thomas & Reed, 1988a, b). The opening of a non-specific pore in the inner mitochondrial membrane during phase transition has been shown to lead to a rapid and nearly complete depletion of the mitochondrial GSH reserve (Savage et al., 1991; Broekemeier et al., 1989; Halestrap et al., 1993).

1.5 Aims and Scope

Apoptosis has been a subject of research in this laboratory for some years now, particularly the induction of death by gliotoxin in macrophages and T blasts. The aims of this study were developed from these existing research interests.

1. Define for the most effective incubation times and concentrations for the induction of apoptosis in thymocytes by gliotoxin and thapsigargin, and in P815 cells by thapsigargin. Confirm the reliability of the assays for apoptosis with the use of dexamethasone as a positive control.

2. Determine if an increase in the intracellular [Ca^{2+}] was a consistent feature of apoptosis in thymocytes as had been reported in the literature. Use thapsigargin, gliotoxin and dexamethasone to determine their effect on [Ca^{2+}], in thymocytes with the use of bulk cell and flow cytometric fluorimetric assays. Compare the results with those obtained from the study of the onset of apoptosis.
3. Investigate the possible relationship between the increase in $[\text{Ca}^{2+}]_i$, stimulated by thapsigargin and the onset of mitochondrial dysfunction and apoptosis.

4. Investigate other effects of thapsigargin, such as protein synthesis inhibition, the effect of thapsigargin on dexamethasone-induced apoptosis, and cell cycle disruption.

5. Inquire into whether glutathione has a role in apoptotic signalling. This includes making measurements of the relative intracellular [GSH] and looking for an increase in S-glutathiolated proteins after apoptosis.

6. Make preliminary investigations into changes in the glycosylation profile of the plasma membrane of apoptotic cells. Use a flow cytometric method with the fluorescein-conjugated lectin wheat germ agglutinin to detect N-acetylglucosamine on apoptotic thymocytes.
Materials and Methods

2. Materials and Methods

2.1 Stock solutions and tissue culture

2.1.1 Solutions stored at -20°C
Stock solutions were prepared as folloew and stored at -20°C:

- 2,5-di-[1-butyl]-1,4-hydroquinone (MBH) (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) as 450 mM in methanol.
- Cyclohexamide (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) as 14.9 mM in CH_{3}CN.
- Dexamethasone as 20 mM in ethanol.
- Hoechst 33342 (Molecular Probes, Eugene, OR, USA) in ethanol (stock made up at 1 mg/mL in DMSO and used immediately).
- Tunicamycin prepared in our laboratory from Penicillium决ree (Vilgalys et al., 1997) and purchased from Sigma (MO, USA).
- Gliotoxin prepared as 25.9 mM in dimethylsulfoxide (DMSO). Gliotoxin from both sources produced equivalent results.
- Hoechst 33342 (Hoe342) (Molecular Probes, Eugene, OR, USA) as 10 mM in ddH2O.
- Malathion (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) as 100 µg/mL in ddH2O.
- Monobromobimane (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) as 63 mM stock in acetonitrile.
- N-Ethylmaleimide (NEM) (Fluka AG, Buchs SG, Switzerland) as 50 mM in ddH2O.
- O-phthalaldehyde (OPT) (Sigma) as 100 mM in DMSO.
- Oxidised glutathione (GSSG) (Sigma) as 2.5 mM in deionised-distilled water (ddH2O).
2.1 Stock solutions and tissue culture

2.1.1 Solutions stored at -20°C

Stock solutions were prepared as follows and stored at -20°C:

- **2,5-di-(t-butyl)-1,4-hydroquinone (tBHQ)** (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) as 450mM in methanol;
- **cyclopiazonic acid** (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) as 14.9mM in CH₃Cl;
- **dexamethasone** as 20mM in ethanol;
- **Fluo-3 AM** (Molecular Probes, Eugene, OR, USA) in individual 50 µg vials, made up at 1µg/µL in DMSO and used immediately;
- **gliotoxin** prepared in our laboratory from *Penicillium billai* (Waring et al., 1987) and purchased from Sigma Chemical Co (St. Louis, MO, USA), prepared as 25.5mM in dimethylformamide (DMF). Gliotoxin from both sources produced equivalent results;
- **Hoechst 33342 (Ho342)** (Molecular Probes, Eugene, OR, USA) as 10mM in ddH₂O;
- **maitotoxin** (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) as 10ug/mL in ddH₂O;
- **monobromobimane** (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) as 50mM stock in acetonitrile;
- **N-ethylmorpholine (NEM)** (Fluka AG, Buchs SG, Switzerland) as 50mM in ddH₂O;
- **o-phthaldialdehyde (OPT)** (Sigma) as 100mM in DMF;
- **oxidised glutathione (GSSG)** (Sigma) as 0.5mM in deionised distilled water (ddH₂O).
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Procysteine® (gift from Dr Cynthia Leaf, Free Radical Sciences Inc., Cambridge, MA, USA) 1M in PBS;

Pronase (Calbiochem) 10mg/mL in ddH2O;

propidium iodide (Molecular Probes, Eugene, OR, USA) as 400µg/mL in phosphate buffered saline (PBS);

reduced glutathione (GSH) (Sigma) as 50mM in PBS;

Rhodamine 123 (R123) (Molecular Probes) as 10^3µg/mL in PBS (26µM)

RNase (Sigma) as 20mg/mL in ddH2O;

[^35]Sglutathione (NEN Research Products, Dupont, De, USA) stored as supplied in ddH2O with 10mM dithiothreitol;

[^35]Smethionine (ICN Biomedicals Inc., Irvine, CA, USA) stored as supplied;

thapsigargin (Calbiochem, La Jolla, CA, USA) as 3mM in dimethylsulphoxide (DMSO);

2.1.2 Solutions stored at room temperature

Stock solutions were prepared as follows and stored at room temperature:

Digitonin (ICN Biochemicals) as 0.9mM in absolute ethanol

Ethidium bromide (Sigma) at 1mg/mL in phosphate buffered saline (PBS), stored wrapped in foil.

2.1.3 Tissue Culture

The incubation medium was Eagle’s minimum essential medium, F15, (GIBCO, Grand Island, NY, USA) plus 5% foetal bovine serum (FBS) (CSL Ltd, Parkville, Vic, Australia And Flow Laboratories Inc., Irvine, CA, USA), except for the estimation protein synthesis inhibition, where minimum essential medium Eagle (modified; without methionine and
cysteine) with Earle’s salts (EMEM) (Flow Laboratories Inc., Irvine, CA, USA) was used.

2.2 Animals
All mice were 10 day old Balb/c obtained from Animal Services at the Australian National University.

2.3 Tissue preparation and cell culture

2.3.1 Thymocytes
Thymus tissue was collected from 10 day old mice in F15 + 5% FBS, passed through a stainless steel mesh, filtered through Nybolt nylon monofilament gauze (pore size 60 micron), suspended in F15 + 5% FBS at 1 x 10^6 cells/mL for treatment in all experiments except where otherwise stated. The cells were incubated with various experimental treatments, for time periods up to 18 hours in Costar 12 well or 6 well tissue culture plates or tissue culture flasks.

2.3.2 P815 cells
P815 cells were cultured in F15 + 5% FBS at 37°C in 5% CO₂ and harvested in log phase growth. These cells were pelleted at 300g for 5 minutes at 4°C and resuspended in fresh F15 + 5% FBS for treatment with a cell concentration of 2 x 10^5 cells/mL. The cells were incubated for time periods up to 24 hours in Costar 12 or 6 well tissue culture plates.

2.4 Estimation of apoptosis

2.4.1 Agarose gel electrophoresis
For DNA fragmentation the thymus was collected from 10 day old mice in ice cold F15 + 5% foetal bovine serum, passed through a stainless steel mesh, filtered through Nybolt nylon monofilament gauze (pore size 60 micron), and centrifuged at 300g for 5 minutes at 0°C. The cells were resuspended in F15 media + 5% FBS at 1 x 10^6 cells/mL and incubated for 6 hours at 37°C in 5% CO₂. The DNA was isolated as previously described (Braithwaite et al., 1987). In brief, after treatment cells were
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2.4.1 DNA analysis

Cells were washed in PBS and then resuspended in 0.5mL PBS followed by an equal volume of Pronase buffer (200mM NaCl, 10mM EDTA, 20mM TrisHCl, 1% sodium dodecylsulphate, pH 8.0) and 50µL of 10mg/mL Pronase solution. After an overnight incubation at 37°C the solution was extracted with phenol/chloroform, the DNA precipitated in 10 volumes of absolute ethanol at -70°C, washed in ice cold absolute ethanol, rehydrated in 100µL 10mM EDTA, and treated with 5µL of 20mg/mL RNase overnight at 37°C. The DNA was fractionated using electrophoresis in 1.5% agarose gels containing 50µg/mL ethidium bromide with Tris-acetate buffer, pH 8.0 at 60-80mA for 4-6 hours. Gels were photographed under UV light (Transilluminator, Ultra-Violet Products Inc., San Gabriel; Polaroid film 57) and negatives scanned using laser densitometry (LKB 2222-010 UltraScan XL or a Nova Line Gel Documentation System with QGel software). Percent DNA fragmentation was determined by estimating the relative areas of the scans (Waring et al., 1988b) and with the QGel software.

2.4.2 Electron microscopy

For electron microscopy, cells were prepared and incubated as for DNA analysis. After incubation the cells were washed once in PBS and then pelleted and fixed with 2% glutaraldehyde in 0.1M sodium cacodylate (pH 7.4) for 2 hours. Preparations were post-fixed in osmium tetroxide for 1.5 hours and en bloc stained in 2% uranyl acetate for 1 hour, then dehydrated in ethanol and embedded in Spurrs resin. Sections were cut and viewed on a Philips 301 electron microscope. The sample fixing, staining and section cutting for electron microscopy was conducted by Cathy Gillespie and her colleagues in the Electron Microscopy Unit at the John Curtin School of Medical Research, Australian National University. Sections chosen were randomly selected. The technician did not view any sections before choice was made.

2.4.3 Ethidium bromide

The cells for flow cytometry were prepared as for DNA analysis. After incubation at 37°C in 5% CO₂ the cells were pelleted at 300g for 5 minutes at 4°C, the supernatant discarded, the cells resuspended in 100µL
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ethidium bromide (EB) stock solution (1mg/mL) and incubated on ice in the dark for 20 minutes. The cells were washed 3 times using PBS with 0.1% w/v BSA and resuspended in approximately 200µL of residual supernatant after the final wash ready for flow cytometric analysis using a Becton Dickinson FACScan. The fluorescence was collected in fluorescence channel 2 (Fl2) (585 /42nm bandpass filter). The method used is based on that of Lyons et al. (1992) with modifications.

2.4.4 Propidium iodide

The percentage of apoptotic cells in a sample was estimated with propidium iodide (PI) staining of 1 x 10^6 ethanol fixed cells (Nicoletti et al., 1991, Darzynkiewicz et al., 1992). The thymocytes after incubation were pelleted at 300g, 5 minutes, 0°C, resuspended in 1mL PBS, then mixed with 3 mL 95% ethanol and left for at least 2 hours or overnight at 4°C. After fixing, the cells were washed twice in PBS, the pellet resuspended in 1780µL PBS, 200µL propidium iodide stock and 20µL RNase stock solution, and incubated for at least 30 minutes at room temperature in the dark. Flow cytometric analysis of the samples was conducted using a Becton Dickinson FACScan. Data was collected on fluorescence detector 2 (Fl2) (585/42nm band pass filter) at 1024 resolution with the mean of the diploid G_0/G_1 peak positioned approximately at channel 200. Apoptotic cells were deemed to be those with a fluorescence of less than those in the G_0/G_1 peak.

2.5 Analysis of flow cytometric data

All flow cytometric data were analysed using WinMDI software kindly provided by Joseph Trotter at the Salk Institute, La Jolla, CA, USA.

2.6 Statistical analysis of results

Statistical analysis was conducted with SYSTAT for Windows, Version 5 (SYSTAT Inc. Evanston, IL, USA).

All error bars on graphs define the range of duplicate results unless otherwise stated in the legend. Statistical analysis of significance was only conducted on results conducted in greater than duplicate.
2.7 Calcium assays

2.7.1 Bulk cell fluorimetry

For measurement of the cytosolic calcium concentrations by bulk cell fluorimetry, the thymus was collected from 10 day old mice in ice cold Puck's saline, passed through a stainless steel mesh, filtered through gauze, and cells pelleted at 300g for 5 minutes at 0°C. The cells were resuspended in F15 media at $2.5 \times 10^7$ cells/mL and incubated with fluo-3 AM at a final concentration of $5 \mu$g/mL for 1 hour at 37°C in 5% CO$_2$. After incubation they were washed twice with PBS (300g, 0°C, 5 minutes) and resuspended in Hepes buffered saline (140mM NaCl, 5mM KCl, 1.8mM CaCl$_2$, 1mM MgCl$_2$, 10mM glucose, 10mM Hepes, pH 7.4) at $2.5 \times 10^7$ cells/mL and kept on ice until used (within 2 hours). Changes in $[\text{Ca}^{2+}]_i$ were measured over total time periods of 12 or 20 minutes. The cells were mixed with 37°C Hepes buffered saline in a 1.5mL Suprasil cuvette to a concentration of $1.25 \times 10^6$ cells/mL and left to equilibrate in the spectrophotometer for at least 90 seconds. An Hitachi F-3000 fluorescence spectrophotometer was used with a thermostatically (37°C) controlled cuvette cradle and magnetic stirrer. To measure the calcium bound fluo-3, the instrument was set with the excitation and emission wavelengths at 505nm and 530nm respectively; the band width setting was 5nm with time points taken at 30 second intervals. The toxin, either thapsigargin or gliotoxin, was added after the background fluorescence had stabilised. Two minutes before the end of the time course digitonin was added at 30µM to obtain $F_{\text{max}}$. The average autofluorescence values for cells incubated without fluo3-AM were used as $F_{\text{min}}$ in calcium concentration calculations (Merritt et al., 1990). Calcium ion concentrations were calculated using $[\text{Ca}^{2+}]_i = K_d(F-F_{\text{min}})/(F_{\text{max}}-F)$; using $K_d = 864$ (Merritt et al., 1990).

2.7.2 Flow cytometric estimations

For measurement of $[\text{Ca}^{2+}]_i$, using flow cytometry, the bulk cell method from (Merritt et al., 1990) was modified. Thymocytes were collected and
prepared as for DNA analysis, except that only 2% FBS was included in the medium because of the bubble free requirements of the flow cytometer. The cells were diluted to 2 x 10^7 cells/mL. Prior to each run, 400μL of cell suspension was incubated with 2.4μL fluo-3 (1μg/μL) for 10 minutes in a 37°C heating block. Immediately thereafter 100μL of the stained cells was mixed with 1900μl F15+2% FBS and loaded onto a Becton Dickinson FACStar Plus. After 2 minutes the toxin or volume of control medium was added to the suspension and data acquisition was continued. The fluo-3 was excited with an argon ion laser of 200mW and its fluorescence collected on Fl1 with a 530nm filter. Time was used as a parameter and the data collected for 5120 seconds (85.3 minutes) and 6 x 10^6 cells were counted. The data was analysed using WinMDI analysis program. Only relative \([\text{Ca}^{2+}]_i\) were estimated using this method because of the difficulties of obtaining Fmax values. Only four assays were completed with each thymus preparation; the treated cells were run first in 50% of pairs of assays and a control was run first in the other assay pairs.

2.8 Glutathione assays

2.8.1 Flow cytometric assay

The proportion of cells with normal or low [GSH], was estimated using the Becton Dickinson FACStar Plus after staining live cells with OPT after the method of Treumer et al., 1986. The cultured cells (1 x 10^6 cells/mL) were washed twice in PBS, resuspended in 500μL of Hank’s buffered saline solution (HBSS) and 5μL OPT stock, incubated for 5 minutes at room temperature and analysed immediately. The instrument was triggered using a 488nm laser line with the OPT being excited using a UV laser on a dual laser bench. Subsequent fluorescence was collected in fluorescence detector 3 (Fl3-2), filter 405/30 band pass (measure of OPT binding to protein) and in fluorescence detector 4 (Fl4) filter 520 long pass (measure of OPT binding to GSH); between Fl3-2 and Fl4 there was a 440nm beam splitter.
2.8.2 HPLC assay
To assay for GSH in cell homogenates by HPLC, 2 x 10^8 cells were harvested after treatment and pelleted. The supernatant was aspirated and after the addition of 300µL of PBS, the pellet was freeze/thawed 3 times using dry ice/ethanol and 37°C water bath. For the sample derivatization 120µL of the cell homogenate was transferred into a microfuge tube, or 120µL PBS for the blank, and pelleted in a microfuge for 5 minutes at 4°C. After 100µL of supernatant were transferred to a clean microfuge tube and 100µL 2.5mM mBBr in 50mM NEM pH 8.0 was added, the tube was incubated in the dark for 5 minutes at 37°C. After incubation, 10µL of 100% TCA was added, vortexed, pelleted for 5 minutes at 4°C and the supernatant taken and 25µL injected. The HPLC separation of the mBBr adducts was performed with a Nova-Pak C18, 4µm cartridge (Waters) using a Waters 600MS System Controller and Waters 717 Autosampler. The fluorescent adducts were detected using a Waters 470 Fluorescence detector. The mobile phase was as described previously (Cotgreave & Moldéus, 1986).

2.8.3 Glutathione labelling of proteins
P815 cells were cultured and harvested in log phase growth as described above in 2.3.2 Tissue preparation and cell culture - P815 cells. The cells (2 x 10^5 cells/mL) were incubated with 42µCi of [35S]glutathione for 24 hours, then for a further 24 hours with added 10nM thapsigargin or solvent control. After the total 48 hour incubation the cells were pelleted at 300g for 5 minutes at 4°C. The pellet was rapidly resuspended in 200µL of lysis solution (6.6% glycerol and 1.3% SDS), boiled for 5 minutes, then cooled and 20uL of DNase/RNase (1mg/mL Dnase I, 0.5mg/mL Rnase A, 50mM MgCl2, 50mM Tris-HCl pH 7.0) added and the mixture stored at -20°C until used for electrophoresis using 12% SDS-PAGE pH 8.3 (Laemmli, 1970). Sample was mixed with sample buffer in a ratio of three parts sample to one part buffer (62.5mM Tris-HCl, pH 6.8, 20% (w/v) glycerol, 2% (w/v) SDS), heated for 5 minutes at 95°C before loading on the gel, and run at
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200V 25mA for a 20cm square gel. The gel was fixed for 30 minutes with 20% acetic acid (Ajax Chemicals, Auburn, Australia), washed in ddH₂O for 30 minutes, enhanced for autoradiography in 1M sodium salicylate (BDH laboratory Supplies, Poole, England) for 30 minutes and dried onto filter paper before exposure for 4 days against Kodak X-OMAT X-ray film.

2.9 Assays of mitochondrial activity

2.9.1 Rhodamine 123
Thymocytes or P815 cells, after incubation at 1 x 10⁶ cells/mL with various concentrations of thapsigargin or solvent control, were pelleted at 300g for 5 minutes at 4°C, and resuspended in the residual supernatant. To this suspension was added 100µL of 1µg/mL R123 for 10 minutes at room temperature in the dark. After staining with R123 the cells were washed three times with cold PBS and then immediately analysed using a Becton Dickinson FACScan; fluorescence was collected in fluorescence detector 1 (Fl1), filter 430/30nm band pass.

2.9.2 Rhodamine 123 and Hoechst 33342
P815 cells, after incubation at 1 x 10⁵ cells/mL with various concentrations of thapsigargin or solvent control, were pelleted at 300g for 5 minutes at 4°C, and resuspended in the residual supernatant. To this suspension was added 100µL of 1µM Ho342 for 30 minutes at 37°C in the dark and then 100µL of 1µg/mL R123 for a further 10 minutes at room temperature in the dark. After staining the cells were washed three times with cold PBS and then immediately analysed using a Becton Dickinson FACStar Plus; fluorescence from R123 was collected in fluorescence detector 1 (Fl1), filter 430/30nm band pass and from Ho342 in fluorescence detector 32 (Fl32) filter 424nm bandpass dichroic ± 44nm.

2.9.3 ATP concentration
After treatment at 2 x 10⁵ cells/mL (10mL total volume) with thapsigargin or dexamethasone, thymocytes were pelleted at 300g 4°C for 5 minutes, washed twice with cold PBS and the pellet then suspended in 500µL cold
PBS. A 50µL aliquot of cells was taken for protein determination using the Biorad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA, USA), then an equal volume of 20% (v/v) trichloroacetic acid (TCA) (Sigma) in water was added and well mixed. After standing on ice for 10-15 minutes, the proteins were pelleted at 14,000 rpm in a microfuge for 10-15 minutes and the supernatant extracted three times with ether (saturated with water), and the ether extract discarded. After the final extraction residual ether was blown off the supernatant with an argon or nitrogen stream, and 100µL of the extract was injected onto a Whatmans Partasil SAX strong anion exchange column using a Beckman System Gold HPLC. A concave gradient over 80 minutes of 100% 10mM KH$_2$PO$_4$ pH 6.4 to 95% 500mM KH$_2$PO$_4$ pH 5.0 was used to elute the nucleotides. Standard ATP solutions were run with each determination. ATP levels were usually expressed as ng/µg protein. In some experiments the ATP was calculated as ng/million cells. Dr Paul Waring conducted the HPLC separation of the cell extract.

2.10 Cell Sorting

2.10.1 Confirmation of ethidium bromide staining method

Thymocytes suspended at 1 x 10$^6$ cells/mL in F15 + 5% FBS were incubated with or without 1µM gliotoxin for 6 hours at 37°C with 5% CO$_2$. After incubation, the cells were pelleted at 300g for 5 minutes at 4°C, stained with ethidium bromide as described above in 2.4.3 Estimation of apoptosis - Ethidium bromide. After being washed three times with PBS + 0.1% w/v bovine serum albumin (BSA) the stained cells were resuspended at 3 x 10$^6$ cells/mL in PBS + 0.1% w/v BSA and immediately sorted at 4°C using a Becton Dickinson FACStar Plus, into two populations according to the relative ethidium bromide FI2 fluorescence of the cells (filter 575/26 bandpass). The set up of the FACStar Plus flow cytometer was as described above in 2.4.3 Estimation of apoptosis - Ethidium bromide. The "normal" cells were designated as those cells with low ethidium bromide staining, and the "apoptotic" cells as those with increased staining. After sorting the cells were pelleted at 300g for 5
minutes at 4°C and then immediately fixed for electron microscopy as described in 2.4.2 Estimation of apoptosis - Electron microscopy.

2.10.2 Cell cycle analysis

P815 cells were collected from culture flasks, pelleted and resuspended in fresh F15 medium with 2% FBS at 4 x 10^6 cells/mL. The cells were sorted on a Becton Dickinson FACStar Plus into two populations according to size. Cells with the lowest forward scatter were designated "small", and the cells with the largest forward scatter were designated "large", and were collected and kept at 4°C until the sorting was complete. The population of intermediate size between small and large was not collected. The cells collected as "small" and "large" were then washed once with room temperature PBS and the resulting pellet resuspended and diluted in F15 + 5% FBS to 1 x 10^5 cells/mL. Aliquots of both the "small" and "large" populations were fixed immediately in ethanol and stained with propidium iodide as described above in 2.4.4 Estimation of apoptosis - Propidium iodide or used in further experiments.

2.11 Inhibition of protein synthesis

After a 1 hour incubation in methionine-free EMEM + 5% FBS at 37°C with 5% CO₂ to deplete the intracellular methionine concentration the cells were pulsed simultaneously with [³⁵S]methionine (50µCi) and thapsigargin or control solution for 1 hour. After this incubation the cell suspension was pelleted at 300g for 5 minutes 4°C, washed once with cold PBS and the supernatants discarded. After the second wash the pellet was resuspended in 2mL of 15% (w/v) trichloroacetic acid (TCA) and the resulting mixture incubated for 15 minutes on ice. After incubation on ice, the mixture was pelleted at 300g for 10 minutes, washed twice with 2mL of 15% (w/v) TCA, the supernatants discarded, and the resulting pellet resuspended in 500µL 15% (w/v) TCA, and the amount of [³⁵S]methionine incorporated determined by scintillation counting. The protein synthesised by the cells was expressed as: % protein synthesis = (cpm of test/cpm of control) x 100.
2.12 Cell surface glycosylation

Thymocytes, collected as described above in 2.3.1 Tissue preparation and cell culture - Thymocytes, were treated with various gliotoxin concentrations to induce apoptosis and then stained with ethidium bromide and the FITC-conjugated lectin wheat germ agglutinin (WGA). After incubation at 37°C in 5% CO₂ the cells were pelleted at 300g for 5 minutes at 4°C, the supernatant discarded, the cells resuspended in 100µl ethidium bromide stock solution (1mg/ml) and incubated on ice in the dark for 20 minutes. The cells were washed once using PBS with 0.1% w/v BSA and resuspended in 50µL stored of lectin solution (25µg/mL) on ice in the dark for 30 minutes, washed twice with PBS with 0.1% w/v BSA and resuspended in 200µL of residual supernatant after the final wash, ready for flow cytometric analysis using a Becton Dickinson FACScan. The fluorescence signal was detected in Fl1 (filter 530/30nm band pass).
3. Defining the onset of apoptosis

3.1 Introduction

The onset of apoptosis in the cell systems used in this project required defining before an attempt could be made to investigate the involvement of bacterial and goat thymus in apoptosis.

3.1.1 Detection of Apoptosis

3.1.1.1 General

The most easily identified changes associated with apoptosis are the condensation of chromatin visible in electron microscopy and DNA fragmentation. For many years, electron microscopy and assessment of electrophoresis were the only methods available for identifying the occurrence of apoptosis. Today, new techniques using flow cytometry and fluorescence microscopy are being used and refined.

When attempting to identify agents that induce apoptosis, care must be taken to distinguish between necrosis and cells. Some agents which prevent DNA fragmentation may in fact be killing the cells by necrosis. This is an important consideration when tests other than electron microscopy and used to detect apoptosis. Methods that rely on DNA fragmentation alone will frequently be unable to differentiate between normal and necrotic because the degradation of DNA within necrotic cells usually takes several days (reviewed in Campbell, 1985).

3.1.1.4.1 Electron microscopy

The identification of condensed chromatin in the nucleus of apoptotic cells using electron microscopy is the only sure way of identifying apoptosis. It was the appearance of chromatin condensation that was first used to define the process (Kerr et al., 1972). Recently, examples have been based in the literature of apoptotic DNA with condensation, fragmented DNA fragmentation, or internucleosomal DNA (Hedges et al., 1982). Therefore, it is important to confirm the results of other more rapid and easily quantified methods for detecting apoptosis with electron microscopic inspection.
3.1 Introduction

The onset of apoptosis in the cell systems used in this project required defining before an attempt could be made to investigate the involvement of calcium and glutathione in apoptosis.

3.1.1 Detection of Apoptosis

3.1.1.1 General

The most easily identified changes associated with apoptosis are the condensation of chromatin, visible in electron micrographs and DNA fragmentation. For many years, electron microscopy and agarose gel electrophoresis were the only methods available for identifying the occurrence of apoptosis. Today new techniques using flow cytometry and fluorescence microscopes are being used and refined.

When attempts are made to identify agents that inhibit apoptosis, care must be taken to differentiate between normal cells and necrotic cells. Some agents, which prevent DNA fragmentation, may in fact be killing the cells by necrosis. This is an important consideration when tests other than electron microscopy are used to detect apoptosis. Methods that rely on DNA fragmentation alone will frequently be unable to differentiate between normal and necrotic because the degradation of DNA within necrotic cells usually takes several days (reviewed in Campbell, 1983).

3.1.1.1.1 Electron microscopy

The identification of condensed chromatin in the nuclei of apoptotic cells using electron microscopy is the one sure way of identifying apoptosis. It was the appearance of chromatin condensation that was first used to define the process (Kerr et al., 1972). Recently examples have been cited in the literature of apoptotic chromatin condensation without DNA fragmentation, as mentioned above in 1.1.2.2.2 Endonuclease identity. Therefore, it is important to confirm the results of other more rapid and easily quantified methods for detecting apoptosis with electron microscopic inspection.
3.1.1.1.2 Agarose gel electrophoresis

Agarose gel electrophoresis provides a simple method of visualising the fragmentation of DNA extracted from a population of cells. The major disadvantage of this technique is the difficulty associated with quantification. With this method, the DNA from all the cells in a treated sample, perhaps a million cells, is run as a single sample on a gel. At best the percentage of fragmented DNA can give a feel for the extent of DNA fragmentation in the entire population, but cannot specify what percentage of the cells contained fragmented DNA.

The other disadvantages with agarose gel electrophoresis include the time consuming nature of the DNA preparation, and the difficulty of distinguishing between normal and necrotic cells; both types of cells will have intact DNA after incubation times of up to at least 24 hours.

3.1.1.1.3 Fluorescence methods

3.1.1.1.3.1 General

The development of fluorescence methods for the detection of apoptosis has improved both the speed and the quantification of the onset of apoptosis. Some methods have been devised specifically for the flow cytometer and others for use with fluorescence microscopes. More recently methods have been adapted to suit both instruments. In general, the fluorescence methods depend either on recognising a small change in membrane permeability or on detecting the fragmentation of DNA.

The major advantage of these methods is the ability to record data about individual cells thus allowing the statistical analysis of the response of thousands of cells to treatment. Some methods are better than others for the differentiation of necrotic and normal cells. The technique chosen must depend not only on the availability of equipment but very importantly on what the experiment is trying to detect. In this project the two fluorescent dyes ethidium bromide and propidium iodide have been used to detect apoptosis with a flow cytometer.
3.1.1.3.2 Differential membrane permeability

Ethidium bromide (EB) is a fluorescent dye which intercalates into the structure of DNA. It has poor membrane permeability in healthy cells but is able to rapidly enter necrotic cells. On this basis, ethidium bromide has been used to differentiate between viable and non-viable cells (Mishell et al., 1980). It has been used in fluorescent microscopy methods to detect apoptosis, especially in conjunction with other dyes that can more easily permeate the plasma membrane. Duke et al. (1994) used EB in conjunction with acridine orange, a dye that readily enters even healthy cells and intercalates into DNA and RNA. They assumed EB was only taken up by non-viable cells (necrotic cells).

Ethidium bromide has the potential to allow differentiation between apoptotic and necrotic cells, and also between cells that have recently entered the apoptotic process and those which have further progressed in the process. Lyons et al. (1992) developed a method that is very effective when used with thymocytes. This method relies on early apoptotic cells showing a slight increase in permeability to EB molecules.

Analysis of EB staining of unfixed cells for flow cytometry involves the placement of three regions on a plot of the EB fluorescence signal versus FSC (see 3.2.1.2 Ethidium bromide staining can distinguish between apoptosis and necrosis and Figure 3-2). Normal cells appear in Region1 with moderate forward scatter and low EB staining. Apoptotic cells appear in Region2. This is a population of cells with slightly increased EB fluorescence and slightly decreased forward scatter, that is, reduced volume. These are the cells that have most recently entered the apoptotic process. Over time this population of cells moves to a position of highly increased EB staining associated with the development of apoptotic bodies that are no longer able to maintain their membrane integrity (Region3). Necrotic cells immediately appear in the highly staining region and generally show increased forward scatter (also Region3). EB staining
to detect apoptosis is best used with thymocytes. This method is further discussed in the Results and Discussion sections of this chapter.

Another method that relies on the change in membrane permeability associated with the onset of apoptosis uses Hoechst 33342 dye to stain the cells before analysis with flow cytometry (Ormerod et al., 1993). This technique does not appear to allow the observation of two separate populations of apoptotic cells and requires UV laser excitation which is not available in all laboratories.

3.1.1.1.3.3 DNA fragmentation
3.1.1.1.3.3.1 DNA histograms

The induction of apoptosis has been shown to be accompanied by an appearance of cells in a sub-G\textsubscript{1} peak in a DNA histogram in cultures of mouse thymocytes (Nicoletti et al., 1991; Compton et al., 1988b; Telford et al., 1991; Walker et al., 1991), a mouse haemopoetic cell line (Rodriguez-Tarduchy et al., 1990), and a pre-B cell line (Ormerod et al., 1992). When the cells are fixed, for example with 70% ethanol, the plasma membrane becomes permeable to smaller molecules. It is thought that the smaller DNA fragments are leached from the cell and the total cell fluorescence of dye-bound-DNA is consequently reduced. Thus, in these assays, the loss of fluorescence is associated with the nucleosomal fragmentation characteristic of apoptosis. In the experiments conducted in this study, propidium iodide has been used as the fluorescent dye and the cells were fixed with 70% ethanol.

The use of DNA histograms to quantitate apoptosis gives quick and reproducible results that are statistically significant because of the large number of cells counted in each assay. This method is able to detect only those cells with fragmented DNA. This can lead to an underestimation of apoptotic cells when the result is compared to the numbers of cells with condensed chromatin counted in electron micrographs. Furthermore in long incubations, when apoptotic bodies undergo secondary necrosis (Wyllie et al., 1980), all DNA is lost from the cell particles as they lyse and
therefore they no longer bind PI and be registered by the cytometer. Another consideration is the inability of this method to differentiate between normal and necrotic cells.

In summary, the use of fluorescent DNA intercalating dyes to stain fixed cells for the quantitation of apoptosis provides an excellent, rapid and reproducible method, and especially useful in a well-defined system. If however, there is some risk of necrosis being an outcome of the cell treatment, the results should be confirmed by other methods, such as EB staining and electron microscopy.

3.1.1.1.3.3.2 Fluorescent labelling of strand breaks

Methods developed to label strand breaks directly are becoming increasing popular. They have two major advantages. Firstly the DNA fragmentation can be directly measured with labelling at each break rather than indirectly as in the DNA histogram method. Secondly, the technique can be readily applied to tissue sections, even after they have been embedded in paraffin.

Labelling of strand breaks can be accomplished in two main ways; either labelling with nick translation using an exogenous DNA polymerase (Gorczyca et al., 1992; Wijsman et al., 1993) or, with a terminal deoxynucleotidyl transferase (Gorczyca et al., 1992, 1993). As with the DNA histogram method described above, these methods cannot recognise the difference between normal and necrotic cells until a long enough time has elapsed to allow for non-specific DNA degradation. Therefore, they cannot be easily used to differentiate between actual inhibition of apoptosis and the alternative inhibition of apoptosis by the necrotic killing of the cells.

3.1.1.1.3.4 DNA structure

An early feature of apoptotic cell death is the condensation of the chromatin. The detection of apoptosis by electron microscopy relies on this hallmark characteristic. To enable a more rapid estimation of apoptotic
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cells, assays have been developed which rely on changes in the fluorescence of the DNA-binding dye that result when the DNA is packed differently in normal and apoptotic cells.

Both bisbenzamide (Hoechst 33342) (Duke et al., 1994) and acridine orange (Hotz et al., 1994; Darzynkiewicz et al., 1993) have been used with fluorescence microscopes and flow cytometry. The efficacy of these methods in each new cell system tested would need to be confirmed by comparison with other techniques.

3.1.2 Cell Types and Agents Used in this Project

3.1.2.1 Cell Types

Thymocytes taken from 10 day old Balb/c mice were used in this project. These mice were still suckling and had large thymuses that had not yet begun the atrophy associated with the onset of sexual maturity (Ritter & Crispe, 1992). Thymocytes provide an excellent model for investigations into the processes associated with the onset of apoptosis because they are the archetypal apoptotic cell. Greater than 80% of the cells die during negative selection (Joel et al., 1977). Apoptosis in these cells is characterised by DNA fragmentation and chromatin condensation (Wyllie, 1980, 1984).

The second cell type used in this project was the mastocytoma P815 cell line. This cell line was chosen because it had a rapid cell cycle and a great many mitochondria per cell. Apoptosis in this cell line also displayed all the characteristics described above for thymocytes.

3.1.2.2 Agents

3.1.2.2.1 Gliotoxin - a fungal toxin

Gliotoxin is a fungal metabolite of the epipolydithiodioxopiperazine (ETP) class of compounds (Taylor, 1971; Nagarajan, 1984). It was isolated from the fungus Gliocladium fimbriatum (Johnson et al., 1943) and has since been isolated from many different fungi including Aspergillus fumigatus (Glister & Williams, 1944) and Penicillium tertlikowskii (Johnson et al.,...
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1953). The structure of gliotoxin was predicted in 1958 (Bell et al., 1958) and confirmed in 1966 (Beecham et al., 1966).

Gliotoxin has been shown to have a number of biological activities. The toxic activities of the molecule include a wide range of antibacterial, antifungal and anti-viral activities (Taylor, 1971). It can inhibit viral replication by inhibiting the activity of reverse transcriptase enzymes from various viral organisms (De Clercq et al., 1978) and by affecting the activity of RNA polymerases (Rightsel et al., 1964, Miller et al., 1968).

In eukaryotic cells it has also been demonstrated to have toxic activities. It has been demonstrated to inhibit the transfer of a farnesyl tail to the Ras protein (Van der Pyl et al., 1992). This prevents proper attachment of the Ras protein to the inner plasma membrane (Hara & Han, 1995) and therefore interferes with Ras-dependent signalling. Gliotoxin has also been shown to be a potent inhibitor of protein synthesis (Eichner et al., 1986).

Gliotoxin has potent immunosuppressive activity. This was first observed when the ability of macrophages to adhere to plastic surfaces was compromised by an accidental contamination of a macrophage culture with a gliotoxin-producing laboratory strain of Aspergillus fumigatus (Mülbacher & Eichner, 1984). Gliotoxin was then shown to inhibit lymphocyte proliferation (Mülbacher et al., 1985), macrophage phagocytic activity (Eichner et al., 1986), and T cell activity (Mülbacher et al., 1986). This immunosuppressive effect was utilised when gliotoxin treatment of lymph node cell-enriched bone marrow was shown to significantly delay the onset of graft versus host disease in fully allogenic bone marrow chimeras. In this case gliotoxin treatment selectively inhibited the proliferation of mature T and B cells, but not the ability of the bone marrow cells to recolonise lethally irradiated recipients (Mülbacher et al., 1987; Kobayashi et al., 1991). Gliotoxin has also been shown to have a marked inhibitory effect on Langerhans cell function. It has been demonstrated to
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inhibit contact hypersensitivity responses and enhance skin graft survival (McMinn et al., 1990).

Gliotoxin appears to exert its immunosuppressive effects through its ability to induce the characteristic cellular changes associated with apoptosis. This phenomenon has been described in macrophages and T lymphocytes (Waring et al., 1988b).

The activity of gliotoxin in cells is related to the presence of a bridge disulphide within the molecule (Müllbacher et al., 1986; Mason & Kidd, 1951; Middleton, 1974). A suggested mechanism for the anti-viral action of gliotoxin involves the formation of mixed disulphides between gliotoxin and proteins (Mason & Kidd, 1951). Evidence for this mechanism is mounting (Waring et al., 1988a; Petty, 1985) especially since it has been shown to inhibit the activity of an enzyme by the formation of a mixed disulphide with a specific cysteine residue (Waring et al., 1995b).

In this chapter I have described the effect of gliotoxin on thymocyte preparations and demonstrated gliotoxin could induce either apoptosis or necrosis depending on the treatment concentration. The mode of cell death was defined by a number of methods; electron microscopy, agarose gel electrophoresis and flow cytometric methods.

3.1.2.2 Modulators of $[\text{Ca}^{2+}]_i$

The calcium ion concentration in the cell's cytosol ($[\text{Ca}^{2+}]_i$) can be modulated in many ways. As part of this study into the role of calcium ion fluxes in the onset of apoptosis, chemical treatments were chosen that would cause increases in $[\text{Ca}^{2+}]_i$ either by inhibition of the endoplasmic reticulum $\text{Ca}^{2+}$-ATPase or through stimulation of a plasma membrane $\text{Ca}^{2+}$ pore.

3.1.2.2.1 Inhibitors of the endoplasmic reticulum $\text{Ca}^{2+}$-ATPase

Chemicals that inhibit the endoplasmic reticulum $\text{Ca}^{2+}$-ATPase cause an increase in the $[\text{Ca}^{2+}]_i$. This occurs because the $\text{Ca}^{2+}$ sequestered in the endoplasmic reticulum continues to leak passively into the cytosol and the
inhibited Ca\(^{2+}\)-ATPase is no longer able to return the ions to the lumen of the endoplasmic reticulum. In this project, three different chemical inhibitors of the endoplasmic reticulum Ca\(^{2+}\)-ATPase (ER Ca\(^{2+}\)-ATPase) were used: thapsigargin, cyclopiazonic acid (CPA) and 2,5-di-(t-butyl)-1,4-hydroquinone (tBHQ). The activity of thapsigargin was investigated in detail. The other two toxins were used to substantiate the conclusions drawn about the relationship between the increases in [Ca\(^{2+}\)] after thapsigargin treatment, mitochondrial damage and the onset of apoptosis.

Thapsigargin is a sesiquiterpene lactone originally isolated as the major skin-irritating principle from the umbrelliferous plant *Thapsia gargancia* (Rasmussen *et al.*, 1978; Christensen & Norup, 1985). Thapsigargin has been described as a promoter of tumorigenesis in mouse skin (Hakii *et al.*, 1986) but not through the activation of protein kinase C (Jackson *et al.*, 1988). It has been shown to cause rapid and irreversible inhibition of the ER Ca\(^{2+}\)-ATPase (Thastrup *et al.*, 1990). In smooth muscle cells, the endoplasmic reticulum remained empty of Ca\(^{2+}\) for seven days after only a 30 minute treatment with thapsigargin followed by extensive washing (Ghosh *et al.*, 1991). The inhibitory effect of thapsigargin on Ca\(^{2+}\)-ATPase activity has been shown to be highly selective. Thapsigargin only acts on the pump in the endoplasmic reticulum. It does not affect the activity of the plasma membrane Ca\(^{2+}\)-ATPase in the plasma membrane of hepatocytes, erythrocytes, or the heart and skeletal muscle cells (Thastrup *et al.*, 1990).

Since the commencement of this project in March of 1992, reports have appeared which described the onset of apoptosis induced by thapsigargin in human thymocytes (Zhivotovsky *et al.*, 1994), human hepatoma cells (Kaneko & Tsukamoto, 1994a, b), T cell line (WEHI 7.2) (Lam *et al.*, 1994), androgen-independent rat prostatic cell line (Furuya & Isaacs, 1994), and in rat thymocytes (Jiang *et al.*, 1994). The induction of apoptosis by thapsigargin has been discussed in more detail in the section 4.3.2 Thapsigargin.
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Cyclopiazonic acid is a nonpolar toxin synthesised by a strain of *Penicillium cyclopium* (Holzapfel, 1968). It is another agent that inhibits the Ca\(^{2+}\)-ATPase in the endoplasmic reticulum and the sarcoplasmic reticulum (Siedler *et al.*, 1989; Deng & Kwan, 1991; Mason *et al.*, 1991a; Demaurex *et al.*, 1992, Goeger *et al.*, 1988). At this time there are no reports in the literature of cyclopiazonic acid inducing apoptosis in any cell type.

2,5-di-(t-butyl)-1,4-hydroquinone (tBHQ) is a synthetic compound, unrelated in structure to thapsigargin or cyclopiazonic acid but which is also inhibits the endoplasmic reticulum Ca\(^{2+}\)-ATPase (Moore, 1987; Kass *et al.*, 1989). Its activity is not as specific as that of thapsigargin and cyclopiazonic acid. When used at concentrations larger than 50µM, tBHQ causes unknown secondary effects at the plasma membrane that lead to much larger increases in [Ca\(^{2+}\)] (Mason *et al.*, 1991b).

### 3.1.2.2.2 Activator of plasma membrane Ca\(^{2+}\) pore

Maitotoxin is a highly polar water-soluble polyhydroxypolyether of large molecular weight (Yokoyama *et al.*, 1988) extracted from the marine dinoflagellate *Gambierdiscus toxicus* and transmitted to fish through the food chain.

Maitotoxin, because of its large molecular weight and high polarity, is unable to cross cell membranes (Gusovsky & Daly, 1990) and acts on the outside of the cell to raise intracellular calcium ion concentrations (Schettini *et al.*, 1984, 1990; Meucci *et al.*, 1988; Gusovsky & Daly, 1990). Maitotoxin stimulates the activity of plasma membrane calcium influx channels, especially receptor-mediated channels (Meucci *et al.*, 1992; Soergal *et al.*, 1992).

### 3.1.2.2.3 Dexamethasone - a glucocorticoid analog

Glucocorticoid analogs have been well defined as inducers of apoptosis in thymocytes (Wyllie, 1980, 1984). This characteristic provides an excellent opportunity for use of these compounds as positive controls, where other agents being used have unknown or poorly defined effects on thymocytes.
or when using unfamiliar assays. Throughout this project, dexamethasone was used as a positive control in many experiments.

### 3.2 Results

#### 3.2.1 Verification of the methods used to identify cell death

##### 3.2.1.1 Ethidium bromide staining identifies apoptotic cells

Thymocytes were treated for 6 hours with 1 µM gliotoxin; a concentration that induces significant levels of apoptosis (see below). After incubation the cells were sorted on the basis of their EB staining. Examination of the cells sorted from Regions 2 and 3 by electron microscopy showed the cells to have condensed chromatin in their nuclei characteristic of apoptosis (Figure 3-1 a, b).

##### 3.2.1.2 Ethidium bromide staining can distinguish between apoptosis and necrosis

When thymocytes were treated for 6 hours with 1 µM gliotoxin a significant proportion underwent apoptosis, however 50 µM gliotoxin induced necrosis within the same time period (see 3.2.2 Gliotoxin). These effects, established with agarose gel electrophoresis and described below, enabled verification of the usefulness of EB staining to distinguish between apoptosis and necrosis. A typical result after EB staining has been shown in Figure 3-2. After the control treatment, only spontaneous apoptosis was observed and a small percentage of cells could be seen in Region 2 and 3 (Panel a). When apoptosis was induced in the thymocytes with a 1 µM gliotoxin treatment there was a large increase in the percentage of cells seen in both Regions 2 and 3 (Panel b). It is important to note that the cells appeared in both Region 2 and Region 3. When necrosis was induced with 50 µM gliotoxin very few cells appeared in either Region 1 or 2, greater than 90% of the cells stained very brightly with EB and were located in Region 3 (Panel c). These cells could be seen to have had slightly increased forward scatter when compared with the apoptotic cells in Region 3 in Panel b.
### 3.2.1.3 Comparison of detection methods using dexamethasone to induce apoptosis in thymocytes

Experiments were conducted to confirm the comparability and usefulness of the four methods used in this project: electron microscopy, agarose gel electrophoresis, ethidium bromide staining of live cells for flow cytometry and propidium iodide staining of fixed cells for flow cytometry.

Thymocytes were treated with 10µM dexamethasone for 6 hours and then the appearance of apoptosis tested by each of the methods. When fields of these cells were counted after electron microscopy, cells with condensed chromatin were counted as apoptotic. The average number of cells in each field was 60.2 and the average percentage of apoptotic cells was 39.8%. Multiple random fields of cells were counted.

<table>
<thead>
<tr>
<th>% apoptotic cells</th>
<th>No. of cells counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>34.4</td>
<td>61</td>
</tr>
<tr>
<td>45.3</td>
<td>64</td>
</tr>
<tr>
<td>42.9</td>
<td>56</td>
</tr>
<tr>
<td>33.3</td>
<td>60</td>
</tr>
<tr>
<td>43.3</td>
<td>60</td>
</tr>
<tr>
<td>average 39.8 ± 5.6</td>
<td>60.2 ± 2.9</td>
</tr>
</tbody>
</table>

When the DNA from these cells was run out on agarose gel a ladder pattern characteristic of internucleosomal DNA fragmentation was observed (Figure 3-3). When the cells were stained with EB for flow cytometric analysis a total of 42.44 ± 0.45% of the cells appeared in a combination of Regions 2 and 3; the total of the “early” and “late” apoptotic cells. When fixed cells were analysed by flow cytometry using PI to stain the DNA 38.63 ± 1.54% of the cells appeared with fragmented DNA. Figure 3-3 includes representative histograms of fluorescence after PI staining of ethanol fixed thymocytes after 6 hours treatment with either 10µM dexamethasone or negative control treatments.
Figure 3-1 The ethidium bromide method allowed the detection of apoptotic cells. This was confirmed by electron microscopy.

a. cells collected by flow cytometric sorting from Region2 after EB staining (smaller size and slightly brighter fluorescence than normal cells) (x13200), b. an example cell collected from Region3 (very brightly fluorescent) (x16800).

A = cells with apoptotic morphology, S = cell with apoptotic nucleus undergoing secondary necrosis.
Figure 3-2 The stages of apoptosis shown by ethidium bromide staining.

Three regions were clearly visible. Region1 were normal cells (dim fluorescence); Region2 were apoptotic cells (lower FSC and dim fluorescence); Region3 cells were dead, either necrotic cells or apoptotic bodies (very bright fluorescence).

a. Control thymocytes incubated in medium for 6 hours;
b. Thymocytes incubated for 6 hours with 1µM gliotoxin (induced apoptosis);
c. Thymocytes incubated for 6 hours with 50µM gliotoxin (induced necrosis).
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3.2.2 Glutathione

A 6-hour incubation with glutathione at concentrations between 0.08 µM and 10 µM induced DNA fragmentation in thymocytes, as evidenced by agaose gel electrophoresis (Figure 3-4, Table 3-2). The fragmentation was observed at concentrations of 50 µM or above, but below 50 µM no significant DNA degradation was evident.

Table 3-2 Percent DNA fragmentation calculated from densitometry scans after agarose gel electrophoresis of DNA from thymocytes treated with glutathione for 6 hours.

<table>
<thead>
<tr>
<th>Glutathione (µM)</th>
<th>DNA fragmentation percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.5 ± 1.0</td>
</tr>
<tr>
<td>0.08</td>
<td>9.3 ± 3.4</td>
</tr>
<tr>
<td>10</td>
<td>19.6 ± 3.8</td>
</tr>
<tr>
<td>50</td>
<td>49.1 ± 3.8</td>
</tr>
</tbody>
</table>

The appearance of apoptosis at concentrations between 0.08 µM and 10 µM and not below (at 50 µM and above) was confirmed using electron microscopy (Figure 3-5) and EP staining (Figure 3-6). An 18-hour incubation with glutathione induced greater levels of DNA fragmentation consistent with increased apoptosis. After an incubation with 0.1 µM glutathione, the DNA was fragmented compared with that of the control-treated thymocytes. Figure 3-7 shows the DNA fragmentation levels in control and 10 µM glutathione-treated thymocytes. The DNA in M1 was fragmented compared with that of the control-treated thymocytes. Figure 3-7, it can be clearly observed that

Figure 3-3 DNA fragmentation in thymocytes detected with agarose gel electrophoresis and PI staining.

a. DNA fragmentation from thymocytes treated with 10 µM dexamethasone for 6 hours could be visualised with agarose gel electrophoresis. PI staining of ethanol fixed cells proved to be faster and more readily quantifiable method for the detection of cells with fragmented DNA; b. negative control, c. 10 µM dexamethasone. Cells with fluorescence in M1 have subdiploid DNA and are therefore apoptotic.
3.2.2 Gliotoxin

A 6 hour incubation with gliotoxin, at concentrations between 0.05\(\mu\)M and 10\(\mu\)M, induced DNA fragmentation in thymocytes characteristic of apoptosis (Figure 3-4, Table 3-2). No fragmentation was observed at concentrations of 50\(\mu\)M or above, nor below 0.05\(\mu\)M compared with the control.

### Table 3-2 Percent DNA fragmentation calculated from densitometry scans after agarose gel electrophoresis of DNA from thymocytes after a 6 hour incubation with gliotoxin

<table>
<thead>
<tr>
<th>[Gliotoxin] (\mu)M</th>
<th>percentage DNA fragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.3 ± 1.0</td>
</tr>
<tr>
<td>0.1</td>
<td>43.0 ± 5.4</td>
</tr>
<tr>
<td>1</td>
<td>38.5 ± 5.0</td>
</tr>
<tr>
<td>10</td>
<td>19.9 ± 2.0</td>
</tr>
<tr>
<td>50</td>
<td>10.7 ± 0.5</td>
</tr>
<tr>
<td>700</td>
<td>12.0 ± 0.5</td>
</tr>
<tr>
<td>10(\mu)M Dex</td>
<td>40.1 ± 2.8</td>
</tr>
</tbody>
</table>

The appearance of apoptosis at concentration (between 0.05\(\mu\)M and 10\(\mu\)M) and necrosis (at 50\(\mu\)M and above) was confirmed using electron microscopy (Figure 3-5) and EB staining (Figure 3-6). An 18 hour incubation with gliotoxin induced greater levels of DNA fragmentation, consistent with increased apoptosis. After an incubation with 0.1\(\mu\)M gliotoxin, 64% of the DNA was fragmented compared with 20% of the DNA from the control treated thymocytes. Ethidium bromide staining of these cells showed 85% of the cells in Region3 (apoptotic bodies) compared with less than 15% of the control treated cells in Region3.

To determine how rapidly gliotoxin was able to induce apoptosis in thymocytes, an hourly time course (0-6 hours) was investigated using 0, 1, 100\(\mu\)M gliotoxin treatments. Previous experiments had shown that these concentrations induced either no change from background, apoptosis or necrosis, respectively. The cells were analysed with both electron microscopy and EB staining. In Figure 3-7, it can be clearly observed that...
Defining the onset of apoptosis

by four hours of treatment with 1 µM gliotoxin a significant proportion of cells had moved into Region2 (Figure 3-7b). Cells did not appear in Region3 until 6 hours after treatment (Figure 3-7c). The appearance of apoptotic cells in Region2 was mirrored by a loss of cells from the region containing normal cells (Region1) (Figure 3-7a).

Electron microscopic examination of the thymocytes after the 1 µM gliotoxin treatment showed cells with condensed chromatin were observed as early as two hours after treatment (Figure 3-7d). By four hours after gliotoxin treatment there was close agreement in the results between the two methods. This result confirmed the usefulness of the EB method.

Treatment with 100 µM gliotoxin induced the rapid onset of necrosis with no increase in the percentage of cells appearing in Region2 at any time during the 6 hour incubation. Cells appeared in the brightly staining Region3 within 1 hour of treatment (Figure 3-7b & c).

3.2.3 Inhibitors of the endoplasmic reticulum Ca\(^{2+}\)-ATPase

3.2.3.1 Thapsigargin

Thapsigargin induced apoptosis in both thymocytes and P815 cells but over an extended time compared with the induction by gliotoxin and dexamethasone. The DNA fragmentation induced by thapsigargin in thymocytes after a 6 hour and an 18 hour incubation is shown in Figure 3-8. Table 3-3 shows the low percentage DNA fragmentation in thymocytes

<table>
<thead>
<tr>
<th>[Thapsigargin] nM</th>
<th>percentage DNA fragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9.2 ± 0.8</td>
</tr>
<tr>
<td>1</td>
<td>16.2 ± 2.2</td>
</tr>
<tr>
<td>10</td>
<td>21.5 ± 2.4</td>
</tr>
<tr>
<td>50</td>
<td>11.6 ± 1.0</td>
</tr>
</tbody>
</table>
Figure 3-4 DNA fragmentation induced by gliotoxin.

Within 6 hours, gliotoxin concentrations of 0.05µM to 10µM induced DNA fragmentation characteristic of apoptosis in thymocytes. Concentrations equal to and greater than 50µM gliotoxin induced necrosis, and no DNA fragmentation was visible.

Gliotoxin treatments shown here are: a. negative control; b. 0.05µM; c. 0.1µM; d. 0.5µM; e. 1µM; f. 5µM; g. 10µM; h. 50µM; i. 100µM; j. 400µM; k. 700µM; and l. 10µM dexamethasone.
Figure 3-5 The morphological changes characteristic of apoptosis and necrosis were observed in thymocytes treated with gliotoxin for 6 hours. 

a. negative control (x2890), b. 1μM gliotoxin (x4000). n = cells with normal nuclear morphology, CC = cells with condensed chromatin characteristic of apoptosis.
Defining the onset of apoptosis

Figure 3-5 (cont.) The morphological changes characteristic of apoptosis and necrosis were observed in thymocytes treated with gliotoxin for 6 hours.

c. 5μM gliotoxin (x2890), d. 10μM gliotoxin (x2890).

CC = cells with condensed chromatin characteristic of apoptosis.

n = necrotic cell
Figure 3-5 (cont.) The morphological changes characteristic of apoptosis and necrosis were observed in thymocytes treated with gliotoxin for 6 hours.

e. 100\mu M gliotoxin (x4000), f. 500\mu M gliotoxin (x22000). n = cells with morphology characteristic of necrosis
Defining the onset of apoptosis

Figure 3-5 (cont.) The morphological changes characteristic of apoptosis and necrosis were observed in thymocytes treated with gliotoxin for 6 hours.

g. 700μM gliotoxin (x4000). n = cells with morphology characteristic of necrosis.
Defining the onset of apoptosis

Figure 3-6 Gliotoxin induced apoptosis in thymocytes after a 6 hour incubation.

The percentage of cells in Region2 detected with the EB method.
Defining the onset of apoptosis

Figure 3-7 The kinetics of appearance of apoptotic cells after treatment of thymocytes with gliotoxin at 0, 1, 100μM detected with EB staining and with electron microscopy.

a. Region 1 EB staining (normal cells), b. Region 2 EB staining (apoptotic cells), ○ 0μM gliotoxin, ▲ 1μM gliotoxin, ■ 100μM gliotoxin.
Defining the onset of apoptosis

Figure 3-7 (cont.) The kinetics of appearance of apoptotic cells after treatment of thymocytes with gliotoxin at 0, 1, 100mM detected with EB staining and with electron microscopy.

c. Region 3 EB staining (late apoptotic cells or necrotic cells) • 0µM gliotoxin, ▲ 1µM gliotoxin, ▼ 100µM gliotoxin;
d. ♦ apoptosis detected with electron microscopy.
after a 6 hour incubation with various thapsigargin concentrations. This result was confirmed by electron microscopy (Figure 3-9).

A similar result was obtained when thapsigargin treatment of thymocytes was studied with EB staining (Figure 3-10). In both cases, 10nM was the most effective at inducing apoptosis within 6 hours of treatment.

Thapsigargin induced apoptosis more effectively over longer time periods. When thymocytes were treated for 18 hours and apoptosis measured by either EB or PI staining the percentage of apoptotic cells was 90.9 ± 1.2% and 65.6 ± 1.6%, respectively. The condensed chromatin characteristic of apoptosis can be seen in electron micrographs taken after 18 hours of 10nM thapsigargin treatment (Figure 3-11c). It is clear from these micrographs that 1nM thapsigargin had little effect at 18 hours when compared with the negative control (Figure 3-11a & b) and that 100nM thapsigargin was causing considerable cellular damage but not as much apoptosis as the 10nM treatment (Figure 3-11d).

Thapsigargin could induce apoptosis in P815 cells but this required higher concentrations and longer incubation times (Figure 3-12).

### 3.2.3.2 Cyclopiazonic acid

Cyclopiazonic acid also induced apoptosis in thymocytes over 18 hours. Cyclopiazonic acid used at 10µM was found to have a similar effectiveness as 10nM thapsigargin for inducing apoptosis in thymocytes over 18 hours (Figure 3-13b and 3-15). The cells treated with 1µM cyclopiazonic acid showed the same percentage apoptosis as the negative and solvent control treatments.
Figure 3-8 Thapsigargin induced DNA fragmentation in thymocytes.
Thymocytes were incubated with thapsigargin for 6 hours at concentrations of a. negative control; b. 1nM; c. 10nM; d. 100nM; or for 18 hours with thapsigargin concentrations of e. negative control; f. 1nM; g. 10nM; h. 100nM.
Figure 3-9 The morphological changes characteristic of apoptosis were observed in thymocytes treated with thapsigargin for 6 hours.

- a. negative control (x2890); b. 1nM thapsigargin (x2200). 

n = cells with normal nuclear morphology
Figure 3-9 (cont.) The morphological changes characteristic of apoptosis were observed in thymocytes treated with thapsigargin for 6 hours.
c. 5nM thapsigargin (x2890); d. 10nM thapsigargin (x2890). n = cells with normal nuclear morphology; cc = condensed chromatin characteristic of apoptosis; m = swollen mitochondria.
Figure 3-9 (cont.) The morphological changes characteristic of apoptosis were observed in thymocytes treated with thapsigargin for 6 hours.
e. 50nM thapsigargin (x2890); f. 100nM thapsigargin (x2890). Note there are noticeably fewer cells with condensed chromatin at these thapsigargin concentrations above 10nM. m = swollen mitochondria.
Defining the onset of apoptosis

3.2.4.1 Maltoxins

Maltoxins did not induce DNA fragmentation (Figure 3-15). In contrast, thapsigargin induced DNA fragmentation as well as maltoxin induced, as indicated by the induction of DNA fragmentation in Region 3. When cells treated with 1 and 5μg/mL maltoxin for 18 hours were stained with EB 66.1 ± 0.4% and 66.8 ± 0.3% of the cells respectively appeared in Region 3 with negligible numbers in Regions 1 and 2 (Figure 3-15). In electron micrographs, these maltoxin treated cells had necrotic morphology (Figure 3-15a & b).

3.3 Discussion

3.3.1 Verification of the methods used to identify cell death

In this project four distinct methodologies were used to identify the occurrence of cell death, either apoptosis or necrosis. Electron microscopy was found to be the most reliable means of detection of both forms of cell death. However, this technique is time consuming and difficult to quantify accurately. Agarose gel electrophoresis is an effective tool for recognizing the DNA fragmentation that so often accompanies apoptosis, but this method is also time consuming and difficult to quantify.

Figure 3-9 (cont.) The morphological changes characteristic of apoptosis were observed in thymocytes treated with thapsigargin for 6 hours.

g. 500nM thapsigargin (x1000).
3.2.3.3 2,5-di-(t-butyl)-1,4-hydroquinone

2,5-di-(t-butyl)-1,4-hydroquinone (tBHQ) was a poor inducer of apoptosis after 6 hours of treatment (Figure 3-13a) but effective at inducing apoptosis by 18 hours in thymocytes (Figures 3-13b and 3-15). As with cyclopiazonic acid, 10µM tBHQ was the most effective concentration and induced apoptosis to a similar extent to 10nM thapsigargin. When the treated thymocytes were inspected with electron microscopy the induction of apoptosis by tBHQ was obvious (Figure 3-14).

3.2.4 Activator of plasma membrane calcium channels

3.2.4.1 Maitotoxin

Maitotoxin did not induce DNA fragmentation at six or 18 hours (Figure 3-13a & b). Indeed, at both six and 18 hours, the rate of DNA fragmentation, as measured by PI staining, appeared to decrease whether induced by either 1 or 5ng/mL maitotoxin (equivalent to 0.29nM and 1.46nM, respectively). When cells treated with 1 and 5ng/mL maitotoxin for 18 hours were stained with EB 98.1 ± 0.4% and 98.8 ± 0.3% of the cells respectively appeared in Region3 with negligible numbers in Regions 1 and 2 (Figure 3-15). In electron micrographs, these maitotoxin treated cells had necrotic morphology (Figure 3-16a & b).

3.3 Discussion

3.3.1 Verification of the methods used to identify cell death

In this project four distinct methods have been used to identify the occurrence of cell death, either apoptosis or necrosis. Electron microscopy was found to be the most reliable means of detection of both forms of cell death. However this technique is time consuming and difficult to quantify accurately. Agarose gel electrophoresis is an effective tool for recognising the DNA fragmentation that so often accompanies apoptosis, but this method is also time consuming and difficult to quantify.
Figure 3-10 Thapsigargin was not a potent inducer of apoptosis in thymocytes after a 6 hour incubation.

The percentage of cells in Region2 detected with the EB method.
Defining the onset of apoptosis

Figure 3-11 The morphological changes characteristic of apoptosis were observed in thymocytes treated with thapsigargin for 18 hours.

a. negative control (x2890), b. 1nM thapsigargin (x2200). n = cells with normal nuclear morphology
cc = cells with condensed chromatin characteristic of apoptosis.
Figure 3-11 (cont.) The morphological changes characteristic of apoptosis were observed in thymocytes treated with thapsigargin for 18 hours.

c. 10nM thapsigargin (x2200), d. 100nM thapsigargin (x2200). cc = cells with condensed chromatin characteristic of apoptosis.
Figure 3-12 Thapsigargin induced apoptosis in P815 cells over a longer time.

Percentage of cells detected with fragmented DNA using the PI method after a. 18 hours; b. 24 hours.
Defining the onset of apoptosis

Figure 3-13 Cyclopiazonic acid and tBHQ induced apoptosis by 18 hours. Maitotoxin did not induce apoptosis.

The percentage of cells with fragmented DNA after a. 6 hours, b. 18 hours treatment with cyclopiazonic acid, tBHQ, maitotoxin and thapsigargin detected with PI method.

(neg. control = negative control, solvent ctl = solvent control; CPA = cyclopiazonic acid; MTX = maitotoxin; Tg = thapsigargin).
Figure 3-14 tBHQ induced apoptotic morphology in electron micrographs.

Condensed chromatin characteristic of apoptosis was observed in electron micrographs after 18 hours of treatment of thymocytes with 10µM tBHQ (x2890).

CC = cells with condensed chromatin characteristic of apoptosis.
Figure 3-15 The EB method allowed the detection of apoptosis induced by cyclopiazonic acid, tBHQ and thapsigargin and also necrosis induced by maitotoxin in thymocytes after an 18 hour incubation.

After an 18 hour incubation with cyclopiazonic acid, tBHQ or thapsigargin, thymocytes displayed the characteristics of late apoptotic cells, with high EB fluorescence. However these cells also contained populations that appeared in the normal and early apoptotic regions. After maitotoxin treatment the cells were entirely located in Region3, consistent with the cells being necrotic. (neg control = negative control; CPA = cyclopiazonic acid; Tg = thapsigargin; MTX = maitotoxin)
Figure 3-15 (cont.) The EB method allowed the detection of apoptosis induced by cyclopiazonic acid, tBHQ and thapsigargin and also necrosis induced by maitoxtin in thymocytes after an 18 hour incubation.
Defining the onset of apoptosis

Figure 3-16 Electron microscopy revealed necrotic morphology in thymocytes after maitotoxin treatment for 18 hours.

a. 1 ng/mL maitotoxin (x8000), b. 5 ng/mL maitotoxin (x28800). nc = cells with necrotic morphology.
Two flow cytometric methods were investigated for the detection of cell death. These methods have the advantages of being readily quantified and yielding rapid results. The EB method, to stain live cells, was effective for thymocytes and had the added advantage of being able to differentiate between two populations of apoptotic cells depending on their plasma membrane permeability. These were designated as the "early" and "late" apoptotic cells. The "early" apoptotic cells were those which still significantly excluded EB. The "late" cells were those cells or apoptotic bodies that had a permeable plasma membrane and therefore stained brightly with EB. These cells in Region2, defined here as "early" should not be confused with the preapoptotic cells described by Cohen et al. (1993) and other workers. In the experiments described here the term "early" only refers to those cells with condensed chromatin and low plasma membrane permeability to EB.

The EB method was also able to distinguish between normal, apoptotic and necrotic cells. The propidium iodide method proved to be reliable, and less subjective than the EB method. The EB method requires an operator choice of regions. The PI method consistently produced a lower percentage of cells undergoing apoptosis than the EB method, however the ease with which it could quantify apoptosis and its reproducibility made it an attractive method. When there was a suspicion that necrosis might have been an outcome of an experiment, samples of cells were prepared with both assay methods to provide extra reliability.

3.3.2 Gliotoxin

The mode of action of gliotoxin in the cell is not yet resolved. The results presented here demonstrate that gliotoxin was a potent inducer of apoptosis within a window of concentrations from 0.05µM to 10µM. At concentrations of 50µM and above, gliotoxin induced necrotic cell death, rapidly and effectively.
3.3.3 Inhibitors of the endoplasmic reticulum \( \text{Ca}^{2+} \)-ATPase

The three toxins, thapsigargin, cyclopiazonic acid and tBHQ are unrelated compounds but each is an effective inhibitor of the endoplasmic reticulum \( \text{Ca}^{2+} \)-ATPase. Thapsigargin is the most potent of these inhibitors. This potency against the \( \text{Ca}^{2+} \)-ATPase was reflected in the thapsigargin concentration of 10nM required to induce apoptosis in thymocytes compared with the 10µM concentration of cyclopiazonic acid and tBHQ required to achieve the same effect.

The only feature these three compounds have in common is their inhibition of the endoplasmic reticulum \( \text{Ca}^{2+} \)-ATPase and yet they induce apoptosis in thymocytes with similar kinetics. This suggests that their mode of action is similar and due to the inhibition of the endoplasmic reticulum \( \text{Ca}^{2+} \)-ATPase pump and the related changes in calcium metabolism in the cell.

3.3.4 Activator of plasma membrane calcium channels

Maitotoxin is known to activate calcium ion inflow into the cell. It is likely that the cell's ability to deal with the large increases in \( [\text{Ca}^{2+}]_i \), resulting from this \( \text{Ca}^{2+} \) inflow, would be rapidly overwhelmed. The expected outcome from the cell's inability to deal with the excess \( \text{Ca}^{2+} \) would be a loss of homeostatic control and death by necrosis. The results presented here support this hypothesis. Maitotoxin induces necrosis not apoptosis in thymocytes within 6 hours of treatment.

3.4 Conclusion

Each of the four techniques used in this project were shown to provide reliable data about the onset of apoptosis. In addition, electron microscopy can be used to detect necrosis in any cell type and EB staining used with flow cytometry can distinguish normal, apoptotic and necrotic thymocytes. The two flow cytometric methods were shown to have the advantages of being more readily quantified and yielding results more rapidly.
Defining the onset of apoptosis

Gliotoxin, a fungal toxin, was demonstrated to induce apoptosis in thymocytes within 6 hours at concentrations of 0.05µM to 10µM. Gliotoxin concentrations of 50µM and above induced the rapid onset of necrosis.

Three unrelated inhibitors of the endoplasmic reticulum Ca\(^{2+}\)-ATPase, thapsigargin, cyclopiazonic acid and tBHQ, were demonstrated to induce apoptosis in thymocytes over 18 hours. These agents were not equally potent. Thapsigargin was the most effective in thymocytes with maximal apoptosis being induced with 10nM treatment. For both cyclopiazonic acid and tBHQ, a concentration of 10µM for 18 hours was the most effective treatment for the induction of apoptosis in thymocytes. Thapsigargin was demonstrated to induce apoptosis in the mastocytoma P815 cell line, however in these cells higher thapsigargin concentrations and longer incubation times (24 hours) were required.

Maitotoxin is an activator of calcium ion inflow channels in the plasma membrane. Treatment of thymocytes with low concentrations of maitotoxin, 1ng/mL and 5ng/mL, induced necrosis within 6 hours.

Having defined the onset of apoptosis in thymocytes with these agents the next chapter will address the involvement of fluxes in [Ca\(^{2+}\)] in the onset of apoptosis.
4. Calcium changes in apoptosis

At that time, DNA fragmentation was perceived as the ultimate outcome of apoptosis and the means by which the cells died. Since then, the hallmark morphological changes of apoptosis have been observed in cells that have not digested their DNA to oligonucleosomal lengths (Zakani et al., 1983; Sun et al., 1994a; Tomel et al., 1993; Falciati et al., 1993) and other irreversible biochemical changes have been demonstrated in cells undergoing apoptosis, including the activation of tri- and tetra-enzyme activity (Peace et al., 1989, 1991) and cell membrane changes (Dutta et al., 1986; Bers et al., 1992; Norris et al., 1994; Foulk et al., 1992; Haskett et al., 1994).
4.1 Introduction

When this project was commenced, evidence had been presented that indicated an early rise in the intracellular calcium ion concentration was an essential part of the apoptotic process (as discussed in detail in 1.3.2 Calcium ions in apoptosis). Much of the early work relied on bulk cell fluorimetry for the detection of increases in $[\text{Ca}^{2+}]_i$, and on the induction of apoptosis by ionophores as evidence of the vital role for calcium ions in the process (Kaiser & Edelman, 1977, 1978a, 1978b; Wyllie, 1980; Cohen & Duke, 1984; McConkey et al., 1988). The observed role of calcium ions in apoptosis appeared to fit well with the concurrent observations that thymocytes and other cell types contained a Ca$^{2+}$-dependent endonuclease capable of digesting DNA into oligonucleosomal lengths (Hewish & Burgoyne, 1973; Burgoyne & Mobbs, 1975, Nakamura et al., 1981). This endonuclease activity was also observed to be activated in isolated nuclei when the calcium concentration in the incubation medium was raised (Cohen & Duke, 1984).

At that time, DNA fragmentation was perceived as the ultimate outcome of apoptosis and the means by which the cells died. Since then the hallmark morphological changes of apoptosis have been observed in cells that have not digested their DNA to oligonucleosomal lengths (Zakeri et al., 1993, Sun et al., 1994a; Tomei et al., 1993, Falcieri et al., 1993) and other irreversible biochemical changes have been demonstrated in cells undergoing apoptosis, including the activation of transglutaminase activity (Fesus et al., 1989, 1991) and cell membrane changes (Duvall et al., 1985; Dini et al., 1992; Morris et al., 1984; Fadok et al., 1992; Haslett et al., 1994).

In more recent times, the role of calcium ions as a universal signal for apoptosis has come into doubt. Many results have been published which have indicated no involvement for calcium ions in apoptosis (Lennon et al., 1992; Duke et al., 1994). Other results have indicated a role for Ca$^{2+}$ in the activation of apoptosis in a particular cell type by some inducing
agents and not by other agents (Whyte et al., 1993). Still other results have shown a decrease in $[\text{Ca}^{2+}]$ during apoptosis (Cohen et al., 1993; Magnelli et al., 1993).

Gliotoxin was already known to induce apoptosis in T blasts and macrophages (Waring, 1988b). Early in this project, gliotoxin was demonstrated to induce apoptosis in mouse thymocytes (see section 3.4.2 Gliotoxin) at concentrations between 0.05µM and 10µM. It was also demonstrated to induce necrosis at concentrations of 50µM and above.

Having demonstrated the range of concentrations within which gliotoxin induced apoptosis, the next step was to determine if there was an increase in the $[\text{Ca}^{2+}]$, associated with this apoptotic cell death. Bulk cell fluorimetry with the membrane permeant fluorescent calcium chelating dye, fluo-3-AM (Merritt et al., 1990) was used initially in this study. Thapsigargin was used in these experiments as a positive control to confirm the method. Thapsigargin is a compound known to inhibit rapidly and irreversibly the endoplasmic reticulum $\text{Ca}^{2+}$-ATPase (Ghosh et al., 1991) and so lead to an increase in the $[\text{Ca}^{2+}]$.

Bulk cell fluorimetry has problems associated with the method as discussed in section 1.3.2 Calcium ions in apoptosis. In essence, a large increase in fluorescence may be observed if few of the cells lose their dye into the incubation medium, which is calcium rich. This may occur as a result of either necrosis, secondary necrosis of apoptotic bodies, or a loss in membrane permeability as a result of rough handling of the cells. To confirm the results obtained using the bulk cell fluorimetry with gliotoxin and thapsigargin, the method was modified for use with flow cytometry. Flow cytometry has the advantage of only detecting the fluorescence associated with the cells that pass in front of the laser beam and not detecting the fluorescence that results from leaked dye reacting with the $\text{Ca}^{2+}$ in the medium.
Previously reported experiments had shown dexamethasone induced an early calcium flux associated with apoptosis in rat thymocytes (McConkey et al., 1989c). These had been conducted using a bulk cell method. This effect of dexamethasone was tested using a flow cytometric method.

Fluo-3-AM was chosen as the fluorescent calcium chelating dye because of its greater fluorescence change, its lower affinity for calcium when compared with fura-2 and Quin-2 (Minta et al., 1989; Lattanzio & Bartschat, 1991) and its localisation in the cytosol after passing through the plasma membrane into the cell (Michelangeli, 1991). These characteristics allow better resolution of raised cytosolic \([\text{Ca}^{2+}]\). Unfortunately the increase in fluorescence associated with the binding of fluo-3 with \(\text{Ca}^{2+}\) is not associated with a wavelength shift so it is unsuitable for measurements using ratios at two wavelengths (Minta et al., 1989). Despite this disadvantage a concentration can be calculated when using bulk cell fluorimetry with the method described by Merritt et al. (1990) but with flow cytometry only relative changes in concentration can be observed.

All the \([\text{Ca}^{2+}]\), estimations reported here were conducted with thymocytes.

4.2 Results

4.2.1 Bulk cell fluorimetry - gliotoxin and thapsigargin

4.2.1.1 High gliotoxin concentrations and thapsigargin elicit an increase in \([\text{Ca}^{2+}]\),

The fluorescence of thymocytes in bulk preparation was observed over a 510 second (8.5 minute) period after the addition of various concentrations of gliotoxin or thapsigargin (Figure 4-1). Neither the negative control, 3µM, 50µM nor 100µM gliotoxin treatments elicited any increase in \([\text{Ca}^{2+}]\), over this time, as determined by measurement of an increase in fluorescence associated with the calcium chelating dye fluo-3AM. The 10nM, 50nM and 100nM thapsigargin treatments provoked a rapid increase in fluorescence which reached a maximum within 180
Calcium changes in apoptosis

Figure 4-1 Changes in $[\text{Ca}^{2+}]$ above normal in mouse thymocytes after treatment with gliotoxin or thapsigargin, observed with bulk cell fluorescence.

Zero seconds represents the time at which the toxin was added.

$\Delta$ Control, $\blacktriangle$ 3$\mu$M gliotoxin, $\blacklozenge$ 50$\mu$M gliotoxin, $\blacklozenge$ 100$\mu$M gliotoxin, $\square$ 500$\mu$M gliotoxin, $\blacklozenge$ 700$\mu$M gliotoxin, $\blacklozenge$ 10nM thapsigargin, $\bigcirc$ 50nM thapsigargin, $\times$ 100nM thapsigargin.

Figure 4-2 The gliotoxin-induced rise in $[\text{Ca}^{2+}]$ is not from the endoplasmic reticulum.

- 30nM thapsigargin at 180 seconds then 400$\mu$M gliotoxin added at 720 seconds, $\square$
- 400$\mu$M gliotoxin added at 720 seconds, $\bullet$ Control buffer added at 720 seconds.
seconds (3 minutes) of treatment. The increase in $[\text{Ca}^{2+}]$, associated with the thapsigargin treatment remained high for the duration of the assay. Both the 500µM and 700µM gliotoxin treatments induced a gradual increase in the $[\text{Ca}^{2+}]$, that was continuing to rise at the end of the assay.

4.2.1.2 Different pools of $\text{Ca}^{2+}$ are mobilised by gliotoxin and thapsigargin

Nine minutes after treatment with 30nM thapsigargin, when the $[\text{Ca}^{2+}]$ was maintained at a plateau, the thymocytes were treated with 400µM gliotoxin. The gliotoxin elicited another increase in calcium concentration, which was similar in kinetics and size to the increase caused by 400µM gliotoxin alone (Figure 4-2).

4.2.2 Flow cytometry - gliotoxin and thapsigargin and dexamethasone

When using flow cytometry to observe $[\text{Ca}^{2+}]$, changes, the agent under study (control, gliotoxin, thapsigargin or dexamethasone solution) was mixed into the cell suspension two minutes after data collection began. The increase in fluorescence associated with calcium binding to fluo-3 was proportional to the total increase in $[\text{Ca}^{2+}]$ (Merritt et al., 1990).

4.2.2.1 Gliotoxin

Concentrations of gliotoxin of less than 400µM had no noticeable effect on $[\text{Ca}^{2+}]$, in thymocytes when measured in the first 85 minutes after treatment of the equilibrated cell suspension (Figure 4-3, 4-4, 4-5). This result reflected the results obtained from bulk cell fluorimetry. Mouse splenocytes were also tested and similarly showed no increase in $[\text{Ca}^{2+}]$, at gliotoxin concentrations up to and including 100µM.

Figure 4-3 is a histogram of the fluorescence distribution of the cells 80 minutes after treatment. Figure 4-4 shows the average fluorescence of the cell population associated with calcium bound fluo-3 at various time points before and after treatment. Figure 4-5 shows the fluorescence distribution of cells, represented as a density plot of time in minutes versus fluo-3.
Figure 4-3 Histogram of fluorescence of Ca$^{2+}$-bound fluo-3 measured by flow cytometry 80 minutes after treatment.

a. Control, 10µM dexamethasone and 10µM gliotoxin; b. Control and 10nM thapsigargin.
Figure 4-4 Flow cytometric measure of the fluorescence of Ca\(^{2+}\)-bound fluo-3.

The average fluorescence for the cells counted at each time point was plotted against time. ■ control, □ 10µM gliotoxin, ◆ 500µM gliotoxin, ◤ 10nM thapsigargin, ▲ 10µM dexamethasone.
Figure 4-5 Low gliotoxin concentrations, which induced apoptosis, did not induce an increase in free intracellular calcium ions. High gliotoxin concentrations, which induced necrosis, stimulated a rapid rise in the $[\text{Ca}^{2+}]$.

Density plots from the flow cytometric assay of $[\text{Ca}^{2+}]$ of time (minutes) versus $\text{Ca}^{2+}$-bound fluo-3 fluorescence (FI-1).
associated fluorescence, for thymocytes after treatment with various concentrations of gliotoxin.

Thymocytes treated with 10µM gliotoxin showed no increase in the average fluorescence over the control at any time up to the end of the assay 83 minutes after treatment (Figures 4-3 and 4-4). The lack of effect on \([\text{Ca}^{2+}]\) of 1µM and 10µM gliotoxin treatments was obvious when density plots of cell fluorescence against time were inspected (Figure 4-5). However gliotoxin concentrations equal to and greater than 500µM caused immediate rises in cytosolic calcium concentrations that began to decrease after 30 minutes although still remained elevated above control after 85 minutes (Figure 4-4, 4-5).

4.2.2.2 Thapsigargin

As had been previously observed using bulk cell fluorimetry, thapsigargin treatment caused a rapid increase in the fluorescence associated with calcium bound fluo-3. Thapsigargin treatments of 10nM and above caused large fluxes in \([\text{Ca}^{2+}]\), in thymocytes (Figure 4-3, 4-4). The fluorescence of thymocytes 80 minutes after the addition of 10nM thapsigargin can be seen in Figure 4-3. The fluorescence of the thapsigargin-treated cells was much higher than for the control-treated cells. When the average fluorescence of thymocytes at various time points after treatment was graphed, 10nM thapsigargin could be seen to have provoked a large and prolonged increase in the fluorescence associated calcium bound to fluo-3 in virtually all the cells in the assay (Figure 4-4).

Figure 4-6 shows the calcium-bound fluo-3 fluorescence distribution for thymocytes after treatment with 0, 1 10 and 50nM thapsigargin. These density plots show time in minutes versus the calcium-fluo-3 associated fluorescence. The majority of the treated thymocytes showed an increase in \([\text{Ca}^{2+}]\), within 5 minutes of the 10nM and 50nM treatments (Figure 4-6). The 1nM thapsigargin treatment induced a much smaller increase in calcium concentration. The prolonged increase in calcium concentration
Figure 4-6 Thapsigargin induced a rapid and prolonged increase in the $[\text{Ca}^{2+}]_i$.

Density plots from the flow cytometric assay of $[\text{Ca}^{2+}]_i$ of time (minutes) versus $\text{Ca}^{2+}$-bound fluo-3 fluorescence (FL-1).
Calcium changes in apoptosis

Figure 4-7 Calcium-free medium abrogated the prolonged increase in \([\text{Ca}^{2+}]_i\) induced in thymocytes by 20nM thapsigargin.

Density plot from the flow cytometric assay of \([\text{Ca}^{2+}]_i\) of time (minutes) versus \(\text{Ca}^{2+}\)-bound fluo-3 fluorescence (Fl-1).
Figure 4-8 Treatment with 10µM dexamethasone did not induce an increase in the $[Ca^{2+}]_i$ within 34 minutes compared with the negative control.

Density plot from the flow cytometric assay of $[Ca^{2+}]_i$ of time (minutes) versus Ca$^{2+}$-bound fluo-3 fluorescence (FI-1).
Calcium changes in apoptosis

stimulated with 20nM thapsigargin was abrogated in calcium-free medium (Figure 4-7).

4.2.2.3 Dexamethasone
Using flow cytometric method, there was no detectable increase in fluo-3 associated fluorescence observed in the dexamethasone-treated thymocytes over the control cells over 34 or 85 minutes. The method was always verified by the use of 10nM thapsigargin as a positive control (Figures 4-3, 4-4 and 4-8).

4.2.3 Fluo-3 and apoptosis
Fluo-3-AM was tested for its ability to induce apoptosis in thymocytes independently to confirm none of the changes in fluorescence were due to effects of the fluo-3 on the cells. Neither the 10 minute nor 1 hour staining protocols used with bulk cell fluorimetry and flow cytometry, respectively induced any apoptosis above background after a 6 hour incubation.

4.3 Discussion
4.3.1 Gliotoxin
As previously observed, gliotoxin induced apoptosis in thymocytes at concentrations between 0.05µM and 10µM and induced necrosis at concentrations of 50µM and above. Using both the bulk cell fluorimetry method and the flow cytometric method, no increase in the \([\text{Ca}^{2+}]\) was detected in thymocytes treated with gliotoxin concentrations that induce apoptosis. Even after 85 minutes (1h 15min) there was no increase in the \([\text{Ca}^{2+}]\), which suggests most strongly that there is no increase in \([\text{Ca}^{2+}]\), associated with the induction of apoptosis by gliotoxin.

Gliotoxin concentrations of 500µM and above rapidly induced necrosis in thymocytes and these concentrations also provoked a rapid increase in the \([\text{Ca}^{2+}]\). This increase was not sustained, however, and within 40 minutes after treatment the fluorescence associated with the \([\text{Ca}^{2+}]\), began to decrease. As this flow cytometry assay measured only the fluo-3-bound
Calcium changes in apoptosis

Ca\(^{2+}\) within the cells, if the plasma membrane had become leaky the dye would have leached out from the cells and their fluorescence would have decreased. This result is consistent with the loss of membrane integrity associated with necrosis.

The initial increase in [Ca\(^{2+}\)], associated with 500µM and 700µM gliotoxin was likely to be the result of a serious derangement of Ca\(^{2+}\) homeostasis provoked by the very high gliotoxin concentrations. Gliotoxin is a thiol reactive compound known to bind covalently to protein thiol groups (Waring et al., 1995b). Some plasma membrane ion channels have been shown to be sensitive to thiolation changes (Nicotera et al., 1985) and it is possible that at gliotoxin concentrations of 500µM and above the functions of these ion channels were severely compromised. This could have led to an extreme disruption of the cell’s ability to maintain ion homeostasis and resulted in necrosis. Preliminary data has indicated that dithiothreitol can abrogate the rise in [Ca\(^{2+}\)], due to treatment with high concentrations of gliotoxin (Waring & Beaver, 1996a).

4.3.2 Thapsigargin

As previously observed, thapsigargin was able to induce apoptosis in thymocytes but the progress of induction was much slower than for gliotoxin. Thapsigargin took close to 18 hours and gliotoxin 6 hours to induce large scale apoptosis. Thapsigargin treatment of thymocytes induced a rapid increase in [Ca\(^{2+}\)], that reached a maximum value within three to five minutes after treatment. This increase was maintained for at least the 85 minutes of the flow cytometry assay in calcium-rich medium. In calcium-free medium the prolonged rise in [Ca\(^{2+}\)], was abrogated. Thapsigargin treatment stimulated store depletion inflow when the external medium was calcium rich and this inflow prolonged the high intracellular [Ca\(^{2+}\)], (Putney, 1986, 1990).

The only recorded direct effect of thapsigargin is inhibition of the endoplasmic reticulum Ca\(^{2+}\)-ATPase and the resultant emptying of the
Calcium changes in apoptosis

endoplasmic reticulum Ca$^{2+}$ stores (Ghosh et al., 1991). Therefore it is reasonable to propose that thapsigargin treatment induced apoptosis as a result of the disruption of the cell's calcium ion homeostasis. Cohen & Duke (1984) found that isolated nuclei underwent DNA fragmentation within 90 minutes of being incubated in an environment of raised [Ca$^{2+}$]. However, because of the extended time before the onset of thapsigargin-induced apoptosis in mouse thymocytes, it is unlikely that the observed increase in [Ca$^{2+}$], resulted in a direct activation of a Ca$^{2+}$-dependent nuclear endonuclease.

Kanedo and Tsukamoto (1994a) demonstrated thapsigargin induced apoptosis in human hepatoma cell line, but both epidermal growth factor and vasopressin caused similar [Ca$^{2+}$] elevations as thapsigargin without inducing DNA fragmentation. They concluded the elevation in [Ca$^{2+}$], was not enough in itself to induce death. They proposed the depletion of the endoplasmic reticulum Ca$^{2+}$ levels led to a depletion of Ca$^{2+}$ in the nucleus and this resulted in a structural change in the chromatin and induction of an apoptotic response. A conclusion supported by the results obtained in HL-60 promyelocytic leukaemia cells (Zhu & Loh, 1995).

On the other hand Jiang et al. (1994) found that thapsigargin concentrations between 1nM and 100nM induced apoptosis in thymocytes from mature male rats within 4 hours. From their experiments they proposed a direct role for the increased [Ca$^{2+}$] in the nucleus in the formation of condensed chromatin. Table 4-1 summarises the results from a number of recent investigations into the action of thapsigargin in various cell types.

Clearly the effect of thapsigargin on cells is not straight forward. Some cells appear to be much more sensitive to its effects than do others. The majority of the publications listed in Table 4-1 did not include measurements of the size of the calcium flux induced by thapsigargin in the cell type under study. Therefore, it is not possible to compare the
Table 4-1 Recently published examples of thapsigargin-induced apoptosis

<table>
<thead>
<tr>
<th>thapsigargin concentration</th>
<th>time of onset of apoptosis</th>
<th>cell type</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.7nM</td>
<td>4 days</td>
<td>hepatoma cell line</td>
<td>Kanedo &amp; Tsukamoto, 1994a</td>
</tr>
<tr>
<td>7.7 - 77nM</td>
<td>48 hours</td>
<td>hepatoma cell line</td>
<td>Kanedo &amp; Tsukamoto, 1994b</td>
</tr>
<tr>
<td>50nM</td>
<td>6 hours</td>
<td>thymocytes (mature male rat)</td>
<td>Chow et al., 1995</td>
</tr>
<tr>
<td>30nM - 1µM</td>
<td>22 hours</td>
<td>fibroblasts - no apoptosis, cell cycle block only</td>
<td>Takuwa et al., 1995a</td>
</tr>
<tr>
<td>1 - 100nM</td>
<td>4 hours</td>
<td>thymocytes (mature male rat)</td>
<td>Jiang et al., 1994</td>
</tr>
<tr>
<td>100nM</td>
<td>2 - 4 hours</td>
<td>W7MG1 mouse lymphoma cells</td>
<td>Lam et al., 1993</td>
</tr>
<tr>
<td>50nM</td>
<td>20 hours</td>
<td>WEHI 7.2</td>
<td>Lam et al., 1994</td>
</tr>
<tr>
<td>500nM</td>
<td>24 - 48 hours</td>
<td>prostate cancer cells</td>
<td>Furuya &amp; Isaacs, 1994</td>
</tr>
<tr>
<td>1 - 50nM</td>
<td>24 hours</td>
<td>WEHI-231 B lymphoma cells</td>
<td>Choi et al., 1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immature phenotype</td>
<td></td>
</tr>
</tbody>
</table>
relative sensitivity to thapsigargin of the endoplasmic reticulum Ca$^{2+}$-ATPases in these cells nor to draw conclusions about the relative intracellular [Ca$^{2+}$] rises thapsigargin treatment provoked in each cell type. Without a comparison of [Ca$^{2+}$], changes the extreme differences in response cannot be readily explained.

Swollen mitochondria were observed in electron micrographs of both thymocytes and P815 cells treated with thapsigargin. The possible role of mitochondrial dysfunction in the onset of apoptosis induced by thapsigargin was investigated and the results are presented and discussed in the next chapter (Chapter 5 Calcium, thapsigargin and the mitochondria).

4.3.3 Dexamethasone

McConkey et al. (1989) described a doubling of the [Ca$^{2+}$], within 30 minutes of treatment in rat thymocytes treated with the synthetic glucocorticoid methylprednisolone. After 90 minutes of treatment the calcium concentration had risen to eight-fold higher than the control concentration. This is comparable with the rise described above caused by thapsigargin treatment and was a significantly different result to the observation made here with flow cytometry and described above.

The difference in technique may account for the difference in result. In the paper by McConkey et al. (1989) the cells were incubated with the dye (Quin-2) for at least 15 minutes before the methylprednisolone was added. Samples of the cell suspension were taken at 0, 30, 60, 90 and 120 minutes, washed and resuspended in fresh buffer. In the flow cytometric method used in this project, the cells were also preincubated with fluorescent dye (fluo-3-AM), but instead of taking aliquots of cells, washing and then measuring their fluorescence intensity, the cells were running continually through the cytometer with no further cell treatment needed.

Another significant difference between these experiments was the species and age of the animals used as the source for the thymocytes. McConkey
et al. (1989) used rat thymocytes taken from rats nearing sexual maturity, whereas in this project the thymocytes were from suckling mice. The population of cells in the thymus at these two ages could be expected to differ fundamentally with respect to the phenotype of the cells and their propensity to die by apoptosis. Suckling mammals still have large thymuses whereas as in mammals near sexual maturity the thymus has undergone a massive involution (Dougherty & White, 1945; Dougherty et al., 1964; Ritter & Crispe, 1992). The calcium response of cells from both suckling mice and rats and mature mice and rats needs to be compared using the flow cytometry technique. A difference in the calcium response either between species or age group could provide further insight into the area of calcium and apoptosis.

4.3.4 Fluo-3 and apoptosis

Fluo-3-AM did not induce apoptosis within 6 hours as a result of its chelation of calcium ions inside the thymocytes. Therefore any changes in calcium associated fluorescence observed with the use of fluo-3 reflect the effect of the treatment applied rather than of the fluorescent dye.

4.4 Conclusion

At gliotoxin concentrations which induced apoptosis within 6 hours there was no observable increase in $[\text{Ca}^{2+}]$, within the first 85 minutes after treatment. At very high concentrations of gliotoxin, which induced necrosis, there was a steady increase in $[\text{Ca}^{2+}]$, followed by a decline. This decline was probably associated with the breakdown to the plasma membrane and the leakage of the fluorescent dye. Thapsigargin induced rapid and large increases in $[\text{Ca}^{2+}]$, at concentrations of 10nM and above, yet at 10nM thapsigargin did not induce significant apoptosis within 6 hours. Dexamethasone, which like gliotoxin, induced considerable apoptosis within 6 hours did not provoke a calcium concentration increase.
Taken together these results show that an early rise in the \([\text{Ca}^{2+}]\) in mouse thymocytes is not a marker for the onset of apoptosis induced by gliotoxin or dexamethasone. Furthermore, as indicated by the longer time for induction of apoptosis by thapsigargin, cell death was unlikely to be the result of a direct activation of the nuclear \(\text{Ca}^{2+}\)-dependent endonuclease. Thapsigargin-induced apoptosis was more likely to be the result of some other effect, in the endoplasmic reticulum, mitochondria, nucleus or cytosol, triggered by the change in calcium metabolism.
5. Calcium, thapsigargin and the mitochondria

5.1 Introduction

Thapsigargin is a lycium of biological origin that inhibits the endoplasmic reticulum Ca\(^{2+}\)-ATPase (Hwang et al., 1992; Strehler et al., 1994) and the Ca\(^{2+}\)-ATPase from the plasma membrane (Thorell et al., 1992). In the previous chapter it was demonstrated that the arginin treatment led to a prolonged release of Ca\(^{2+}\) from the endoplasmic reticulum (Punnett, 1988; Hillman et al., 1993) and the irreversible nature of the release event by the kainate.

Both, Ca\(^{2+}\) and cytochrome C release have been demonstrated to preferentially inhibit the endoplasmic reticulum Ca\(^{2+}\)-ATPase (Berridge et al., 1989; Kish et al., 1991; Kish et al., 1992) and the plasma membrane Ca\(^{2+}\)-ATPase (Kishi et al., 1993). MacLennan et al. (1991) has shown that a prolonged increase in the [Ca\(^{2+}\)] of the cytosol led to a dramatic release of Ca\(^{2+}\) from the endoplasmic reticulum.

The calcium leakage in the cytosol of the cell has several roles, which have been discussed in detail previously in the studies of A. J. T. S. (Goding, 1993). Calcium ions, which are released from the plasma membrane, are reseed in the endoplasmic reticulum. This influx of calcium, by analogy, sensitizes the mitochondrial matrix membrane (Berridge et al., 1989; Carrell et al., 1989; Carrell et al., 1991). When the mitochondrial matrix concentration of calcium becomes above 250 μM, phase transition can be triggered (Carrell et al., 1991).

Mitochondrial phase transition induces mitochondrial swelling, a loss of cristae structure and an associated disruption of the electron transport chain, leading to a decrease in the ATP/ADP ratio (Wolfgang et al., 1970; Helewood, 1969; Dillman & Dillman, 1989; Krebs et al.,...
5.1 Introduction

Thapsigargin is a toxin of botanical origin that inhibits the endoplasmic reticulum Ca$^{2+}$-ATPase (Thastrup et al., 1990; Ghosh et al., 1991) but not the Ca$^{2+}$-ATPase from the plasma membrane (Thastrup et al., 1990). In the previous chapter it was demonstrated that thapsigargin treatment led to a prolonged rise in [Ca$^{2+}$], when thymocytes were suspended in normal calcium-rich medium. The [Ca$^{2+}$] elevation was prolonged as a result of constant refilling of the endoplasmic reticulum from the extracellular medium (Putney, 1986; Mason et al., 1991a) and the irreversible nature of the pump inhibition by the toxin.

Both tBHQ and cyclopiazonic acid treatments also have been demonstrated to preferentially inhibit the endoplasmic reticulum Ca$^{2+}$-ATPase (Goeger et al., 1988, Siedler et al., 1989, Mason et al., 1991a; Kass et al., 1989) and to result in increases in the [Ca$^{2+}$]. Maitotoxin on the other hand has been demonstrated to activate calcium inflow channels in the plasma membrane (Meucci et al., 1992, Soergal et al., 1992) which led to a direct increase in the [Ca$^{2+}$] of the cytosol.

The calcium released in the cytosol of the cell has several fates, which have been discussed in detail previously in the sections 1.3.1.1 Buffering calcium ion fluxes and 1.3.3 Mitochondria and calcium. Generally, the calcium ions in excess of the cell’s requirement are exported across the plasma membrane, sequestered in the endoplasmic reticulum, or, in times of prolonged calcium overload, sequestered in the mitochondria (Altin & Bygrave, 1986; Carafoli, 1987; Carafoli et al., 1971). When the mitochondrial matrix concentration of calcium ions rises above 20µM, phase transition can be triggered (Compton et al., 1988a).

Mitochondrial phase transition causes mitochondrial swelling, a loss of cristae structure and an associated disruption of the electron transport chain, leading to a decrease in the ATP/ADP ratio (Wrogemann & Pena, 1976; Halestrap, 1989; Duncan & Shamsadeen, 1991; Corton et al.,
The mode of mitochondrial uptake of calcium ions appears to be dependent on the mechanism of release of calcium from the intracellular stores, rather than simply on a rise in cytosolic \([\text{Ca}^{2+}]\). Rizzuto et al., (1994) found that a proportion of cellular mitochondria reacted rapidly to treatment of permeabilised cells with inositol-1,4,5-triphosphate (IP₃) by a rapid transient increase in mitochondrial \([\text{Ca}^{2+}]\). Treatment with tBHQ induced no such rapid transient. This work gave no insight into the longer term effect of prolonged elevation of cytosolic \([\text{Ca}^{2+}]\) caused by agents that inhibit the endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase, such as thapsigargin and 2,5-di-(t-butyl)-1,4-hydroquinone (tBHQ). The results of other experiments on phase transition effects after cytosolic calcium overload suggest these inhibitors of the endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase could be expected to induce phase transition (Halestrap, 1989; Halestrap et al., 1993) and this in turn could induce apoptosis (Richter, 1993).

Rhodamine 123 (R123) staining can give a measure of the relative activity of mitochondria in whole cells. In this project, R123 uptake by cells was quantified using flow cytometry in an attempt to show a correlation between the loss of mitochondrial activity and the appearance of swollen mitochondria. This dye will traverse membranes that have an electrochemical gradient (Darzynkiewicz et al., 1981; Papadimitriou et al., 1994). Accordingly, mitochondria with a functioning electron transport chain will take up R123, whereas mitochondria with defective electron transport will not. The R123 accumulated in active mitochondria fluoresces when excited by the laser of a flow cytometer, and cells with fewer active mitochondria will have a reduced total fluorescence when compared with cells with mostly active and fully functioning mitochondria. In this study, the R123 method yielded clear results when used with cells that contained many large mitochondria, such as the P815 mastocytoma cell line. The
thymocytes had fewer smaller mitochondria and it proved difficult to differentiate between the background and mitochondrial fluorescence.

Another approach to the investigation of the role of mitochondria in the apoptosis induced by thapsigargin used here was the measurement of the intracellular ATP concentrations ([ATP]). Thymocytes were assayed for their [ATP] after they had been treated with different agents but at time points where a low amount of DNA fragmentation but similar in both treatments could be expected (12 hours for 10nM thapsigargin and 4 hours for 10µM dexamethasone). This allowed a comparison of the [ATP] at approximately the same stage in the development of apoptotic characteristics. Therefore, any change in [ATP] after thapsigargin treatment but not after dexamethasone treatment was likely to be a specific effect of thapsigargin rather than being a general outcome of apoptosis.

5.2 Results

5.2.1 Swollen mitochondria appear after thapsigargin treatment

Swollen mitochondria were observed in both thymocytes and P815 cells treated with thapsigargin. Figures 5-1 and 5-2 show electron micrographs of thymocytes after a 6 hour and an 18 hour incubation, respectively, with various thapsigargin concentrations. Enlarged mitochondria with disrupted cristae structure are especially visible in the cells treated with thapsigargin at 10nM and above. Figure 5-3 shows electron micrographs of P815 mastocytoma cells treated with 0, 10 or 100nM thapsigargin for 24 hours. In all these cases the thapsigargin treated cells displayed highly enlarged mitochondria with an appearance similar to that observed in calcium ion-induced injury and phase transition (Duncan & Shamsadeen, 1991; Savage et al., 1991). Swollen mitochondria could be seen in cells which also contained condensed chromatin in both thymocyte and P815 cell preparations (see examples in Figures 3-9d, 3-11c & d, 5-2d and 5-3d). The appearance of enlarged mitochondria appeared to precede the onset of the chromatin condensation in all thapsigargin treated cells.
Figure 5-1 After 6 hours of incubation with thapsigargin thymocytes had swollen mitochondria.

a. negative control (x22000), b. 1nM thapsigargin (x22000), m = mitochondria.

Note: the mitochondria in cells treated with control or 1nM thapsigargin did not have enlarged or swollen matrix.
Figure 5-1 (cont.) After 6 hours of incubation with thapsigargin thymocytes had swollen mitochondria.

c. 5nM thapsigargin(x22000), d. 10nM thapsigargin(x28800). m = mitochondria.

Note: The mitochondria in cells treated with 5 and 10nM thapsigargin had enlarged and distorted shapes.
Figure 5-1 (cont.) After 6 hours of incubation with thapsigargin thymocytes had swollen mitochondria.
e. 50nM thapsigargin(x22000), f. 100nM thapsigargin(x16800) m = mitochondria. Note: The mitochondria in thymocytes treated with 50nM and 100nM thapsigargin had enlarged and distorted shapes.
Figure 5-1 (cont.) After 6 hours of incubation with thapsigargin thymocytes had swollen mitochondria.

Note: The mitochondria in thymocytes treated with 500nM thapsigargin had swollen and distorted shapes.
Figure 5-2 The swollen mitochondria in thapsigargin treated thymocytes were obvious after 18 hours of incubation.

a. negative control (x22000), b. 1nM thapsigargin (x22000). m = mitochondria

Note: the mitochondria in thymocytes treated with control or 1nM thapsigargin were intact and not swollen.
Figure 5-2 (cont.) The swollen mitochondria in thapsigargin treated thymocytes were obvious after 18 hours of incubation.

c. 10nM thapsigargin(x22000), d. 100nM thapsigargin(x22000)  m = mitochondria

Note: The mitochondria in thymocytes treated with 10nM and 100nM thapsigargin showed swollen and distorted shapes.
Figure 5-3 Thapsigargin induces mitochondrial changes in P815 cells treated for 24 hours.

a. negative control (x13200), b. 10nM thapsigargin (x16800), m = mitochondria.

nm = normal mitochondria; sm = swollen distended mitochondria.
Thapsigargin and the mitochondria

Figure 5-3 (cont.) Thapsigargin induces mitochondrial changes in P815 cells treated for 24 hours.

c. 100nM thapsigargin(x13200), d. 1000nM thapsigargin(x16800), sm = mitochondria, swollen and distended
Figure 5-4 Both normal and swollen mitochondria occur within the same cell after thapsigargin treatment of P815 cells

This electron micrograph shows a P815 cell treated for 24 hours with 1000nM thapsigargin (10400x). s = swollen mitochondria, n = normal mitochondria.
When electron micrographs of thapsigargin treated P815 cells were inspected many cells could be observed which contained both mitochondria of normal appearance and others that were swollen and distorted (Figure 5-4).

5.2.2 Thapsigargin treated cells had decreased mitochondrial activity

5.2.2.1 Rhodamine 123 fluorescence

Rhodamine 123 (R123) was used to measure mitochondrial integrity. The initial experiments with R123 staining were conducted with thymocytes. Subsequently P815 cells were found to offer less ambiguous results as they have numerous large mitochondria (Figure 5-3a). P815 cells, therefore, yield a signal from mitochondrial R123 uptake large enough to be readily quantified above background (cytosolic) fluorescence.

Figure 5-5 shows a comparison of two typical fluorescence histograms. Two distinct populations were clearly visible, with control P815 cells mostly appearing in the brightly staining population, as would be expected for cells with normally functioning mitochondria. When compared with the control cells, those P815 cells treated with 100nM thapsigargin for 24 hours showed a greater proportion of cells in the low staining population.

Thymocytes were incubated for 6 hours with various concentrations of thapsigargin, 10µM dexamethasone, or 1µM gliotoxin. When R123 staining was plotted against forward scatter those cells with diminished R123 staining also had reduced forward scatter. This indicated the low R123 staining cells had reduced size and were apoptotic (Figure 5-6). Table 5-1 presents a summary of the percentage of cells with low R123 fluorescence for each of the plots presented in Figure 5-6.
Figure 5-5 Thapsigargin treated P815 cells had a lower fluorescence after R123 staining than control treated cells.

Presented here is a typical fluorescence histogram for cells stained with R123 as an indicator of the average mitochondrial membrane potential within the cell. The lower staining population has a reduced or collapsed membrane potential. Control-treated cells are shown with a **solid line** and the thapsigargin-treated cells with a **dashed line**.
Thapsigargin and the mitochondria

Figure 5-6 Thymocytes with dim R123 fluorescence were also reduced in size.

Thymocytes were incubated for 6 hours with various treatments then stained with R123. The contour plots shown here display R123 fluorescence on the x-axis, FSC (a measure of cell size) on the y-axis and the cell count on the z-axis. The population of cells with normal R123 staining form the main peak on each graph. The cells with reduced R123 staining form a discrete population with lower forward scatter.
Table 5-1  Summary for thymocytes of the percentage of cells with low R123 fluorescence for each of the plots presented in Figure 5-6.

<table>
<thead>
<tr>
<th>Treatment for 6 h</th>
<th>% cells with dim R123 fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative control</td>
<td>6.19</td>
</tr>
<tr>
<td>1nM thapsigargin</td>
<td>5.59</td>
</tr>
<tr>
<td>10nM thapsigargin</td>
<td>9.49</td>
</tr>
<tr>
<td>100nM thapsigargin</td>
<td>10.21</td>
</tr>
<tr>
<td>10µM dexamethasone</td>
<td>18.15</td>
</tr>
<tr>
<td>1µM gliotoxin</td>
<td>15.06</td>
</tr>
</tbody>
</table>

The 10nM and 100nM thapsigargin treatments, which have been shown not to induce apoptosis by 6 hours, stimulated an increase in the number of thymocytes with dim R123 fluorescence. The gliotoxin and dexamethasone treatments, which have been shown to induce large-scale apoptosis by 6 hours, stimulated a much larger increase in the percentage of R123 dim cells but no swollen mitochondria (Figure 3-5) (Wyllie, 1981).

Comparable results were obtained with P815 cells. Figure 5-7 shows the effect of thapsigargin on R123 staining in P815 cells after an 18 hour incubation and Figure 5-8 the results after a 24 hour incubation. Table 5-2 summarises the percentage of cells observed with dim R123 fluorescence for each plot in Figures 5-7 and 5-8: The increase in the percentage apoptotic cells after 100nM thapsigargin treatment for 18 hours is reflected in an increase in the percentage of cells with dim R123 fluorescence.

Similarly, after 24 hours of incubation with both 100nM and 1000nM thapsigargin, the increase in apoptotic P815 cells was accompanied by an increase in the percentage of cells with dim R123 fluorescence. The increase in apoptotic cells after 10nM thapsigargin was not as large as for the higher concentrations, nor was the increase in dim R123 cells. Figure
5-9 shows the correlation between the percentage of cells with dim R123 fluorescence and the percentage of apoptotic cells for P815 cells treated with negative control, 10nM, 100nM or 1000nM thapsigargin for 24 hours (r = 0.935, p=<0.001).

Table 5-2  Summary for P815 cells of the percentage of cells with low R123 fluorescence for each of the plots presented in Figures 5-7 and 5-8.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation time</th>
<th>% cells apoptotic</th>
<th>% cells dim R123 fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative control</td>
<td>18h</td>
<td>3.7</td>
<td>11.90</td>
</tr>
<tr>
<td>1nM thapsigargin</td>
<td>18h</td>
<td>5.1</td>
<td>18.75</td>
</tr>
<tr>
<td>10nM thapsigargin</td>
<td>18h</td>
<td>7.0</td>
<td>13.01</td>
</tr>
<tr>
<td>100nM thapsigargin</td>
<td>18h</td>
<td>28.4</td>
<td>21.55</td>
</tr>
<tr>
<td>negative control</td>
<td>24h</td>
<td>12.1</td>
<td>8.63</td>
</tr>
<tr>
<td>10nM thapsigargin</td>
<td>24h</td>
<td>29.8</td>
<td>21.6</td>
</tr>
<tr>
<td>100nM thapsigargin</td>
<td>24h</td>
<td>59.6</td>
<td>29.05</td>
</tr>
<tr>
<td>1000nM thapsigargin</td>
<td>24h</td>
<td>57.3</td>
<td>27.98</td>
</tr>
</tbody>
</table>

Figure 5-10a & b shows a comparison of the average percentage of apoptotic cells with the percentage of low R123 staining cells, for P815 cells at 18 hours and 24 hours, respectively. This indicates a relationship between the loss of R123 bright cells and the onset of apoptosis.

To simultaneously quantify the apoptotic cells and the cells with dim R123 fluorescence, attempts were made to stain P815 cells with both R123 and Hoechst 33342 (Ho342). Ho342 is a stain that is normally excluded from
Figure 5-7 After an 18 hour incubation P815 cells with dim R123 fluorescence were also reduced in size.

P815 cells were incubated for 18 hours with various treatments then stained with R123. The contour plots shown here display R123 fluorescence on the x-axis, FSC (a measure of cell size) on the y-axis and the cell count on the z-axis. The population of cells with normal R123 staining form the main peak on each graph. The cells with reduced R123 staining form a discrete population with lower forward scatter.
Figure 5-8 After an 24 hour incubation P815 cells with dim R123 fluorescence were also reduced in size.

P815 cells were incubated for 24 hours with various treatments then stained with R123. The contour plots shown here display R123 fluorescence on the x-axis, FSC (a measure of cell size) on the y-axis and the cell count on the z-axis. The population of cells with normal R123 staining form the main peak on each graph. The cells with reduced R123 staining form a discrete population with lower forward scatter.
Figure 5-9 Correlation between dim R123 staining and apoptosis in P815 cells after thapsigargin treatment.

There is a correlation between the percentage of P815 cells that appear with dim R123 fluorescence and the percentage of apoptotic cells.
healthy cells, but has been reported to be able to permeate apoptotic cells (Ormerod et al., 1993). Figure 5-11 shows the results of a representative experiment when the P815 cells were subjected to a 23 hour incubation with 100nM thapsigargin or negative control treatment and then stained with both R123 and Ho342.

In the plot for the control cells, there was a large population of cells that were both Ho342 bright and R123 bright with high forward scatter (blue on the dot plot). This was unexpected, as normal cells would be expected to stain brightly with R123 and dimly with Ho342 and to have a high FSC. There was also a substantial population stained Ho342 bright and R123 dim with slightly reduced forward scatter; the staining pattern expected of cells late in apoptosis (red on the dot plot). This population of cells was much greater than could reasonably expected from spontaneous apoptosis in this cell type. There was also a small population with much reduced forward scatter and dim staining for both the Ho342 and R123 stains (black in the dot plot).

When the P815 cells treated with 100nM thapsigargin were considered, the population of apoptotic cells with Ho342 bright and R123 dim staining increased to be the largest cell population (red). The population with much reduced forward scatter and dim staining for both the Ho342 and R123 stains was also increased (black).

5.2.2.2 ATP concentrations

To clarify the relationship between thapsigargin treatment and loss of mitochondrial function the total intracellular ATP concentration was measured in thymocytes after a 12 hour incubation with thapsigargin. This is the time at which the percentage of cells with fragmented DNA begins increasing dramatically. See section 7.2.2 A reduction in [GSH]; accompanied the onset of apoptosis for details of the kinetics of thapsigargin- and dexamethasone-induced apoptosis in thymocytes. Both
Figure 5-10 As the average percentage of apoptotic P815 cells increased so did the average percentage of dim R123 staining cells.

The cells were stained and analysed after incubation times of a. 18 hours, b. 24 hours. **Diagonal hatched bars** show percentage of apoptotic cells (PI method), **open bars** the percentage of cells with reduced R123 staining.
Figure 5-11 The results from dual staining with R123 and Ho342 was difficult to interpret.

P815 cells were incubated for 23 hours with negative control or 100nM thapsigargin. The blue population were the Ho342 and R123 bright cells, the red population the Ho342 bright and R123 dim cells and the black population were the cells with low forward scatter and dim Ho342 and R123 staining.
10 and 100nM thapsigargin led to a large decrease in the concentration of ATP (Figure 5.1). To confirm that this decrease in ATP was specific to thapsigargin treatment, and not a more general effect associated with the onset of apoptosis, the ATP concentration of thymocytes 4 hours after 10µM dexamethasone treatment was tested. This was the time point, after dexamethasone treatment, at which a large increase in the percentage of cells with fragmented DNA was consistently observed. Treatment for 4 hours with 10µM dexamethasone caused no decrease in the ATP concentration (control treatment 4.2 ± 0.3 nmol ATP/10^6 cells compared with 10µM dexamethasone treatment 4.8 ± 0.6 nmol ATP/10^6 cells).

5.2.3 Other inhibitors of the endoplasmic reticulum Ca^{2+} - ATPase

The results presented in the previous sections indicate that a relationship exists between the inhibition of the ER Ca^{2+}-ATPase by thapsigargin, the appearance of mitochondrial swelling, and the onset of cell death. To test this proposed relationship, other agents that are known to inhibit the ER Ca^{2+}-ATPase were tested for their ability to induce swollen mitochondria and apoptotic cell death over a similar time scale.

The rate of leakage of Ca^{2+} from the endoplasmic reticulum after inhibition of the Ca^{2+}-ATPase is regulated by the rate of inflow through store-depletion activated refilling from outside the cell. However, treatment with an agent that activated the influx calcium channels in the plasma membrane would more likely lead to larger calcium ion inflow as the normal cellular mechanism for regulating inflow would be overridden. Such a treatment would be more likely to lead to necrosis as the external [Ca^{2+}] overwhelms the cell's ability to maintain the normal very low [Ca^{2+}]i (Farber 1982; Thor et al., 1984; Cobbold & Rink, 1987).

The agents chosen to test this relationship between inhibition of the endoplasmic reticulum Ca^{2+}-ATPase and the onset of mitochondrial swelling and apoptosis included two inhibitors of the ER Ca^{2+}-ATPase, cyclopiazonic acid (CPA) (Siedler et al., 1989; Mason et al., 1991) and 2,5-di-(t-butyl)-1,4-hydroquinone (tBHQ) (Kass et al., 1989; Mason et al.,
Figure 5-12 Thapsigargin treatment provoked a decrease in the intracellular concentration of ATP.

Treatment with both 10nM and 100nM thapsigargin induced a significant fall in the [ATP] in thymocytes after a 12-hour incubation when compared with the control.
Thapsigargin and the mitochondria

1991), and one activator of the influx calcium channels in the plasma membrane, maitotoxin (MTX) (Gusovsky & Daley, 1990; Soergel et al., 1992).

Treatment with cyclopiazonic acid and tBHQ induced apoptosis in thymocytes over the same time scale as thapsigargin (Sections 3.4.3.2 Cyclopiazonic acid and 3.4.3.3 2,5-di-(t-butyl)-1,4-hydroquinone and Figure 3-12). Unfortunately, only the induction of swollen mitochondria by tBHQ treatment of thymocytes was confirmed because of inadequate supplies of cyclopiazonic acid. Thymocytes treated for 18 hours with 10µM tBHQ showed the swollen mitochondria and the characteristic apoptotic morphology that previously had been observed after an 18 hour 10nM thapsigargin treatment (Figure 5-13 # Figure 3-14).

Maitotoxin, which stimulated entry of calcium ions into the cytosol from the extracellular medium, induced the thymocytes to undergo necrotic cell death rather than apoptosis (previously described in section 3.4.4.1 Maitotoxin and Figures 3-14). The influx of calcium induced by maitotoxin treatment was confirmed with the fluorescent calcium chelating dye fluo-3 and flow cytometric analysis. Within 3 minutes there was a dramatic rise of between 600 and 800nM in \([\text{Ca}^{2+}]\). This increase was of similar magnitude to that induced by 500µM gliotoxin, and as after the gliotoxin treatment it was not prolonged. Coincident with the calcium rise was a dramatic drop in FSC. Within 20 minutes all the dye was lost from cells, which was consistent with the previous observations of necrosis after MTX treatment. No such dye loss or change in FSC was observed after thapsigargin treatment. No swollen mitochondria could be detected in the thymocytes treated with maitotoxin when inspected with electron microscopy. The mitochondria appeared either normal or condensed, whilst the cells appeared necrotic (Figure 5-14, 5-15).
Figure 5-13 tBHQ treatment of thymocytes induced swollen mitochondria qualitatively similar to the changes induced by thapsigargin.

Thymocytes were incubated with various concentrations of tBHQ for 18 hours a. negative control (x64000). *nm* = mitochondria with normal morphology.
Figure 5-13 (cont.) tBHQ treatment of thymocytes induced swollen mitochondria qualitatively similar to the changes induced by thapsigargin.

Thymocytes were incubated with various concentrations of tBHQ for 18 hours. 

b. 10μM tBHQ (x22000), c. 10μM tBHQ (x40000). Sm = mitochondria with swollen dilated shape.
Figure 5-13 (cont.) tBHQ treatment of thymocytes induced swollen mitochondria qualitatively similar to the changes induced by thapsigargin.

Thymocytes were incubated with various concentrations of tBHQ for 18 hours.  

d. 100µM tBHQ(x22000), e. 100µM tBHQ(x52000). sm = mitochondria which have swollen dislended shape.
Figure 5-14 Maitotoxin induced necrosis but no swollen mitochondria in thymocytes after an 18 hour incubation.

Thymocytes were incubated with maitotoxin for 18 hours a. negative control, a. 1ng/mL maitotoxin (x22000), b. 5ng/mL maitotoxin (x16800) = mitochondria with normal structure.
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5.3 Discussion

The results presented here showed that thymocytes developed swollen mitochondria after treatment with either thapsigargin or 5-H1C and before the onset of apoptosis. P815 cells also developed qualitatively similar morphologic changes and apoptosis after treatment with thapsigargin. In previous studies, these workers showed that the use of this compound mimicked apoptosis in thymocytes. The current results support the hypothesis that thapsigargin inhibits the participation of a calcium mobilization increase in the death of thymocytes.

A substantial increase in mitochondrial swelling and the changes in mitochondrial morphology of thapsigargin-induced apoptosis were also observed in thymocytes. In contrast with the P815 cells, the thymocytes did not exhibit typical electron microscopic changes. Nevertheless, the results presented in electron micrographs of thymocytes treated with thapsigargin and 5-H1C also included rapid necrosis with no mitochondrial swelling.

The calcium ion inflow after inhibition of the endoplasmic reticulum Ca\(^{2+}\)-ATPase has been shown to be regulated through G-protein-linked mechanisms (Fernando & Bannat, 1993). These mechanisms are not affected by thapsigargin treatment and therefore the thapsigargin-induced Ca\(^{2+}\) inflow would be expected to proceed at a rate which was within physiologically normal limits. However, both maitotoxin and 500\(\mu\)M maitotoxin probably facilitated calcium ion influx at a rate greater than that which would occur in normal physiological activity. As a result these treatments induced necrosis not apoptosis.

The prolongation of the increase in the [Ca\(^{2+}\)] is likely to be important in the death of thymocytes treated with thapsigargin and 5-H1C.

Figure 5-15 Higher magnification of a thymocyte treated with 1ng/mL maitotoxin shows no mitochondrial swelling.

magnification = x40000. m = mitochondria with normal structure.
5.3 Discussion

The results presented here showed that thymocytes developed swollen mitochondria after treatment with either thapsigargin or tBHQ and before the onset of apoptosis. P815 cells also developed qualitatively similar morphologic changes and apoptosis after treatment with thapsigargin. In previous sections these agents were also demonstrated to induce apoptosis in thymocytes, along with cyclopiazonic acid, another inhibitor of the endoplasmic reticulum Ca\(^{2+}\)-ATPase. Taken together, these results support the hypothesis that there is a causal relationship between the inhibition of the endoplasmic reticulum Ca\(^{2+}\)-ATPase, the development of swollen mitochondria and the onset of apoptosis.

A substantial rise in \([\text{Ca}^{2+}]_i\) was not in itself enough to provoke the changes in mitochondrial structure and the onset of apoptosis. Maitotoxin induced a large increase in the internal calcium ion concentration but the cells died rapidly by necrosis, with no mitochondria swelling evident in electron micrographs. Furthermore, as described previously in Chapters 3 and 4, gliotoxin treatments of 500\(\mu\)M and greater stimulated a rapid increase in the \([\text{Ca}^{2+}]_i\), similar in extent to that provoked by maitotoxin, and also induced rapid necrosis with no swollen mitochondria.

The calcium ion inflow after inhibition of the endoplasmic reticulum Ca\(^{2+}\)-ATPase has been shown to be regulated through G-protein-linked mechanisms (Fernando & Barritt, 1995). These mechanisms are not affected by thapsigargin treatment and therefore the thapsigargin stimulated Ca\(^{2+}\) inflow would be expected to proceed at a rate which was within physiologically normal limits. However, both maitotoxin and 500\(\mu\)M gliotoxin probably facilitated calcium ion influx at a rate greater than that which would occur in normal physiological regulation. As a result these treatments induced necrosis not apoptosis.

The prolongation of the increase in the \([\text{Ca}^{2+}]_i\) is likely to be important in the development of the swollen mitochondria. The toxins thapsigargin, cyclopiazonic acid and tBHQ, are all recognised as irreversible inhibitors
of the endoplasmic reticulum Ca\(^{2+}\)-ATPase. The irreversible nature of the inhibition lead to prolonged calcium increases inside the cell. Short term calcium fluxes are usually dealt with by sequestration of calcium ion in the endoplasmic reticulum and export from the cell through the plasma membrane Ca\(^{2+}\)-ATPase. When these toxins are used, endoplasmic reticulum sequestration is no longer an option available to the cell and the mitochondria would be likely to act as a sink for Ca\(^{2+}\), which would inevitably lead to phase transition and the loss of mitochondrial function.

The loss of mitochondrial function was investigated here with two methods. Firstly the uptake by mitochondria of the fluorescent dye R123 was measured and secondly the cellular production of ATP was assayed. Both these methods showed that thapsigargin treatment lead to a decrease in mitochondrial functioning before the majority of cells were apoptotic. Six hour treatments with either 10\(\mu\)M dexamethasone or 1\(\mu\)M gliotoxin treatments of thymocytes stimulated an even larger increase in the percentage of cells with low R123 staining. At this time many of these cells would be in the advanced stages of apoptosis. The observed decrease in R123 staining could be a result of these late apoptotic changes. On the other hand, it may also suggest that R123 is not a reliable assay for mitochondrial membrane potential.

When the results from the use of fluorescent dyes for the measurement of mitochondrial membrane potential are considered, special attention must be paid to the staining protocol used; it is vitally important to wash the cells thoroughly. The inner mitochondrial membrane is not the only cellular membrane with a membrane potential. These cationic fluorescent dyes, such as R123, travel down any electrochemical gradient and accumulate on the negatively charged side of the membrane (Darzynkiewicz et al., 1981; Papadimitriou et al., 1994). Thorough and repeated washing of the cells after staining enables the removal of background staining due to accumulation of dye in the cytosol (Darzynkiewicz et al., 1981, 1992).

R123 has been shown to be taken up by mitochondria with either lowered
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or normal membrane potentials. The uptake of the dye by the functioning mitochondria is higher but differentiation between these and the dysfunctional mitochondria can be difficult (Chen & Smiley, 1993). This observation explains the existence of a population of cells after thapsigargin treatment, which showed many swollen mitochondria per cell in the vast majority of the cells by electron microscopy but that did not show the expected dramatic decrease in R123 staining.

R123 is perhaps not the best fluorescent dye available to detect changes in mitochondrial functioning. The use of other dyes such as JC-1 (Reers et al., 1991; Smiley et al., 1991; Chen & Smiley, 1993) would allow more precise estimations of the average cellular mitochondrial health because they form J-aggregates with differing absorption and fluorescence wavelength maxima under conditions of changed pH and membrane potential. These J-aggregates have been demonstrated to only form in mitochondria which are maintaining a membrane potential.

Other workers have recently published results using the fluorescent dyes JC-1 and DiOC₆(3) (Chen, 1988) to detect changes in mitochondrial membrane potential in relation to the onset of apoptosis. Cossarizza et al. (1995) found that cycloheximide treatment facilitated the induction of apoptosis by TNFα in a human myelomonocytic cell line. TNFα is thought to induce apoptosis through an oxidative process involving free radical generation. Uncoupling of the mitochondrial electron transport chain can lead to production of free radicals. These workers found that after a 6 hour incubation with both cycloheximide and TNFα, 70% of the cells had fragmented DNA and 85% had depolarised mitochondria, and concluded the generation of free radicals from the depolarised mitochondria induced the cell death. They used the dye JC-1 and washed the cells twice before analysis.

Petit and her co-workers have conducted an extensive series of experiments on thymocytes, splenocytes and rat embryo cell lines transvected with SV40 investigating the effect of dexamethasone-induced
death on various cellular functions including mitochondrial function (Vayssière et al., 1994, Zamzami et al., 1995a, b; Petit et al., 1995). In all of these papers they described a decrease in the mitochondrial membrane potential as measured by DiOC6(3) fluorescence prior to the onset of apoptosis. This result does not accord with other previous published results that suggested that mitochondrial structure and activity was maintained throughout dexamethasone-induced apoptosis (Wyllie 1981; Del Bino et al., 1991; Darzynkiewicz et al., 1992; Weis et al., 1995) nor the normal ATP concentration found in this study after 4 hours of dexamethasone treatment.

Cossarizza et al. (1994) made a thorough investigation of the kinetics of onset of apoptosis, loss of mitochondrial membrane potential and reduction in mitochondrial mass in thymocytes after either dexamethasone or heat shock treatment using both JC-1 and R123 dyes. Their results indicate the fragmentation of DNA occurred (at 4 - 6 hours) before the loss of mitochondrial membrane potential (at 6 - 8 hours) and the loss of plasma membrane integrity occurred later again (at 8 - 12 hours). These results accord well with the observation in this study of no decrease in [ATP] after 4 hours of dexamethasone treatment and an increase in the R123 dim staining cells after 6 hours.

In the experiments reported by Petit and co-workers the cells were not washed after staining with the fluorescent dye to remove background fluorescence. The fluorescence detected could therefore have contained a significant contribution from dye in the cytosol and therefore have been a measure of changes in the plasma membrane potential as much as changes in the mitochondrial membrane potential (Darzynkiewicz et al., 1991, 1992). Furthermore, in none of their papers did they measure the [ATP] in the treated cells to confirm the presence of mitochondrial damage or dysfunction.

The measurement of the mitochondrial membrane potential is not enough in itself to determine whether mitochondrial dysfunction is preceding cell
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dead - it is also important to measure the ATP concentration. As explained before, cells often have heterogeneous mitochondrial populations. Indeed, Chen & Smiley (1993) showed that some cell types contain mitochondria with regions of high membrane potential and others with reduced membrane potential within the same mitochondria. In this study, P815 cells were observed to contain both mitochondria of normal appearance and others that were swollen and distorted. In cells with both normal and distorted mitochondria the fluorescence of the R123 would be likely to appear within the broad “normal” staining peak. However, such cells may have only a limited capability for ATP synthesis, perhaps producing less ATP than is required for the anabolic needs of the cell and below a threshold at which apoptosis is provoked. Therefore, these results with R123 staining can only be used to indicate a relationship between the onset of thapsigargin-induced apoptosis and the loss of mitochondrial function.

The [ATP] was observed to be substantially decreased when the ATP concentration was measured in thymocytes after 12 hours of thapsigargin treatment. The time point of 12 hours was chosen because at that point there was usually around 40% of the cells with fragmented DNA, and the effect of thapsigargin could then be compared with the effect of dexamethasone treatment on [ATP] at 4 hours (when a similar proportion of cells had fragmented DNA). The dexamethasone treatment lead to no decrease in [ATP] and no swelling of mitochondria. More recent work has shown that as early as 6 hours after thapsigargin treatment there is a lowering of [ATP] in thymocytes (Waring & Beaver, 1996b). These results suggest the decrease in [ATP] associated with thapsigargin treatment must be specific to thapsigargin’s effects on calcium ion homeostasis, rather than as a result of the apoptotic process.

Other workers have also proposed a relationship between the loss of cellular ATP and the onset of apoptosis, using inhibitors of components of the electron transport chain. A fall in ATP production was associated with
the induction of apoptosis after 48 hours in a dopaminergic neuron cell culture by the inhibition of the mitochondrial electron transport chain Complex I with 1-methyl-4-phenylpyridinium (Hartley et al., 1994). Cultured human lymphoblastoid cells were provoked to apoptotic death after incubation with a Complex I inhibitor (rotenone), a Complex III inhibitor (antimycin A) and with an inhibitor of the ATP synthase (oligomycin), each of which would have led to a decrease in the intracellular [ATP] (Wolvetang et al., 1994).

5.4 Conclusion

In this chapter the results presented showed thapsigargin treatment induced the appearance of swollen mitochondria before the onset of DNA fragmentation in both thymocytes and P815 cells. These thapsigargin treated cells also fluoresced less brightly after R123 staining which is indicative of mitochondrial damage.

A drop in [ATP] was observed after a thapsigargin treatment which induced low levels of DNA fragmentation (12 hours), dexamethasone-treated cells at a similar stage (4 hours) showed no such drop in [ATP]. These observations are consistent with the onset of mitochondrial damage occurring before the onset of apoptosis. By 6 hours, when dexamethasone had induced large scale DNA fragmentation the thymocytes exhibited decreased R123 staining. This drop in R123 staining is likely to reflect the final stages of the death of the cell.

In conclusion, the mitochondrial swelling and decrease in [ATP] observed after thapsigargin treatment is more likely to be a trigger for apoptosis rather than a result. This supports the hypothesis that thapsigargin-induced apoptosis is related to a loss of mitochondrial activity in the cells, which in turn is a result of the increase in [Ca\(^{2+}\)] (Wrogemann & Pena, 1976; Duncan, 1991) caused by the inhibition of the ER Ca\(^{2+}\)-ATPase.
6. The effect of thapsigargin on dexamethasone-induced apoptosis and the cell cycle
6.1 Introduction

6.1.1 Inhibition of apoptosis

To gain insight into the processes involved in the regulation of apoptosis, attempts have been made to inhibit its onset and progression. In some cells, methods that increase intracellular glutathione concentrations and those which alter calcium ion homeostasis have been found to inhibit apoptosis. The role of glutathione in apoptosis will be discussed in the next chapter *Glutathione in apoptosis*. Calcium has been demonstrated to inhibit dexamethasone-induced apoptosis in a variety of cell types (Iseki *et al.*, 1991; Zhao *et al.*, 1995; Motyka *et al.*, 1995; Lampe *et al.*, 1995). As thapsigargin is an agent which causes increases in the cytosolic [Ca$^{2+}$], its use with dexamethasone-treated thymocytes could provide insight into the role of calcium in glucocorticoid-induced apoptosis.

6.1.2 Protein synthesis, the cell cycle and calcium

Thapsigargin has been demonstrated to inhibit protein synthesis in many cell types (Wong *et al.*, 1993; Preston & Berlin, 1992; Ghosh *et al.*, 1991; Delpino *et al.*, 1994; Tinton *et al.*, 1995). Wong *et al.* (1993) observed an inhibition of protein synthesis within 10 minutes of treatment and a lack of polysome accumulation, indicative of impaired translational initiation. They also observed a decrease in the concentration of actin mRNA, despite a recovery of translational activity within 3 hours of treatment. Thapsigargin treatment of DD1MF-2 smooth muscle cells inhibited both DNA and protein synthesis for at least 4 hours and 50 hours respectively (Ghosh *et al.*, 1991). In a human rhabdomyosarcoma cell line, overall protein synthesis activity was inhibited by thapsigargin treatment, but partially restored when fresh medium replaced the thapsigargin treatment solution (Delpino *et al.*, 1994). The calcium ion sequestered by the endoplasmic reticulum has been proposed to be essential for optimal protein synthesis and processing (Preston & Berlin, 1992; Wong *et al.*, 1993; Clark *et al.*, 1994). Other agents that deplete the endoplasmic reticulum Ca$^{2+}$ store have also been shown to inhibit protein synthesis (Dreher *et al.*, 1995;
Fleming & Mellow, 1995; Clark et al., 1994). Differential inhibition of the expression of proteins has been observed after thapsigargin treatment and this appeared to involve an inhibition of the intracellular transport or maturation of membrane proteins (Ono & Kawakita, 1995). A comparative examination of the expression of proteins that are synthesised into the lumen of the rough endoplasmic reticulum and those synthesised entirely within the cytosol may yield interesting answers to the question of the relative importance of the endoplasmic reticulum calcium ion store to protein synthesis.

Thapsigargin has also been demonstrated to impair DNA synthesis and proliferation in cultured smooth muscle cells (Short et al., 1993; Ghosh et al., 1991) as a result of the depletion of the IP₃-sensitive calcium pools in the endoplasmic reticulum. In serum-stimulated human fibroblasts, thapsigargin introduced immediately before the cells entered the G₁/S boundary completely inhibited the expression of cyclin A, activation of p33 (cdk2) cyclin dependent kinase and the initiation of DNA synthesis (Takuwa et al., 1995a). This is in contrast to the Ca²⁺ mobilising ionophore ionomycin that did not provoke any of these effects. Takuwa et al., 1995a suggest this result demonstrates the endoplasmic reticulum Ca²⁺ stores play a pivotal role in the traverse of cells across the G₁/S transition point. A similar effect has been observed in androgen-independent prostatic cancer cells, which had decreased expression of G1 cyclins, arrested in G₀ after thapsigargin treatment and eventually became apoptotic (Furuya et al., 1994). As a result of calbindin studies, this inhibition was determined to be dependent on a rise in the cytosolic [Ca²⁺] and not on store depletion, unlike the examples of protein synthesis inhibition and the cell cycle block in muscle cells reviewed above. In this study, a preliminary investigation was conducted into the inhibitory effects of thapsigargin on protein synthesis in thymocytes and cell cycle block in P815 cells.
6.2 Results

6.2.1 Thapsigargin inhibited dexamethasone-induced apoptosis

Thymocytes were treated simultaneously with 10μM dexamethasone and various concentrations of thapsigargin for 6 or 18 hours. Apoptosis was quantified with the EB method using flow cytometry. Thapsigargin concentrations of 10nM and 100nM significantly inhibited dexamethasone-induced apoptosis over 6 hours (Figure 6-1a) but not as effectively as cycloheximide treatment, a well-known inhibitor of protein synthesis (Oleinick, 1977) and of dexamethasone-induced apoptosis. Cycloheximide was included in this experiment as a positive control for the inhibition of dexamethasone-induced apoptosis in thymocytes.

When the treatment was extended to 18 hours, 10nM and 100nM thapsigargin induced large scale apoptosis in their own right (Figure 6-1b). After treatment of the thymocytes with both dexamethasone and either 10nM or 100nM thapsigargin the percentage of apoptotic cells fell below that induced by dexamethasone alone. When 100nM thapsigargin was considered in particular, it was noted that the percentage of apoptotic cells after treatment with dexamethasone alone was the same as when both dexamethasone and 100nM thapsigargin were used together.

Thapsigargin had no inhibitory effect on the apoptosis induced by gliotoxin at 6 hours nor at 18 hours. Indeed at 6 hours the combination of thapsigargin and gliotoxin resulted in increased apoptosis with 1 and 10nM thapsigargin (Figure 6-2a), whereas, at 18 hours thapsigargin appeared to have no effect on the level of induction of apoptosis by gliotoxin (Figure 6-2b).

6.2.2 Thapsigargin partially inhibited protein synthesis

Thapsigargin was observed to be a poor inhibitor of protein synthesis in thymocytes (Figure 6-3) being only partially effective at concentrations equal to or greater than 100nM.
Figure 6-1 Thapsigargin inhibited dexamethasone-induced apoptosis in thymocytes at 6 hours but not at 18 hours.

The EB method was used to quantify the total percentage of apoptotic cells (R2 + R3) after 6 hours or 18 hours when thymocytes were incubated with
- thapsigargin alone;
- thapsigargin and 10µM dexamethasone together;
- cycloheximide alone; or
- cycloheximide and 10µM dexamethasone together.

Thapsigargin concentrations expressed in nM and cycloheximide concentrations in µM.
Thapsigargin has other effects

Figure 6-2 Thapsigargin treatment does not inhibit gliotoxin induced apoptosis.

The EB method was used to quantify the total percentage of apoptotic cells (R2 + R3) after 6 hours or 18 hours when thymocytes were incubated with

- thapsigargin alone; or ▲ thapsigargin and 1µM gliotoxin together.

The graph shows the percentage of apoptotic cells (independent variable [thapsigargin] nM) for 6 hours and 18 hours.
Thapsigargin has other effects

8.2.3 Thapsigargin Interrupts the cell cycle in P815 cells

When analysing PI stained thymocytes after thapsigargin treatment, a reduction in the already small proportion of cells appearing in the S and G2/M phase had been observed (data not shown). The percentage of mitotic thymocytes was always observed to be very small and so a more rapidly cycling cell type (P815 mastocytoma cell line) was chosen as a tool in the investigation of the effect of thapsigargin on the cell cycle.

To describe this phenomenon more precisely, resting P815 cells were sorted into two populations dependent on size: "small" and "large".

The two populations were then rechallenged to choose cells with the most difference in size. The "large" population was assayed for thapsigargin effects (Figure 6-4); the "small" population was largely in S or G2/M phases and engaged in mitosis. Samples of cells were fixed and stained immediately after sorting with no incubation in thapsigargin or control solution ("time zero"), and also after 24 hours. No other thapsigargin or control treatment of the unsorted cells ("unsort") or the "small" or "large" sorted cell populations were done.

After 24 hours treatment of the "large" (mitotic) cells with 100 nM thapsigargin the percentage of cells in the S/G2/M phases fell from 68.4% before treatment to 13.1% after treatment. This value was similar to the 15.7% of cells in S/G2/M after the same thapsigargin treatment of unsorted cells (Figure 6-4). When the "large" (mitotic) cells were treated with the solvent control for 24 hours, they were found to have achieved a similar percentage in the S/G2/M phase (68.2%) to the unsorted control population (68.2%), that is returned to a normal cell cycle distribution (Figure 6-4).

When the sorted "small" (G0) cells were treated with the solvent control for 24 hours the percentage of cells entering the S phase was not affected.

Figure 6-3 Thapsigargin is a poor inhibitor of protein synthesis in thymocytes.

The incorporation of $^{35}$S-methionine after treatment of thymocytes with various concentrations of thapsigargin for 1 hour was expressed as a percentage of the counts per minute (CPM) incorporated by the control cells.
6.2.3 Thapsigargin interrupts the cell cycle in P815 cells

When analysing PI stained thymocytes after thapsigargin treatment, a reduction in the already small proportion of cells appearing in the S and G2/M phase had been observed (data not shown). The percentage of mitotic thymocytes was always observed to be very small and so a more rapidly cycling cell type (P815 mastocytoma cell line) was chosen as a tool in the investigation of the effect of thapsigargin on the cell cycle.

To describe this phenomenon more precisely, rapidly cycling P815 cells were sorted into two populations dependent on size, “small” and “large”. The sort regions were designed to choose cells with the most difference in size. The “small” cell population was depleted of cells in S/G2/M phases and the “large” population was enriched for S/G2/M phases (Figure 6-4); therefore the “small” cells were largely in G1 and the “large” cells were in S or M phases or engaged in mitosis. Samples of cells were fixed and stained immediately after sorting with no incubation with thapsigargin or control solution (“time zero”), and also after 24 hours of either thapsigargin or control treatment of the unsorted cells (“unsort”) or the “small” or “large” sorted cell populations.

After 24 hours treatment of the “large” (mitotic) cells with 10nM thapsigargin the percentage of cells in the S/G2/M phases fell from 68.4% before treatment to 13.1% after treatment. This value was similar to the 15.7% of cells in S/G2/M after the same thapsigargin treatment of unsorted cells (Figure 6-4). When the “large” (mitotic) cells were treated with the solvent control for 24 hours, they were found to have achieved a similar percentage in the S/G2/M phases (46.3%) as the unsorted control population (49.2%), that is returned to a normal cell cycle distribution (Figure 6-4).

When the sorted “small” (G1) cells were treated with the solvent control for 24 hours, the percentage of cells in the S/G2/M phases returned to the normal distribution, from 19.2% before treatment to 49.7% after 24 hours compared with 49.2% in the unsorted control treated cells. However, the
Thapsigargin has other effects

Figure 6-4 The cell cycle in P815 cells is blocked before S/G2/M phase after 10nM thapsigargin treatment.

The percentages of cells in S/G2/M phases after various cell sorting and thapsigargin treatments are presented here.  
"unsort" were the cells that had not been sorted and had been treated with solvent control or 10nM thapsigargin.  
"sm time zero" and "lg time zero" were the cells sorted in the "small" G1 population and "large" mitotic population respectively, that were fixed and stained immediately with PI for cell cycle analysis. These samples allowed a determination of the extent of enrichment of the two cell populations.  
"sm 24h 0nM" and "sm 24h 10nM" were the "small" G1 cells after a 24 hour incubation with the solvent control or 10nM thapsigargin respectively.  
"lg 24h 0nM" and "lg 24h 10nM" were the "large" mitotic cells after a 24 hour incubation with the solvent control or 10nM thapsigargin respectively.
percentage of cells in the S/G2/M after 10nM thapsigargin treatment of the “small” cells fell further below the already depleted proportion (from 19.2% to 7.6%).

From these results it is clear that P815 cells, sorted to deplete (“small”) or enrich (“large”) the mitotic population, returned to the cell cycle distribution typical of unsorted cells after 24 hours of the control treatment. However, thapsigargin treatment lead to a block in the cell cycle that prevented the P815 cells moving from G1 into the S/G2/M phases but did not prevent the normal traverse of cells through S/G2/M.

6.2.4 Apoptosis occurs in all phases of cell cycle
Irrespective of whether the P815 cells were originally in the “small” G1 population or in the “large” mitotic population, there was a two-fold increase in the percentage of apoptotic cells after thapsigargin treatment when compared with the control treated cells of the same size (Figure 6-5). These results indicate thapsigargin induced apoptosis in P815 cells during all phases of the cell cycle.

6.3 Discussion

6.3.1 Thapsigargin inhibits dexamethasone-induced apoptosis
Some treatments, which result in increases in the [Ca^{2+}], have been shown able to inhibit apoptosis. Treatments that increase the cytosolic calcium concentration have been demonstrated to inhibit dexamethasone-induced apoptosis in a wide variety of cell types (Iseki et al., 1991; Zhao et al., 1995; Motyka et al., 1995; Lampe et al., 1995). In this study, thapsigargin has been demonstrated to inhibit dexamethasone-induced apoptosis in thymocytes. This inhibition was obvious at 6 hours but, after 18 hours incubation with both agents, the inhibitory effect was much reduced. Indeed, after 18 hours incubation with 10nM or 100nM, thapsigargin alone was enough to induce apoptosis in the majority of cells. It is difficult to ascertain whether the cells treated with both thapsigargin and dexamethasone died as a result of apoptosis induced by the
Thapsigargin has other effects

Figure 6-5 Apoptosis induced by thapsigargin in P815 cells occurs in all phases of the cell cycle.

The percentages of cells in S/G2/M phases after various cell sorting and thapsigargin treatments are presented here. 

"unsort" were the cells that had not been sorted and had been treated with solvent control or 10nM thapsigargin.

"sm time zero" and "lg time zero" were the cells sorted in the "small" G1 population and "large" mitotic population respectively, that were fixed and stained immediately with PI for cell cycle analysis. These samples allowed a determination of the extent of enrichment of the two cell populations.

"sm 24h 0nM" and "sm 24h 10nM" were the "small" G1 cells after a 24 hour incubation with the solvent control or 10nM thapsigargin respectively.

"lg 24h 0nM" and "lg 24h 10nM" were the "large" mitotic cells after a 24 hour incubation with the solvent control or 10nM thapsigargin respectively.
Thapsigargin and the cell cycle

dexamethasone stimulus or by the thapsigargin. The 100nM thapsigargin may have completely abrogated the dexamethasone effect and then proceeded to kill the cells.

This ability of an agent, which raises intracellular $[\text{Ca}^{2+}]_i$, to inhibit dexamethasone-induced apoptosis supports the results described in Chapter 4 Calcium changes in apoptosis, that dexamethasone-induced apoptosis is not a calcium-dependent process.

6.3.2 Thapsigargin was a poor inhibitor of protein synthesis

Thapsigargin has been described as an inhibitor of protein synthesis in a range of cell types (Ghosh et al., 1991; Preston & Berlin, 1992; Wong et al., 1993). Dexamethasone-induced apoptosis has been recorded as able to be inhibited by protein synthesis inhibitors (Wyllie et al., 1984; Yamada & Ohyama, 1988; Christ et al., 1993; Evans & Dive, 1993; Chow et al., 1995).

As stated above, thapsigargin inhibited dexamethasone-induced apoptosis in thymocytes at 6 hours. It might have exerted its inhibitory activity because of its ability to suppress protein synthesis. However, in the results presented here, it is clear that thapsigargin acted as a poor inhibitor of protein synthesis in mouse thymocytes. A thapsigargin concentration of 10nM was able to significantly abrogate the apoptosis induced by dexamethasone, but this concentration caused no inhibition of protein synthesis. The 100nM thapsigargin treatment decreased protein synthesis to only 80% of the control but inhibited dexamethasone-induced apoptosis only a little more than the 10nM thapsigargin treatment. Taken together these results suggest the inhibition of dexamethasone-induced apoptosis by thapsigargin was not as a result of its ability to inhibit overall protein synthesis. This does not rule out the possibility that the synthesis of a protein specific to dexamethasone-induced apoptosis was inhibited.

Similarly, thapsigargin's ability to inhibit protein synthesis was unlikely to be related to its ability to induce apoptosis in thymocytes, as protein
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synthesis inhibitors have been demonstrated not to induce apoptosis in thymocytes (Wyllie et al., 1984) nor in P815 cells within 18 hours (Waring et al., 1995a). At the most effective concentration of thapsigargin for the induction of apoptosis in thymocytes (10nM) it did not significantly inhibit protein synthesis.

6.3.3 Thapsigargin interrupts the cell cycle in P815 cells

Thapsigargin is only known to directly block the activity of one cellular protein, the ER Ca^{2+}-ATPase, although its ability to initiate a cell cycle block in P815 cells during G1 could possibly be the result of direct action of thapsigargin on a cellular protein. More likely, the observed cell cycle block was a result of changes in cellular calcium ion homeostasis. This could involve the emptying of the endoplasmic reticulum calcium ion pools with a resultant decrease in the cell's ability to fold proteins in the lumen of the endoplasmic reticulum or to initiate calcium signals. On the other hand, the large and prolonged increase in the cytosolic [Ca^{2+}] could result in mitochondrial dysfunction and lowered cellular ATP concentrations or the disruption of calcium sensitive processes.

The endoplasmic reticulum calcium ion pools have a role in cell cycle progression from G1 to the S phase transition (Takuwa et al., 1995b; Waldron et al., 1994). The endoplasmic reticulum calcium ion stores play an important role in the processing and trafficking of proteins that have been synthesised into the lumen (Sambrook, 1990) and the appropriate production of nuclear cell cycle related proteins, such as cyclin A (Takuwa et al., 1995a), may be affected by store depletion (Takuwa et al., 1995b). In addition, thapsigargin may also affect nuclear [Ca^{2+}], directly as the Ca^{2+}-ATPase on the nuclear membrane has been reported to be identical to the endoplasmic reticulum Ca^{2+}-ATPase (Lanini et al., 1992). Many intranuclear processes in the replication of DNA require calcium, including the synthesis and activation of DNA polymerase α and the DNA replicating replitase complex (Subramanyam et al., 1990; Takuwa et al., 1995b). Therefore, a decrease in the [Ca^{2+}] in the endoplasmic reticulum
or nucleus could lead to the disruption of processes required by the cell for its progression from G\textsubscript{1} to S phase.

The reduction in the total ATP concentration of thapsigargin treated P815 cells could also contribute to the cell cycle block observed in G\textsubscript{1}. An actively dividing cell requires a substantial supply of ATP for the processes associated with the synthesis of DNA and the proteins required for cell cycle progression. ATP also is required for the production of dNTPs by phosphorylation of dNDPs. Several enzymes involved in the replication of DNA involve the use of ATP as a source of free energy, including helicase, which unwinds the DNA before the synthesis of the daughter DNA strand. ATP is also required for the control of microtubule polymerisation (Pariente \textit{et al.}, 1987), a process important in cell cycle progression. A mitochondrial electron transport chain inhibitor that prevents the efficient synthesis of ATP (Wolvetang \textit{et al.}, 1994) in the cell has been demonstrated to inhibit cell cycle progression at the G\textsubscript{1}/S boundary (Loffler, 1985). In this project, thapsigargin-treated cells were demonstrated to have dysfunctional mitochondria and reduced intracellular ATP concentrations. A lack of adequate supplies of ATP for DNA replication may have contributed to the cell cycle block induced by thapsigargin treatment.

The high cytosolic [Ca\textsuperscript{2+}] associated with thapsigargin treatment may also affect the processes of mitosis more intimately. Raised [Ca\textsuperscript{2+}] has been shown to cause depolymerisation of microtubules \textit{in vitro} (Weisenberg, 1972), and spindle microtubules \textit{in vivo} and \textit{in vitro} (Izant, 1983; Kiehart, 1981; Salmon & Segall, 1980; Zhang \textit{et al.}, 1992). The prolonged elevation of [Ca\textsuperscript{2+}], after thapsigargin treatment may have prevented the formation of an appropriate polymerized microtubule assembly or resulted in a depolymerization of the assembly that is required for the mitotic separation of the chromosomes. Whilst this may not contribute directly to the cell cycle block observed here before S phase, these changes may affect the cell's ability to re-enter the cell cycle and may also play a part in
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the triggering of apoptosis.

The role of the fragments of endoplasmic reticulum membrane structures that are associated with spindle microtubules (reviewed in Hepler & Wolniak, 1984) may have also been detrimentally affected by thapsigargin treatment. These membrane structures contain Ca\(^{2+}\)-ATPase pumps that are part of a system able to maintain [Ca\(^{2+}\)] micro-environments in the vicinities of the centrosome and the mitotic spindle. Antibodies against the Ca\(^{2+}\)-ATPase in these vesicles have been shown to inhibit their activity and led immediately to an increase in cytosolic [Ca\(^{2+}\)], destruction of the mitotic apparatus and irreversible blockage of the cell cycle (Silver, 1986; Hafner & Petzelt, 1987). Silver (1986) suggested that any factors which regulate the Ca\(^{2+}\)-ATPase would play a part in the regulation of the mitotic apparatus.

6.3.4 Apoptosis occurs in all phases of cell cycle

The onset of apoptosis and the block of the cell cycle do not appear to be causally related. The results presented here indicate that the rate of apoptosis in P815 cells after thapsigargin treatment is similar in both the G\(_1\) and S/G\(_2\)/M phases. Also a causal relationship between the cell cycle block and apoptosis would not explain the very high apoptotic rate of thymocytes treated with thapsigargin for 18 hours (near 80%), as nearly 90% of thymocytes are small non-proliferating cells (Snoeij et al., 1988).

6.4 Conclusion

Thapsigargin is a poor inhibitor of protein synthesis in thymocytes but despite this is able to inhibit dexamethasone-induced apoptosis in thymocytes after 6 hours of incubation by an unknown mechanism that involves calcium ions. Thapsigargin was also observed to inhibit the cell cycle of P815 cells in G\(_1\). The mechanism of this inhibition is not clear from this work and further investigations need to be performed to separate the role of decreased ATP concentrations, increased cytosolic [Ca\(^{2+}\)] and decreased calcium ion stores in the process.
7.1 Introduction

Methods which lead to an increase in the intracellular glutathione concentration have been shown to inhibit antioxidant-induced apoptosis (Malorni et al., 1994; Sandstrom et al., 1994; Forrest et al., 1994). Glutathione has been recognised as an important intracellular antioxidant and is present in millimolar concentrations in most cell types (Meister & Anderson, 1983).

Glutathione also has a role in the cell in the formation of mixed disulfides with both small molecules such as Coenzyme A and with proteins. Two roles for the ability of glutathione to form mixed disulfides have been proposed. Firstly, as a protective mechanism to provide an "emergency" pool of glutathione that can be mobilised according to need (Moore, 1968; Kaeberer & Kaeberer, 1978). Secondly, the formation of mixed disulfides with proteins has been shown to increase the activity of enzyme activity (Gilbert, 1952; Bragdon, 1961). During various types of stress, mixed disulfides have been demonstrated to be formed in cells and this was shown to be associated with a drop in the [GSH]/[GSSG] ratio from its normal value of close to 100/1 (Dellamore et al., 1987).

The agents used in this study, dexamethasone, thapsigargin and glutethimide (with the exception of glutethimide at very high concentrations), generally have not been considered to act as proapoptotics. Here they were tested for their ability to affect the intracellular glutathione concentration ([GSH]) during the process of apoptosis in thymocytes. Attempts were made to modulate the onset of apoptosis by treating the cells with reduced or oxidised glutathione. Lastly, an attempt was made to detect an increase in the presence of S-glutathiolated proteins in P815 cells after apoptosis stimulated with thapsigargin.
7.1 Introduction

Methods which lead to an increase in the intracellular glutathione concentration have been shown to inhibit prooxidant-induced apoptosis (Malorni et al., 1994; Sandstrom et al., 1994a; Forrest et al., 1994). Glutathione has been recognised as an important intracellular antioxidant and is present in millimolar concentrations in most cell types (Meister & Anderson, 1983).

Glutathione also has a role in the cell in the formation of mixed disulphides with both small molecules such as Coenzyme A and with proteins. Two roles for this ability of glutathione to form mixed disulphides have been proposed. Firstly, as a protective mechanism to provide an “emergency” pool of glutathione that can be mobilised according to need (Modig, 1968; Kosower & Kosower, 1978). Secondly, the formation of mixed disulphides with proteins has been shown to have a role in the modulation of enzyme activity (Gilbert, 1982; Brigelius, 1985). During oxidative stress, mixed disulphides have been demonstrated to be formed in cells and this was shown to be associated with a drop in the $[\text{GSH}]/[\text{GSSG}]$ ratio from its normal value of close to 100/1 (Bellomo et al., 1987).

The agents used in this study, dexamethasone, thapsigargin and gliotoxin (with the exception of gliotoxin at very high concentrations), generally have not been considered to act as prooxidants. Here they were tested for their ability to affect the intracellular glutathione concentration ($[\text{GSH}]$) during the process of apoptosis in thymocytes. Attempts were made to modulate the onset of apoptosis by treating the cells with reduced or oxidised glutathione. Lastly an attempt was made to detect an increase in the presence of S-glutathiolated proteins in P815 cells after apoptosis stimulated with thapsigargin.
7.2 Results

7.2.1 Verification of the OPT assay
The use of OPT as a specific fluorescent dye for GSH has been well established (Treumer & Valet, 1986; and references therein). The relationship between the fall in GSH-OPT fluorescence observed in the flow cytometric assay and a decrease in \([\text{GSH}]_i\) was verified by two methods. Firstly, thymocytes were treated with diamide, a substance well known to lower \([\text{GSH}]_i\) (Kosower & Kosower, 1969, 1987). A dramatic reduction in GSH-OPT fluorescence was observed after the diamide treatment (Figure 7-1b) compared with the control cells (Figure 7-1a). This change was qualitatively similar to the fall in fluorescence observed after treatment of the thymocytes with 10µM dexamethasone (Figure 7-1c).

Secondly, an HPLC assay for the monobromobimane adduct of GSH (Cotgreave & Moldéus, 1986) was used. After a 6 hour treatment with 10µM dexamethasone the amount of GSH in the thymocytes had fallen to 55.9% of the control (Figure 7-2). This is in excellent agreement with the percentage of cells with low \([\text{GSH}]_i\) calculated by the OPT flow cytometry assay after the same treatment (Figure 7-1c).

7.2.2 A decrease in \([\text{GSH}]_i\) accompanied the onset of apoptosis
Dexamethasone, thapsigargin and gliotoxin were used to induce apoptosis in thymocytes over varying periods up to 18 hours. The apoptotic cells were defined as those which appeared in the subdiploid region when ethanol fixed cells were stained with propidium iodide, and the percentage of cells with fluorescence reduced below $G_0/G_1$ calculated from flow cytometry data. These results correlated with observed DNA fragmentation visualised after electrophoresis on agarose gels (data not shown here). The percentage of cells with low intracellular glutathione concentrations was calculated from flow cytometry data obtained after OPT staining of unfixed cells. These 18 hour time course studies were carried out using three overlapping seven-hour time courses on different
Figure 7-1 Verification of the OPT flow cytometric method.

The OPT flow cytometric method accurately reflected the relative changes in [GSH]i in intact live thymocytes. R1 represents cells that displayed low GSH-OPT fluorescence, that is, lower than normal [GSH].

The y-axis (FL4) represents the GSH-OPT fluorescence and the x-axis (F32) the potential fluorescence of OPT bound to protein thiols.

A. thymocytes treated for 6 hours with the solvent control.
B. the dramatic reduction in [GSH], induced by 0.5 mM diamide treatment for 6 hours in thymocytes.
C. the effect on the GSH-OPT fluorescence of a 6 hour treatment with 10 µM dexamethasone.
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When thymocytes were treated with 10 µM dexamethasone, a decrease in glutathione was observed within 4 hours and the percentage of apoptotic cells remained constant. Dexamethasone treatment induced a decrease in GSH at 6 hours as determined by HPLC. CONTROL and DEX represent the solvent control and 10 µM dexamethasone treatment, respectively.

Figure 7-2 Dexamethasone and GSH levels.

Dexamethasone treatment induced a decrease in [GSH] at 6 hours as determined by HPLC. CONTROL and DEX represent the solvent control and 10 µM dexamethasone treatment, respectively.
days with different mice. The data in Figures 7-3a, b & c are a collation of one set of representative time courses.

When thymocytes were treated with 10µM dexamethasone, which is a saturating concentration with respect to the glucocorticoid receptor, apoptosis was apparent within 4 hours and the percentage of apoptotic cells continued to rise until 18 hours after treatment. The [GSH] fell before the onset of DNA fragmentation. The percentage of cells with low [GSH] reached a plateau of 96% near 15 hours (Figure 7-3a).

Earlier it was reported that 10nM thapsigargin induced very low levels of apoptosis at 6 hours. In this series of experiments, the percentage of apoptotic cells rose steadily after 8 hours to over 60% by 18 hours. Again the [GSH], fell before the onset of DNA fragmentation. Thus at all time points the percentage of cells with low [GSH] was higher than the percentage of apoptotic cells. (Figure 7-3b)

The control cells, which were treated with a solvent control, showed an increase in the percentage of cells undergoing spontaneous apoptosis over the 18 hour period, and as with the drug treatments the cells had lowered [GSH], before they exhibited fragmented DNA (Figure 7-3c).

Gliotoxin is a potent inducer of apoptosis in thymocytes. After a 7 hour treatment with 1µM gliotoxin, a large percentage of the cells had fragmented DNA characteristic of apoptosis and an even larger percentage of the cells had their [GSH], decreased below the control. This again is consistent with these cells undergoing a reduction in their [GSH], before their DNA is fragmented (Figure 7-4).

There was a correlation between the percentage of cells with low [GSH], and the percentage of apoptotic cells after a variety of treatments. The data from the 18 hour time courses using dexamethasone, thapsigargin and the solvent control were combined (including the data from Figures 7-3a, b & c) into a correlation graph of the percentage of apoptotic cells.
Figure 7-3 [GSH] decreases before DNA fragmentation occurs.

- O percentage of apoptotic cells; ■ percentage of cells with low [GSH],
  a. 10µM dexamethasone; b. 10nM thapsigargin; c. solvent control.
versus the percentage of cells with low [GSH]_i (Figure 7-5). A clear positive correlation was observed (r = 0.977, p < 0.000001) between the percentage of apoptotic cells and the percentage of cells with low [GSH]_i. The slope of this line was greater than one and this supported the hypothesis that the [GSH]_i was reduced in the cells before the DNA was fragmented.

7.2.3 Inhibition of dexamethasone-induced apoptosis was accompanied by a restoration of [GSH]_i

As previously reported (Chapter 6 Thapsigargin has other effects), both thapsigargin and cycloheximide treatments were observed to inhibit dexamethasone-induced apoptosis. Another interesting observation, was a decrease in the percentage of cells with decreased [GSH]_i, associated with the decrease in apoptosis.

Thymocytes treated with cycloheximide at 1, 10 and 100µM for 6 hours showed no increase in apoptosis above background and also no change in the percentage of cells with low [GSH]_i. When 10 or 100µM cycloheximide and 10µM dexamethasone were used concurrently to treat thymocytes for 6 hours, both the dexamethasone-induced apoptosis, and the associated decrease in the percentage of cells with low [GSH]_i were inhibited by the cycloheximide treatment (Figure 7-6).

Thymocytes were also treated simultaneously with 10µM dexamethasone and various concentrations of thapsigargin for 6 hours. Thapsigargin concentrations of 5 to 100nM dramatically inhibited dexamethasone-induced apoptosis (Figure 7-7). Simultaneous with the decrease in apoptosis was a reduction in the percentage of cells with low [GSH]_i.
Figure 7-4 Lowering of [GSH] \textsubscript{i} precedes apoptosis.

The onset of apoptosis was accompanied by an increase in the percentage of cells with lowered [GSH], when various agents were used to induce apoptosis in thymocytes during a 7 hour incubation. The percentage of cells with fragmented DNA (open bars) and the percentage of cells with lowered [GSH], (diagonal hatched bars).

The bar labels control, \textit{d}ex\textsubscript{10}uM, GSSG\textsubscript{1}mM, GSSG\textsubscript{10}mM and GT\textsubscript{1}uM represent the solvent control, 10µM dexamethasone, 1mM GSSG, 10mM GSSG and 1µM gliotoxin, respectively.
Figure 7-5 Correlation between drop in $[\text{GSH}]_i$ and apoptosis.

There is a correlation between the percentage of cells with low $[\text{GSH}]_i$ and percentage of apoptotic cells after treatment with solvent control, 10\muM dexamethasone and 10nM thapsigargin for times between 0 and 18 hours.
Figure 7-6 When cycloheximide inhibited dexamethasone-induced apoptosis in thymocytes at 6 hours the percentage of cells with lowered [GSH]i also fell.

- percentage of cells with fragmented DNA; ● percentage of cells with low [GSH]i; closed symbols cycloheximide alone, open symbols cycloheximide together with 10µM dexamethasone.
Figure 7-7 Thapsigargin antagonised dexamethasone effects in thymocytes.

Thapsigargin inhibited dexamethasone-induced apoptosis in thymocytes at 6 hours and also inhibited the increase in the percentage of cells with lowered [GSH]; △ percentage of cells with fragmented DNA; ○ percentage of cells with low [GSH]; closed symbols show response to control treatment, open symbols show response to thapsigargin together with 10µM dexamethasone.
Figure 7-8 Pretreatment for 24 hours with Procysteine® reduced the amount of apoptosis induced by 10µM dexamethasone.

a. negative control; b. 10µM dexamethasone; c. 10µM dexamethasone with 25mM Procysteine®; d. 10µM dexamethasone with 50mM Procysteine®

M1 marks the cells with sub-diploid DNA, that is the apoptotic cells.
Figure 7-9 Diamide induced apoptosis and caused an increase in the percentage of thymocytes with lowered [GSH].

This bar graph shows the percentage of cells with fragmented DNA (open bars) and the percentage of cells with lowered [GSH], (diagonal hatched bars).
Figure 7-10 Apoptosis induced with thapsigargin in P815 cells involved S-glutathiolation of proteins.

In this figure two densitometry scans are presented of an autoradiograph of $^{35}$S-labelled proteins extracted from P815 cells after they were treated for 24 hours with $^{35}$S-glutathione and for a further 24 hours with 10nM thapsigargin. **Peaks 1 to 6** represent proteins which showed greater radioactive labelling after the thapsigargin treatment. **Panel A** control cells; **Panel B** 10nM thapsigargin treated cells. **a, b** and **c** represent migration of the molecular mass markers of 14.3, 30 and 46kD, respectively.
1. Glutathione in apoptosis

Thymocytes were treated for 1 hour with GSH, then dexamethasone was added at 10 μM in the culture medium and the cells incubated for a further 6 hours. After this treatment, the percentage of apoptotic cells was reduced from 59.6% with dexamethasone alone to 32.5% with added 1 mM GSH, compared with a background of 18.5%. Lower concentrations of GSH did not inhibit apoptosis, whilst at concentrations above 10 μM the activity of the GSH solution reduced the effects of dexamethasone in a non-specific manner and no protection was afforded against the effects of dexamethasone (data not shown).

Pre-treatment of thymocytes with GSH for 24 hours also delayed the apoptotic profile in response to 10 μM dexamethasone. The percentage of apoptotic cells decreased from 23.1% ± 0.1% (GSH) to 18.8% ± 0.1% (dexamethasone + 50 μM GSH) and 16.2% ± 0.1% (dexamethasone + 100 μM GSH), respectively, from 21.3% ± 0.1% in the control (Figure 7-9). At higher concentrations of thymocytes and lower late viability, the cells became necrotic (data not shown).

Treatment of thymocytes for 7 days with GSH at 1 and 10 mM induced significant apoptosis (33.1%) and 62.2%, respectively, compared with a background level of 12.1% in the control. Conversely, the induction of cell death with lowered [GSH] was decreased to 65.4% and 71.9% at 1 and 10 mM GSH, respectively, from 32.5% in the control (Figure 7-9).

2. Appearance of glutathione induced proteins

PS13 cells stimulated with 10 μM glutathione for 24 hours and then with 10 mM GSH for a further 24 hours showed no proteins more cytotoxicity.
7.2.4 Modulation of the intracellular GSH/GSSG ratio affects apoptosis

Thymocytes were treated for 1 hour with GSH, then dexamethasone was added at 10µM in the culture medium and the cells incubated for a further 6 hours. After this treatment, the percentage of apoptotic cells was reduced from 59.6% with dexamethasone alone, to 35.8% with added 1mM GSH, compared with a background of 19.9%. Lower concentrations of GSH were ineffective at inhibiting dexamethasone induced apoptosis, whilst at concentrations above 1mM the acidity of the GSH solution reduced the pH of the cell culture medium and no protection was offered against the effects of the dexamethasone treatment (data not shown). Pre-treatment of thymocytes with Procysteine® for 24 hours also decreased the amount of apoptosis induced by a 6 hour 10µM dexamethasone treatment (Figure 7-8). The percentage of apoptotic cells were negative control 23.7 ± 0.1%, 10µM dexamethasone 63.0 ± 0.0%, 10µM dexamethasone + 25mM Procysteine® 49.0 ± 2.1%, 10µM dexamethasone + 50mM Procysteine® 46.8 ± 3.0%.

Treatment for 6 hours with diamide concentrations of 5 and 10µM induced both apoptosis and an increase in the percentage of cells with low [GSH]i (Figure 7-9). At higher concentrations of diamide and longer time periods the cells became necrotic (data not shown).

Treatment of thymocytes for 7 hours with GSSG at 1 and 10mM induced significant apoptosis (53.9% and 63.4%, respectively, compared with a background level of 12.7% in the control). Simultaneously, the population of cells with lowered [GSH]i was increased to 65.4% and 71.5% for 1mM and 10mM GSSG, respectively, from 22.0% in the control (Figure 7-4).

7.2.5 Appearance of glutathione labelled proteins

P815 cells incubated with 35S-glutathione for 24 hours and then with 10nM thapsigargin for a further 24 hours, showed six proteins more radioactively labelled than the same proteins in the control treatment (Figure 7-10a & b). The labelled protein peaks numbered 1 to 6 in Figure 7-10 had
molecular masses of 42.8, 40.8, 39.1, 37.5, 32.9, and 12.9kD respectively. Figure 7-11 shows the standard curve used to calculate the molecular masses of the labelled proteins.

7.3 Discussion

Apoptosis has been shown to be induced by substances that cause oxidative stress or that generate free radicals (Hockenbery et al., 1993; Buttke & Sandstrom, 1994). To study the relationship between the cellular redox status, as reflected by the [GSH], and the onset of apoptosis in mouse thymocytes, the cells were treated with agents not considered to directly induce oxidative stress. These agents, dexamethasone, thapsigargin and gliotoxin, are thought to have very different modes of action inside the cell, therefore any changes they cause in common are likely to be part of a generalised pathway to apoptosis.

The apoptotic cells were quantified by staining ethanol fixed cells with propidium iodide and measuring their fluorescence with flow cytometry (Darzynkiewicz et al., 1992; Nicoletti et al., 1991). The GSH concentration was also measured with a flow cytometric method, using o-phthaldialdehyde (OPT). OPT binds the thiol group and the amino group on the γ-glutamyl group on the GSH molecule, making it more specific than other thiol indicators such as the bromobimanes (Treumer & Valet, 1986). A comparison of the OPT assay and the HPLC method demonstrated the OPT assay gave an accurate reflection of the decrease in [GSH], occurring in the thymocytes. Furthermore, the addition of diamide to the cells to chemically remove GSH from the cell (Kosower & Kosower, 1969, 1987) led to a dramatic decrease in the GSH-OPT fluorescence.

When the effects of dexamethasone, thapsigargin and gliotoxin were considered, there was found to be a strong correlation between the percentage of cells with low [GSH], and the percentage of apoptotic cells after all treatments, concentrations and time points. With all treatments the
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Drop in [GSH]\_i\_ preceded the appearance of DNA fragmentation by at least 1 to 2 hours. Recently Slater et al. (1995) have also described a decrease in the intracellular [GSH]\_i\_ before the appearance of DNA fragmentation. This result is in agreement with the findings of Wu et al. (1994) who observed, using entirely different methods, a drop in the [GSH]\_i\_ before the onset of apoptosis in methylprednisilone treated rat thymocytes.

Glutathione is an important defence against oxidant stress in cells (Halliwell & Gutteridge, 1989; Meister, 1994). The [GSH]\_i\_ in normal cells is between 2 and 20mM (in the spleen, a lymphoid organ, the [GSH]\_i\_ is 4-5mM), and the ratio of [GSH]/[GSSG] is usually greater than 100/1. Glutathione has two major roles as an antioxidant. Firstly, through the action of glutathione-S-transferases, GSH reacts with xenobiotics to produce their mercapturic acid derivatives as part of a detoxification pathway. Secondly, it reacts with peroxides, using the enzyme glutathione peroxidase, to convert organic peroxides to their equivalent alcohol, and hydrogen peroxide to water. These reactions produce GSSG. Glutathione-S-transferase also has peroxidase activity. The GSSG produced as a result of glutathione peroxidase activity can be recycled by GSH reductase and requires NADPH (Halliwell & Gutteridge, 1989).

Dexamethasone has been demonstrated to induce the upregulation of glutathione-S-transferase in both a thymocyte and a lymphocyte cell line (Flomelerfelt et al., 1993). Glutathione-S-transferase expression has also been shown to increase in rat ventral prostate soon after androgen withdrawal but before the time at which maximum cell death occurred (Briehl & Miesfeld, 1991). The action of glutathione-S-transferase could have been a possible cause of the decrease in [GSH], observed after the treatments administered in these experiments. However, GSSG treatment induced both apoptosis and an increase in the percentage of cells with low [GSH], and there is no obvious reason why treatment with GSSG should upregulate glutathione-S-transferase, as it is not a xenobiotic compound.

When dexamethasone-induced apoptosis was inhibited, by both
cycloheximide and thapsigargin, the rise in the number of cells with decreased $[\text{GSH}]_i$ was also inhibited. Cycloheximide is a recognised inhibitor of dexamethasone-induced apoptosis and an inhibitor of protein synthesis (Wyllie et al., 1984; Oleinick, 1977). The fall in $[\text{GSH}]_i$ associated with dexamethasone-induced apoptosis occurred at least 1-2 hours before the onset of DNA fragmentation. Cycloheximide inhibited this drop. This indicated that the inhibitory effect of cycloheximide acted early in the apoptosis signalling pathway before the onset of fragmentation.

Protein synthesis inhibitors have been demonstrated to inhibit apoptosis by increasing the supply of intracellular cysteine available for the synthesis of GSH (Ratan et al., 1994b) as a result of its diversion away from protein synthesis. This suggests strongly that GSH has a role early in the apoptotic pathway.

When thapsigargin was used to inhibit the dexamethasone-induced apoptosis the percentage of cells with decreased $[\text{GSH}]_i$ fell but not to the background level. As demonstrated previously (Chapter 6 Thapsigargin has other effects) the concentrations of thapsigargin that inhibited dexamethasone-induced apoptosis at 6 hours went on to induce significant apoptosis at 18 hours. Therefore, the drop in the percentage of the cells with low $[\text{GSH}]_i$ to levels above background when thapsigargin acted as an apoptosis inhibitor, probably reflected the proportion of cells affected by thapsigargin alone, rather than a lack of inhibition of dexamethasone-related changes. That is, at 6 hours the thapsigargin inhibited the dexamethasone-induced apoptosis, but the $[\text{GSH}]_i$ did not fall to background because the cells were being induced to die by apoptosis as a result of the thapsigargin treatment.

Faced with the possibility of extensive peroxidation of cellular macromolecules, with the possibility of subsequent necrosis and local inflammation, a preventative course of action for the cell could be to trigger the apoptosis pathway before such widespread damage occurs. This would allow the cell to dismember itself neatly without causing harm.
to its neighbours. Cellular responses to stress include the lowering of $[\text{GSH}]_i$ through the oxidation of GSH to GS$\text{G}$ and subsequently the formation of mixed disulphides with protein (GS-protein). The extent of the fall in $[\text{GSH}]_i$ may be a trigger for apoptosis to prevent subsequent necrosis. Alternatively, the observation made here that some proteins became labelled with $^{35}\text{S}$-GSH during thapsigargin-induced apoptosis suggested the modification of proteins by S-glutathiolation by GS$\text{G}$ may have an important role in apoptotic signalling.

In all the experiments described here, the fall in $[\text{GSH}]_i$ occurred before the onset of DNA fragmentation and this would be consistent with $[\text{GSH}]_i$ acting as a trigger for apoptosis. The observations that pre-treatment with GSH, Procysteine® and also cycloheximide-treatment, each known to increase $[\text{GSH}]_i$, inhibited dexamethasone-induced apoptosis suggested that the $[\text{GSH}]_i$ was vitally involved in dexamethasone-induced apoptosis signalling.

On the other hand, GS$\text{G}$ itself may be playing a direct role in the observed induction of apoptosis signalling as a result of a decrease in the $[\text{GSH}]_i$ or through the production of S-glutathiolated proteins with altered activity. The interaction of GS$\text{G}$ with cellular structures would be expected to decrease the $[\text{GSH}]_i$ because the GSH would be used to reduce the mixed disulphides formed by the GS$\text{G}$. Results presented here show an increase in S-glutathiolated proteins with the onset of apoptosis in P815 cells induced by thapsigargin. As yet these proteins have not been identified, but it is interesting to speculate that one or more of these proteins may have a role in apoptotic signalling. Taken together, these results indicate that the induction of apoptosis after treatment with GS$\text{G}$ is consistent with the proposal that the glutathione redox defence system is involved in apoptosis signalling.

Furthermore, the previous observations that the GSH precursor $N$-acetylcysteine can confer protection against apoptosis (Malorni et al., 1993), and that the depletion of intracellular GSH stores by inhibiting its
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synthesis with buthionine sulfoximine (BSO) can render cells more susceptible (Zhong et al., 1993), supports the hypothesis that GSH has a central role in the onset of apoptosis.

To test whether a drop in $[\text{GSH}]_i$ alone was enough to trigger apoptosis, thymocytes were treated with diamide, an agent well known to drastically reduce the $[\text{GSH}]_i$. Low concentrations of diamide induced apoptosis, whilst higher concentrations induced necrosis. This suggested that the onset of apoptosis was related to subtle fluxes in GSH metabolism in mouse thymocytes, whilst large decreases in $[\text{GSH}]_i$ swamped the cell's ability to respond in a controlled fashion and necrosis was the outcome. The previously discussed evidence that at low concentrations gliotoxin and diamide induced apoptosis in thymocytes, but at high concentrations they both induced necrosis, supports this conclusion. The damage induced in the cells at the high gliotoxin and diamide concentrations is probably a result of direct interaction of the chemicals with plasma membrane proteins through thiol groups.

7.4 Conclusion

The results presented here support the hypothesis that an oxidative event, which produces GSSG, occurs as an early step in the onset of apoptosis induced by a variety of agents not generally recognised as being pro-oxidants. Furthermore, this decrease in the $[\text{GSH}]_i$ may have a role in apoptosis signalling possibly through the formation of mixed disulphides between proteins and GSSG, which may cause an alteration in the activity of significant proteins. These proteins might be active either in the central signalling pathway of apoptosis or be among the proteins that are early effectors of the apoptotic changes. GSSG has been previously shown to form such mixed disulphides (McConkey et al., 1989; Zhong et al., 1993) and to alter protein activity. It is interesting that gliotoxin is also capable of forming mixed disulphides with cellular proteins.
8. Cell surface changes

It is well understood that apoptotic cells are rapidly removed from tissues by phagocytes, either by neighbouring cells or by professional phagocytes. However, the mechanism by which the apoptotic cell signals its readiness for removal has not been detailed. Early reports suggested changes in the plasma membrane glycoprotein, called phosphatidylserine (Morris et al., 1988; Osh et al., 1992). More recently, integrin and thrombospondin integrin mechanisms have also been suggested (Ouchi et al., 1992; Haiden et al., 1994).

In the preliminary investigations, only the changes in expression of N-acetylglucosaminyl (N-acetylglucosaminyl) were studied. The N-linked sugar normally occurs as part of larger N-linked oligosaccharide tree-like structures on plasma membrane glycoproteins or glycolipids. The N-acetylglucosaminyl residues are usually found when they are not exposed. Basic factors and mannose residues are common to many sugars (Figure 8.1). These glycosylated structures are synthesized in the lumen of the endoplasmic reticulum and modified in the Golgi complex.

The N-acetylglucosaminyl residues would become exposed on the surface of the plasma membrane only if either the terminal state and glycopeptide or mannose residues were removed and consequently the N-acetylglucosaminyl residues would become exposed on the surface of the cell. Distelberg et al. (1995) suggested that a single catalytic entity gives surface expression of sugars in necrotic apoptosis. Incompletely manufactured glycosaminoglycan could be exposed on the plasma membrane during the extensive plasma membrane rearrangements that accompany apoptosis, especially those associated with apoptotic body formation. These rearrangements would be likely to result in the
8.1 Introduction

It is well understood that apoptotic cells are rapidly removed from tissues by phagocytosis, either by neighbouring cells or by professional phagocytes. However, the mechanism by which the apoptotic cell signals its readiness for removal has not been defined. Early reports suggested changes in the plasma membrane glycosylation stimulated phagocytosis (Morris, et al., 1984; Duvall et al., 1985; Dini et al., 1992). More recently, integrins and thrombospondin mediated mechanisms have also been suggested (Savill et al., 1992; Haslett et al., 1994).

In this preliminary investigation, only the changes in expression of N-acetylglucosamine (glc-NAc) were studied. This N-linked sugar normally occurs as part of larger N-linked oligosaccharide tree-like structures on plasma membrane glycoproteins or glycolipids. The glc-NAc residues are usually found within the oligosaccharide structure and therefore are not exposed. Sialic acid and mannose sugars are the most common terminal sugars (Figure 8-1). These oligosaccharide structures are synthesised in the lumen of the endoplasmic reticulum and modified in the Golgi complex.

The glc-NAc residues would become exposed on the surface of the plasma membrane only if either the terminal sialic acid galactose or mannose residues were removed enzymatically in situ or if incompletely assembled oligosaccharide structures were exposed on the surface of the cell. Dransfield et al. (1995) suggested that sialidase activity may alter surface expression of sugars in neutrophil apoptosis. Incompletely manufactured oligosaccharides could be exposed on the plasma membrane during the extensive membrane rearrangements that accompany apoptosis, especially those associated with apoptotic body formation. These rearrangements would be likely to result in the
Figure 8-1 Structure of typical N-linked oligosaccharides

sialic = N-acetylneuraminic acid (sialic acid); Gal = galactose; glcNAc = N-acetylglucosamine; Man = mannose; Fuc = fucose.
incorporation of both endoplasmic reticulum and Golgi complex membranes into the plasma membrane. This proposal is supported by the appearance of phosphotidylserine in the outer membrane leaflet during apoptosis (Fadok et al., 1992).

In these experiments, fluorescein-labelled wheat germ agglutinin (WGA-FITC) was used to probe for the expression of glc-NAc on the plasma membrane of cells, using flow cytometry. To confirm that any observed changes were the result of generalised apoptotic changes and not specific to the means of induction, these changes were compared using spontaneous, dexamethasone- and gliotoxin-induced apoptosis in thymocytes.

8.2 Results

8.2.1 Apoptosis affected WGA binding

Thymocytes were incubated for either six or 18 hours with either the negative control treatment, 1 µM gliotoxin or 10 µM dexamethasone, and then stained with both ethidium bromide and WGA-FITC to identify the apoptotic cells and to detect glc-NAc on the cell surface, respectively. The analysis involved selecting representative samples of cells in each of the three regions previously defined in EB staining (as defined in Chapter 4 Defining the onset of apoptosis) and then determining the mean WGA-FITC fluorescence for the cells in each of these representative regions. These EB regions were defined as being Region1 (normal cells), Region2 (cells early in the apoptotic process), and Region3 (cells well progressed through the process).

In all cases, no matter the treatment, the cells in Region2 had the lowest WGA-FITC fluorescence and the cells in Region3 had a considerably raised WGA-FITC fluorescence. Dot plots of data collected at 6 hours are presented in Figure 8-2a - f. The representative regions, selected on the basis of EB staining, are displayed in the plots of EB versus FSC.
Figure 8-2 Changes in glc-NAc expression on thymocytes after 6 hour incubation

Dot plots a, c, and e display the small representative regions selected after EB staining for apoptosis. Dot plots b, c, and f display WGA-FITC staining of the small representative regions versus cell size (FSC).

a, b. control treatment; c, d: 1µM gliotoxin; and e, f. 10µM dexamethasone.
Figure 8-3 Changes in glc-NAc expression on thymocytes after 18 hour incubation

Dot plots a, c, and e display the small representative regions selected after EB staining for apoptosis. Dot plots b, c, and f display WGA-FITC staining of the small representative regions versus cell size (FSC).

a, b. control treatment; c, d; 1µM gliotoxin; and e, f. 10µM dexamethasone.
Figure 8-4 Average WGA-FITC fluorescence after 6 hours and 18 hours incubation

This bar graph displays the average WGA-FITC fluorescence associated with each of the regions described in Figures 8-2 and 8-3 above. Region1 open bar; Region2 diagonal hatched bar; Region3 cross hatched bar. 

a. 6 hours; b. 18 hours.
Cell Surface Changes

(Figure 8-2a, c, e). The WGA-FITC staining of the cells in each of these regions is shown in the dot plots of FSC versus WGA-FITC (Figure 8-2b, d, f). It was clear that the cells in Regions 1, 2 and 3 progressively decreased in size (FSC) with Region 1 having, on average, the larger cells and Region 3 the smallest. Also evident was the increase in WGA-FITC fluorescence associated with Region 3.

In Figure 8-3 a-f the data from the 18 hour incubation has been presented in a similar fashion, and qualitatively similar changes are obvious. The biggest difference between the 6 hour and 18 hour data is the absence of cells in Region 1 following treatment with the 1 µM gliotoxin or 10 µM dexamethasone. This is consistent with other observations made previously that both of these treatments induce massive apoptosis in thymocytes at 18 hours and that very few normal cells remain. The average WGA-FITC fluorescence for each of the three regions after each treatment has been summarised in Figure 4a & b.

The relative fluorescence detected in the 18 hour experiment was much higher than that recorded in the 6 hour experiment. This was not taken as significant because the magnitude of the fluorescence in a particular experiment depended largely on the set up of the flow cytometer on the day. Only comparisons between fluorescence measurements taken within a single experiment were able to be compared.

8.2.2 WGA binding increased as cells progressed through the apoptotic process

After incubation with 1 µM gliotoxin for 6 hours, thymocytes were stained with ethidium bromide. These stained cells sorted by flow cytometry and the cells in Region 2 were collected. These collected cells, which were already committed to apoptosis but not yet forming apoptotic bodies, were then incubated for various times, up to 4 hours 35 minutes, in fresh medium and finally stained with both ethidium bromide and WGA-FITC.
Figure 8-5 Glc-NAc expression increased over time.
Displayed here are representative WGA-FITC staining histograms for thymocytes at three times after being sorted to collect Region2 cells from EB staining:
Time zero (thin solid line); 2h 10m after sorting (thick solid line); 4h 35m after sorting (thin dashed line).
Over the time the EB staining increased consistently with the time in Region 3 progression. Thymocytes were sorted at various time points and the average fluorescence of WGA-FITC binding increased over time. This increase was also seen in the representative cell images (Figure 8-6) and in the trend of average WGA-FITC fluorescence against time (Figure 8-6).

8.3 Discussion

The results presented here indicate the dynamic balance in the expression of glycosaminoglycans on the plasma membrane of thymocytes during the apoptotic process. In our EB staining, the pattern was used to sort the EB stained cells, and the trend in the time of PI was determined. In our study, we observed a significant increase in the amount of PI at various time points. This increase in PI was consistent with the EB staining data, indicating that the cells were undergoing apoptosis. The average WGA-FITC fluorescence increased with time, suggesting reduced cell volume. The increase in WGA fluorescence is likely to be simply a result of smaller cell size, as the decrease in staining is still gradual even when the reduction in cell size is minimal. The increase in fluorochrome staining of the cells on Region 2 decreased at the later time as the average cell size was further decreased. The trend at later time points shows that glycosaminoglycans are dispersed on the plasma membrane just before or during the later stages of the apoptotic break up of the cell.

Figure 8-6 Average WGA-FITC fluorescence associated with glc-NAc binding increased over time.

Thymocytes were sorted after EB staining and Region 2 cells were collected. This graph displays the average fluorescence of these cells at various time points after sorting. WGA-FITC fluorescence increased with time.
Over the time the EB staining increased, consistent with the cells in Region2 progressing onto Region3. Simultaneously there was an increase in the average WGA-FITC staining of the cells. This increase can be seen in the histograms of WGA-FITC fluorescence from three representative time points (Figure 8-5) and in the graph of average WGA-FITC fluorescence against time (Figure 8-6).

8.3 Discussion

The results presented here indicate that changes occur in the availability of glc-NAc on the plasma membrane of thymocytes during the apoptotic process. The use of EB to stain the fresh cells, as opposed to the use of PI to stain fixed cells, allowed a differentiation between the changes associated with relatively early apoptotic changes (Region2 in EB staining) and those that occurred later when the cells had lost their ability to maintain their plasma membrane integrity (Region3 in EB staining).

It was noteworthy that the cells in Region2 showed a small but consistent decrease of glc-NAc on the plasma membrane. It has been previously demonstrated that these cells have condensed chromatin (in 3.4.1.1 Ethidium bromide staining identifies apoptotic cells), but only slightly reduced cell volume. The decrease in glc-NAc is unlikely to be simply a result of smaller cell size, as the decrease in staining is still obvious even when the reduction in cell size is trivial, such as after the 6 hour gliotoxin treatment. The increase in glc-NAc on the plasma membrane of the cells in Region3 occurred at the same time as the average cell size was further decreasing. This result suggests most strongly that glc-NAc is displayed on the plasma membrane just before or during the later stages of the apoptotic break up of the cell.

These changes appeared to be associated with the apoptosis process rather than the result of a particular treatment and occurred whether the apoptosis was spontaneous background apoptosis or induced by such disparate agents as gliotoxin or dexamethasone.
The time course experiment provided further evidence that the cells in Region2 with lower glc-NAc and lower EB staining progressed over a few hours to take on the Region3 characteristics of raised glc-NAc and EB staining.

No investigation has yet been made into the stage of EB staining at which the cells are phagocytosed. The cells in Region2 have condensed chromatin but it is not yet clear if they all have fragmented chromatin. Lyons et al. (1992) showed that after 3 hours incubation with methylprednisilone a considerable population of cells had appeared in Region2 and this was associated with the development of fragmented DNA. However, the proportion of those cells in Region2 with fragmented DNA was not explicitly investigated. Only sorting for these cells and then assaying for DNA fragmentation would clarify this question. The cells in Region3 do have fragmented DNA. Previous experiments have shown that the 18 hour treatments that cause greater than 90% of the cells to appear in Region3 also caused greater that 90% of the cells to have fragmented DNA as detected by PI staining. It is likely that the cells would not be phagocytosed until after DNA fragmentation, to reduce the chance of large pieces damaged DNA being taken up by the phagocytic cell. The incorporation of damaged DNA into the phagocytic cell could be potentially harmful to the cell. In summary, it is possible that the cells in Region3 are ready for phagocytosis and an increase in glc-NAc is part of the signalling mechanism to phagocytic cells.

8.3.1 Conclusion

Changes occurred in the expression of glc-NAc on the plasma membrane of thymocytes as they went through the apoptotic process. The changes appeared to be a general outcome of apoptosis, no matter the means of induction. These results support the previous experiments by Duvall and his colleagues that suggested the possibility that altered surface glycosylation could act as a trigger for phagocytosis. If an increase in glc-NAc is a trigger for phagocytosis, it is interesting that the cells in Region2,
with condensed but possibly not oligonucleosomally fragmented DNA, displayed decreased glc-NAc. This may prevent cells that have recently entered the apoptotic pathway from being phagocytosed before their DNA is entirely fragmented.
9. Final Conclusions and Future Directions

The major issue of this project was the role of cancer chemotherapeutic agents in the regulation of apoptosis, especially in thymocytes. These agents were used to promote apoptosis.

Dexamethasone, a synthetic glucocorticosteroid analog of high potency, was used to induce apoptosis. Caspase activation and apoptotic cell death have been described in detail in the literature and it provided a benchmark by which the efficacy of other agents could be measured.

Glutamine, a basic compound, whose exact mode of action is still unknown, was found to induce apoptosis in thymocytes at concentrations between 50 μM and 100 μM. At concentrations equal to and greater than 50 μM, glutamine induced the rapid onset of necrosis. Glutamine was observed to induce apoptosis in thymocytes in a manner similar to the treatment, where 90% of the cells had fragmented DNA.

The effects of thapsigargin were investigated in much detail. Thapsigargin is an inhibitor of the endoplasmic reticulum Ca²⁺-ATPase. It was found to induce apoptosis even at a much lower time period, 2 hours in thymocytes. It was also found to be effective at very low concentrations. A 10 μM thapsigargin solution could induce more than 90% of thymocytes to lose fragmented DNA after 24 hours incubation. When thapsigargin was used with the P388 drug-resistant cell line, symptoms similar to those observed in thymocytes were observed longer were noted. The same proportion of apoptotic cells as observed in thymocytes.

The injection of cytokines by these chemotherapeutic agents was confirmed by multiple methods. The cells were inspected with electron microscopy, and DNA was run on agarose gel electrophoresis and the cells were stained with two different dyes: cytochrome oxidase, ethidium bromide (EB) and propidium iodide (PI). Of these methods provided comparable results. Of the two methods proved most useful as they were rapid to
9.1 Final Conclusions

The major focus of this project was the role of calcium ions and glutathione in the regulation of apoptosis, primarily in thymocytes. Three very different agents were used to provoke apoptosis.

Dexamethasone, a synthetic glucocorticosteroid analog of high potency, was used to induce apoptosis. Dexamethasone-induced apoptosis has been described in detail in the literature and it provided a benchmark by which the effects of other agents could be measured.

Gliotoxin, a toxic fungal metabolite, whose direct mode of action is still unknown was found to induce apoptosis in thymocytes at concentrations between 50nM and 10µM. At concentrations equal to and greater than 50µM, gliotoxin induced the rapid onset of necrosis. Gliotoxin was observed to induce apoptosis in thymocytes with similar kinetics to the induction by dexamethasone. After an incubation for 6 hours with either treatment, near 50% of the cells had fragmented DNA.

The effects of thapsigargin were investigated in much detail. Thapsigargin is an inhibitor of the endoplasmic reticulum Ca$^{2+}$-ATPase. It was found to induce apoptosis over a much longer time period; 18 hours in thymocytes. It was also found to be effective at very low concentrations. A 10nM thapsigargin treatment could induce more than 80% of thymocytes to have fragmented DNA after 18 hours incubation. When thapsigargin was used with the P815 mastocytoma cell line, concentrations ten-fold higher and incubations six hours longer were needed to observe the same proportion of apoptotic cells as observed in thymocytes.

The induction of apoptosis by these three agents was monitored by several methods. The cells were inspected with electron microscopy, the DNA was run on agarose gel electrophoresis, and the cells were stained with two different flow cytometry methods, ethidium bromide (EB) and propidium iodide (PI). All of these methods provided comparable results. The flow cytometry methods were especially useful as they were rapid to
conduct, reproducible and simple to analyse. The EB method allowed the
detection of three populations of cells; normal cells, cells early in the
apoptotic process and those well progressed through apoptosis. The PI
method involved the staining of fixed cells and an estimation of the
percentage of cells with fragmented DNA. This was a rapid and
reproducible method but did not allow for the differentiation between
normal and necrotic cells. Indeed the only two methods that did enable a
distinction to be made between normal and necrotic cells were electron
microscopy and the EB flow cytometry method.

After the induction of apoptosis with these agents was defined, their effect
on the calcium ion homeostasis of the cell was investigated. At the
concentrations that induced apoptosis, neither dexamethasone nor
gliotoxin was found to have any effect on the cytosolic $[\text{Ca}^{2+}]$ over 85
minutes. Gliotoxin at very high concentrations (500µM and above) caused
increases in $[\text{Ca}^{2+}]$, but these increases were relatively short-lived as the
cells rapidly became necrotic and lost their membrane integrity. Thapsigargin, which is an irreversible inhibitor of the endoplasmic
reticulum $\text{Ca}^{2+}$-ATPase, caused a rapid and prolonged increase in the
$[\text{Ca}^{2+}]$. Whilst concentrations of thapsigargin which induced apoptosis also
induced large rises in the $[\text{Ca}^{2+}]$, there was not a simple relationship
between these occurrences. Previous reports of DNA fragmentation in
isolated nuclei incubated calcium rich medium described the DNA
fragmentation as occurring within 90 minutes. Thapsigargin induced
apoptotic DNA fragmentation over 12 to 18 hours. Therefore a direct
activation of a $\text{Ca}^{2+}$-dependent endonuclease was an unlikely explanation
of thapsigargin’s apoptosis inducing activity.

The effect of thapsigargin on the mitochondria of the cells was studied in
detail. Thapsigargin-treated thymocytes and P815 cells were observed to
have swollen mitochondria with disrupted cristae structure. When these
cells were stained with Rhodamine 123 (R123), a dye that will move into
an organelle with a transmembrane potential, thapsigargin treated cells
were found to stain less brightly than normal cells. The ATP concentration of thymocytes treated with either dexamethasone or thapsigargin was measured at time points where an approximately equivalent amount of DNA fragmentation had occurred. The dexamethasone treatment did not cause any decrease in the cellular ATP concentration, but the thapsigargin treatment caused a significant drop. It was concluded that the prolonged increase in the \([Ca^{2+}]\) lead to \(Ca^{2+}\) uptake by the mitochondria that eventually resulted in phase transition swelling and a loss of mitochondrial function. This loss of function led to decreased \([ATP]\) and provided an apoptotic stimulus for the cell.

Thapsigargin also had other effects related to the increase in \([Ca^{2+}]\). It was found to be a poor protein synthesis inhibitor in thymocytes. It was able to inhibit dexamethasone-induced apoptosis at least until 6 hours. After an 18 hour incubation the cells still died, most likely from the effects of the thapsigargin, which normally kills the cells over the longer time course. Thapsigargin was shown to be a potent inhibitor of the cell cycle in rapidly cycling P815 cells. It induced a block before S phase. When the onset of apoptosis was studied in terms of the cell cycle it was clear that the apoptosis and cell cycle block were under separate control as the cells died from all phases of the cycle to the same extent.

The role of glutathione was investigated. The intracellular glutathione concentration ([GSH]) in thymocytes was found to decrease before the onset of DNA fragmentation, no matter the method of induction of apoptosis. There was a very high positive correlation between the loss of intracellular GSH and the onset of apoptosis. Dexamethasone-induced apoptosis could be partly inhibited by addition of GSH to the medium and apoptosis could be induced by GSSG alone. An increase in the abundance of some S-glutathiolated proteins was found to accompany the onset of apoptosis induced by thapsigargin in P815 cells. Taken together these results suggested that the GSH/GSSG couple has an important role in the early stages of apoptosis signalling.
Finally, preliminary investigations were conducted into the changes in expression of $N$-acetylglucosamine (glc-NAc) on the outer surface of the plasma membrane. The expression of this sugar was seen to drop slightly after the induction of apoptosis. As apoptosis continued, glc-NAc expression increased to levels much above that observed on normal cells. This observation agreed well with previous reports that alterations in glc-NAc expression may play a role in signalling that the apoptotic cell should be phagocytosed.

**9.2 Future directions**

The results presented in this project open up many possibilities for future experimentation.

Whilst there does not appear to be a major flux in the $[\text{Ca}^{2+}]$, during either dexamethasone- or gliotoxin-induced apoptosis this does not rule out the possibility that small localised calcium transients may be occurring in the cells during the onset of apoptosis.

Investigation of this would require the use of confocal imaging and the new generation of fluorescence calcium ion chelating dyes that are organelle specific. One example is, fura-C$_{18}$, which has a lipid tail and therefore becomes localised inside the plasma membrane of the cell and allows detection of $[\text{Ca}^{2+}]$ which are localised near the plasma membrane. Also of interest, would be the detection of calcium transients in or near the endoplasmic reticulum, nucleus and mitochondria.

Further investigation of thapsigargin's ability to inhibit dexamethasone-induced apoptosis could provide more information on the role of calcium signalling after dexamethasone treatment. It would be interesting to test if the calmodulin inhibitors could interfere with the effect of thapsigargin on dexamethasone-induced apoptosis.

The effects of thapsigargin on cells require much more study. In this study thapsigargin was observed to induce apoptosis in thymocytes from
suckling mice over 18 hours but other groups have observed it induce apoptosis in mature rat thymocytes within 6 hours. An immediate priority would be to determine if it is an age or species difference that led to such a difference in response. If a difference based on age was found, this could provide interesting insights into the changing phenotype of thymocytes as they develop, and into their changing sensitivity to increases in \([\text{Ca}^{2+}]\). Reports in the literature show that thapsigargin can induce apoptosis in many different cell types but over a wide range of times from a few hours to a few days. An investigation into the differences between these cell types in terms of the distribution and abundance of the endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPases, and their sensitivity to thapsigargin inhibition, may provide explanations for these different times of apoptosis induction. In addition, in all the cell types tested with thapsigargin, other inhibitors of the endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase, such as cyclopiazonic acid and tBHQ, should be tested for their ability to induce apoptosis, and the kinetics of induction.

The involvement of mitochondrial function in the onset of apoptosis is currently being investigated in several laboratories with the use of fluorescent dyes. More exact methods of measurement of mitochondrial health need to be used. An analysis of \([\text{ATP}]\) and apoptosis over a short-interval time course with both dexamethasone and the agents which inhibit the endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase would be useful. An oxygen electrode would also provide useful results from such a time course. To investigate the universal nature of mitochondrial changes after thapsigargin treatment, a diverse variety of cell types should be investigated, such as hepatocytes and neurons.

Thapsigargin was found in this work to be a poor inhibitor of protein synthesis within the first hour of incubation. The effect of thapsigargin on protein synthesis could be looked at in more detail. The following questions could be addressed. Does thapsigargin inhibit protein synthesis over longer time periods? Does it lead to a differential expression of
proteins? That is, is the expression of proteins synthesised into the lumen of the endoplasmic reticulum more inhibited than the expression of proteins synthesised in the cytosol? Is there a specific protein that is involved in apoptosis inhibition or cell cycle progression, which has its expression inhibited by thapsigargin's emptying of the endoplasmic reticulum calcium stores? Do the other inhibitors of the endoplasmic reticulum Ca\(^{2+}\)-ATPase also inhibit protein expression in a similar manner?

Thapsigargin was shown here to inhibit the cell cycle causing a block before S phase. The source of this block is unknown. It could result from the emptying of the endoplasmic reticulum calcium stores, which might lead to a disruption of calcium ion signalling or a change in protein expression. Alternatively, it could be an outcome from the prolonged increase in the cytosolic \([\text{Ca}^{2+}]\) which has been demonstrated to cause mitochondrial dysfunction and a decline in ATP production. A third possible cause of the block might be an emptying of the nuclear calcium stores, either directly through the Ca\(^{2+}\)-ATPase in the nuclear membrane or through the endoplasmic reticulum lumen. A comparison of the effect of calcium ionophore action and the effects of cyclopiazonic acid and tBHQ could begin to differentiate between these possibilities. Another avenue of investigation would be to treat the cells to decrease the [ATP] and look for a cell cycle block.

A drop in [GSH], before the onset of DNA fragmentation was a common feature of apoptosis with the agents used in this project. It would be interesting to confirm this as a universal effect by testing the [GSH], in a range of cell types with diverse induction mechanisms. Also interesting to investigate would be the calcium sensitivities of the enzymes that synthesise glutathione. If any of these enzymes is calcium-activated in mouse thymocytes, this could explain both the inhibition of dexamethasone-induced apoptosis at 6 hours and the later onset of thapsigargin-induced apoptosis. Procysteine\(^\circ\) treatment has been shown to be an effective method to raise the intracellular [GSH], and was
demonstrated in this project to reduce the level of apoptosis induced by dexamethasone. Its ability to inhibit apoptosis induced by other agents should be investigated, so as to contribute to a better understanding of the universality of GSH involvement in apoptosis. The observation made here of an increase in abundance of six glutathiolated proteins was interesting. It is a priority to identify these proteins and determine if their activities are altered by the glutathiolation. It is intriguing to note that one of the labelled proteins (33kD) had the same molecular mass as pro-ICE. This suggests an important experiment would be to determine if any of these labelled proteins is an inactive form of an ICE family protease. Perhaps glutathiolation is a signal to cleave and activate these proteins.

The final experiments discussed in this project investigated changes in the expression of glc-NAc on the external surface of the plasma membrane during apoptosis. A survey of the changes in expression of all the sugars involved in protein and lipid glycosylation should be conducted. It would also be interesting to stain cells with lectins and PI and compare the results with the results obtained from the lectin-EB method. An investigation of the DNA fragmentation and relative rates of phagocytosis of the cells in the different EB staining regions would be important.

In conclusion, apoptosis is a fascinating and rapidly developing area of research. Its investigation has allowed the formation of bridges between previously distinct topics of research. There are so many interesting experiments to be done I will have to put down my pen right now and go back to the laboratory bench.
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