THE CYTOTOXIC EFFECTS OF T-2 TOXIN ON NORMAL HUMAN LYMPHOCYTES

BY

THERISHNEE MOODLEY

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ABSTRACT

T-2 toxin is an immunosuppressive mycotoxin that has been conjoined with several symptoms and diseases as early as the turn of the century, but whose mechanisms of action are still being investigated. Accordingly, this study was an attempt to determine the cytotoxic effects of T-2 toxin on normal human lymphocytes *in vitro*, with particular emphasis on mitochondrial viability, cellular and nuclear morphology as well as the localisation of the subcellular sites of toxin interaction. The cytotoxicity of T-2 toxin was assessed with the use of a methylthiazol tetrazolium (MTT) assay. This assay targeted the succinate dehydrogenase activity of the lymphocytic mitochondria, over a range of concentrations of T-2 toxin at various incubation times. The morphology of treated lymphocytes was analysed with the use of transmission electron microscopy and the localisation of the toxin was accomplished via immunocytochemistry. DNA fragmentation studies formed an integral part of the analyses. The cytotoxicity assay indicated that not only was cell viability inversely proportional to both the dose and exposure time, but that the effects of the different doses were only evident at prolonged incubation times (12-24 hours). The electron microscopy studies showed that T-2 toxin (1,56 μg/ml) induced apoptosis (cell suicide) in normal human lymphocytes. This was determined by the observation of chromatin condensation and nuclear disintegration within the toxin treated lymphocytes. Apoptosis seemed to occur independently of mitochondrial damage at 6 hours of exposure to T-2 toxin. The presence of polyribosomes within the treated lymphocytes indicated that protein synthesis was not inhibited. Anti-T-2 toxin conjugated gold label was present in all areas of damage, particularly within the nuclei of the T-2 toxin treated lymphocytes. The DNA fragmentation results showed that T-2 toxin induced fragmentation in lymphocytes, the extent of which was directly proportional to the exposure time. It appears that the early signs of T-2 toxin induced apoptosis in normal human lymphocytes can be determined by damage to the nucleus.

PREFACE

This study represents the original work by the author and has not been submitted in any form to another University. The use of work by other authors has been duly acknowledged in the text.

The research described in this study was carried out in the Department of Physiology, Faculty of Medicine, University of Natal, Durban, under the supervision of Mr. A.A. Chuturgoon and Professor M.F. Dutton.

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ABBREVIATIONS AND ACRONYMS

A-549	-	Human lung carcinoma cell line
AFB_1	-	Aflatoxin B_1
Bcl-2	-	B-cell lymphoma/leukaemia-2 gene
BSA	-	Bovine serum albumin
C-	-	Carbon group
Ca^{2+}	-	Calcium ions
CCM	-	Complete culture medium
CDKs	-	Cyclin-dependent protein kinases
cGy	-	CentiGreys
CHO	-	Chinese hamster ovary cell line
CKIs	-	Cyclin kinase inhibitors
CMI	-	Cell mediated immunity
ConA	-	Concanavalin A
DMSO	-	Dimethylsulfoxide
DPM	-	Dose per minute (of radioactivity incorporated)
EDTA	-	Ethylenediaminetetra acetic acid
ER	-	Endoplasmic reticulum
EtOH	-	Ethanol
Fig.	-	Figure
FPP	-	Farnesyl pyrophosphate
GC	-	Gas chromatography
[^{3}H]	-	Tritiated radiolabel
HBSS	-	Hank's balanced salt solution

HeLa	-	Human cervical carcinoma cell line
IC_{50}	-	Inhibition concentration 50%
ICC	-	Immunocytochemistry
Ig	-	Immunoglobulin
K_a	-	Mean affinity constant
LA	-	Leukoagglutinin
LD_{50}	-	Lethal dose (50%)
LDH	-	Lactate dehydrogenase
mCon	-	Mean optical density of control cells
MDBK	-	Madin Darby bovine kidney cell line
Mg^{2+}	-	Magnesium ions
MRC-S	-	Human lung fibroblast cell line
mT-2	-	Mean optical density of T-2 toxin treated cells
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
n	-	Sample number
NADPH	-	Nicotinamide adenine dinucleotide phosphate (reduced)
NGS	-	Normal goat serum
nM	-	Nanomolar
OAc	-	Acetyl group
OD	-	Optical density
OsO_4	-	Osmium tetroxide
PBS	-	Phosphate buffered saline
PCD	-	Programmed cell death
pg/ml	-	Picograms per millilitre
PHA	-	Phytohaemagglutinin

PSI	-	Protein synthesis inhibition
PWM	-	Pokeweed mitogen
rER	-	Rough endoplasmic reticulum
RPMI-1640	-	Roswell Park Memorial Institute
SCGE	-	Single cell gel electrophoresis
sER	-	Smooth endoplasmic reticulum
S_n	-	Standard deviation of group n
SRBC	-	Sheep red blood cells
$TCID_{50}$	-	Tissue culture 50% inhibitory dose
TdR	-	Thymidine
TEM	-	Transmission electron microscopy
Tris	-	Tris (hydroxymethyl) aminomethane
UdR	-	Uridine
μM	-	micromolar
UV	-	Ultraviolet
V	-	Volts
VERO	-	African green monkey kidney cell line
Waf 1	-	Wild-type p53-activated fragment 1
X_n	-	Mean absorbance of group n

UNITS OF MEASUREMENT USED IN THE METHODS

Unit		
cm	-	centimetre
g	-	(as in 400xg) acceleration due to gravity (relative centrifugal force)
M	-	Molar
mg/ml	-	Milligram per millilitre
ml	-	Millilitre
mM	-	Millimolar
N	-	Normality
nm	-	Nanometre
µg	-	Microgram
µg/ml	-	Microgram per millilitre
µl	-	Microlitre
µm	-	Micrometre
V	-	Volts

CHAPTER 1

1.0 INTRODUCTION

In third world countries, where hunger is rampant and where climate and agricultural practices cause moulds to flourish, the likelihood that humans and their animals will consume mould-damaged crops is greatest (Bennett, 1989). Malnourished organisms are least able to resist the onslaught of environmental toxins.

Since antiquity, moulds were recognised as agents of spoilage and decay. Gangrenous ergotism (St. Anthony's fire), a human mycotoxicosis, was known in medieval times, and its aetiology is associated with the consumption of grains contaminated with *Claviceps*. However, it took the discovery of the aflatoxins to alert the modern public health officials of the potent significance of the problem (Bennett, 1989).

Fusarium contamination of cereals has been reported in South Africa. Symptoms of head blight (scab) of wheat were observed on four farms in the Southern Cape region of South Africa in 1987. *Fusarium graminearum* was found to be the causative fungus of this disease. The chemical analyses of these wheat samples revealed the presence of the trichothecene mycotoxins deoxynivalenol and nivalenol and the oestrogenic metabolite zearalenone (Sydenham *et al.*, 1989).The toxic constituent that was examined and reported upon in this thesis was the *Fusarium* produced T-2 toxin. The T-2 toxin was isolated from a culture of *Fusarium tricinctum* (*F. sporotrichiodes*) strain T-2 (Ueno and Ishii, 1985). This mycotoxin belongs to a group of sesquiterpenes referred to as trichothecenes. T-2 toxin is produced on cereals in many parts of the world and is associated with prolonged wet weather at harvest time. T-2 toxin has been implicated as the causative agent of "alimentary toxic aleukia", a disease that resulted in the death of thousands in Siberia during the second world war (Coker, 1995). The significance of this toxin is that it alters several immune parameters (Coulombe, 1993).

Although T-2 toxin is a known immuno-suppressive mycotoxin, little is known of its effects on normal human lymphocytes, particularly with respects to the mechanisms of cell death. In establishing the purpose of this study, the following questions were asked:

a) and Is T-2 toxin cytotoxic to normal human lymphocytes? If so, is the cytotoxicity dose time related?

b) Does T-2 toxin induce apoptosis (cell suicide) or necrosis in normal human lymphocytes? If so is this related to length of incubation time?

c) Does T-2 toxin inhibit protein synthesis?

d) Does T-2 toxin induce DNA fragmentation in normal human lymphocytes?

e) If T-2 toxin does induce DNA fragmentation in normal human lymphocytes, is this consistent with apoptotic nuclear morphology such as chromatin condensation?

f) Is there an incubation time relationship between treatment of lymphocytes with T-2 toxin and DNA fragmentation?

As yet, many contradictory suggestions have been put forward with regards to T-2 toxin and its induction of apoptosis. The point of contention is that T-2 toxin is reported to induce apoptosis, whilst on the other hand it is said to inhibit protein and RNA synthesis, both of which are necessary for the apoptotic process.

The hypothesis (based upon work conducted by Ueno *et al.* (1995)) was that T-2 toxin induces apoptosis in normal human lymphocytes and does not inhibit protein synthesis. Instead it follows an alternate pathway of precise and lethal action, leading to the ultimate death of the cell.

Traditionally the study of cell death belonged solely to the realm of the pathologist, but in recent years refined understanding of the importance of the field and increasing use of cell culture models, have attracted the attention of biochemists and cell biologists. Shier (1988) attributes the use of cell culture models to the inherent reductionist philosophy of biologists, i.e., that the single cultured cell is the simplest mammalian system capable of expressing death. The question of why cell death remains an interesting area of study, possibly lies in the need of scientists to understand life or as pointed out by Shier (1988) "the potential benefits of being able to control cell death are enormous".

The methods employed in this study included a rapid cytotoxicity assay, electron microscopy, immunocytochemistry and DNA fragmentation analyses. These were engaged with the thought of assessing the influence of dosage and especially incubation time on the cytotoxic effects of T-2 toxin. The incubation time aspect of the study was deemed necessary in the hopes that it would provide insight into the pathways of the induction of cell death by T-2 toxin.

Thoughts and work done by other researchers follows in the literature review. The remainder of this thesis will comprise a detailed description of the methods employed, the results and discussion and a conclusion. Appendices and a list of references form an integral part of these chapters, and are included ultimately.

1.1 Literature Review

The cytotoxicities of mycotoxins have been tested *in vitro* on several cellular systems. Among these systems are the blood and the lymphatic systems, which are clinically the key intermediates in the functioning of all organs. They can therefore be implicated in the transportation of toxins, thus establishing a link between systemic diseases and their respective aetiological factors.

1.1.1 White blood cells

The leukocytes are of particular importance because of the role they play in the body's immune response. This response requires the action of specialised types of white blood cells. Lymphocytes, neutrophils and monocytes are examples of such cells and their roles range through specific and non-specific immuno-defence.

1.1.1.1 Lymphocytes

Lymphocytes primarily produce antibodies and therefore function as immunocompetent cells in the blood and the connective tissue (Leonhardt, 1977). The life span of the B-lymphocyte is 10-20 days and the T-lymphocyte can survive for several months. The T-lymphocytes are thymus dependent, thus making up 100% of the lymphocytes in the

thymus and 70% in the blood. The T-lymphocytes were also able to bind antigen and were effector cells in delayed hypersensitivity. The T-lymphocytes do not produce antibody. The B-lymphocytes on the other hand are thymus independent and make up 0,3% and 19% in the thymus and the blood respectively. They are also capable of binding antigen and do have immunoglobulin (Ig) receptors as well as produce antibody. The B-cells are not effector cells in delayed hypersensitivity (Bessis, 1973).

1.1.1.2 The ultrastructural characteristics of the lymphocytes

The lymphocyte populations are also differentiated into "small" and "large" populations (Fig. 1.1). The small lymphocyte has a diameter on a smear of 6-9 μm and a volume of 200-300 μm^3, whilst the large lymphocyte has a smear diameter of 9-15 μm and a volume of 3 000-9 000 μm^3. Although it is difficult to establish the maturity of the lymphocyte by gauging the size, it is usually safe to assume that the smaller lymphocyte, upon stimulation transforms into the larger lymphoblast which in turn may evolve into three different types of cells. These are plasma cells (responsible for humoral immunity), conditioned lymphocytes carrying cellular immunity or small quiescent memory lymphocytes that are conditioned (Bessis, 1973).

Lymphoblasts have a diameter of approximately 15-20 μm. The nucleus is round or oval and is large in relation to the amount of cytoplasm (Fig. 1.2). One or two nucleoli may be present. The large lymphocytes have a central or slightly eccentric nucleus that is always completely encircled by cytoplasm. The nucleus is slightly smaller than that of the lymphoblast and contains clumped chromatin masses with lighter zones. The nucleolus is usually barely visible. The nucleus of the small lymphocyte is usually oval or sometime kidney shaped. The nucleus is dense and comprises dark chromatin masses and is generally 9/10ths of the cell diameter (Bessis, 1973).

The organelles of the blood cell are not dissimilar to those of other cells in the body. The mitochondria, make up the chondriome and are observed as either oval or rounded, long and bent and sometimes bifurcated, depending on the present activity of the organelle. Their sizes vary from 0,3-0,4 μm in length and from 0,2-0,8 μm in width. There are usually 10-20 mitochondria per lymphocyte. The mitochondrial cristae may be perpendicular to the long axis of the mitochondrion, but they can also have different orientations, either tortuous or

parallel to the axis of the organelle. The pathology of the mitochondrion can often be ascertained by the orientation or the deformation of the cristae. The volume of the mitochondrion is also subject to changes, usually referred to as mitochondrial swelling, sometimes up to 10-60 times during fasting or ischaemia. Mitochondrial vacuolation (cavulation) is also an observed distortion (Bessis, 1973).

The endoplasmic reticulum (ER) of blood cells are a system of canaliculi and flattened sacs that are usually joined together, and under pathologic conditions the canaliculi may become fragmented into smaller vesicles. The ER hypertrophy, swell and become transformed into vacuoles of variable size and regularity. The large lymphocyte is characterised by an almost total absence of granular or ribosomal ER. The transformation of the lymphocytes that might be stimulated with phytohaemagglutinin (PHA) for example, consists of an enlarged mitochondrial volume, structural changes of the cristae as well as enlarged nucleoli. Lymphocytes that were stimulated with pokeweed mitogen (PWM), showed an increase in the endoplasmic reticulum content (Bessis, 1973). Thus it can be assumed that the cytochrome P_{450} monooxygenase system of the mitochondria of blood cells is fully functional and may thus play a role in xenobiotic metabolism. However lymphocytes in comparison to hepatocytes contain low cytochrome P_{450} activity (Tingleff Skaanild and Clausen, 1991).

1.1.1.3 Other white blood cells

The lymphocytes are aided in their function by various other leukocytes, among which are the neutrophils and the macrophages. The neutrophils are referred to as the "professional" phagocytes of the immune system and make up approximately 70% of the leukocytes. Their total lifespan comprises 48 hours in circulation before migrating into the tissues. This migration is as a result of the influence of chemotactic signals; and it is in the tissue that they phagocytose particles by means of opsonization before they die (Male, 1991).

The monocytes are circulating cells that comprise 5% of the total blood leukocytes. These migrate into the tissue to become blood macrophages. Macrophages are large phagocytic cells that are found in most tissues and the linings of serous cavities. They may either remain in the tissues and linings for years or circulate in the secondary lymphoid tissues as antigen presenting cells to the lymphocytes (Male, 1991).

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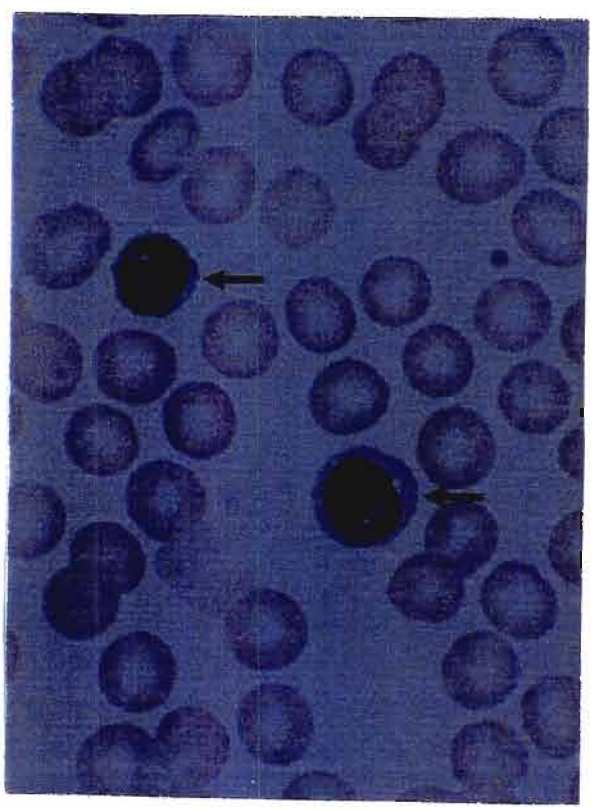

Figure 1.1 (Wheater *et al.*, 1979) Lymphocytes (arrowed) in peripheral blood. The lymphocytes in this figure are of different sizes but are otherwise morphologically similar.

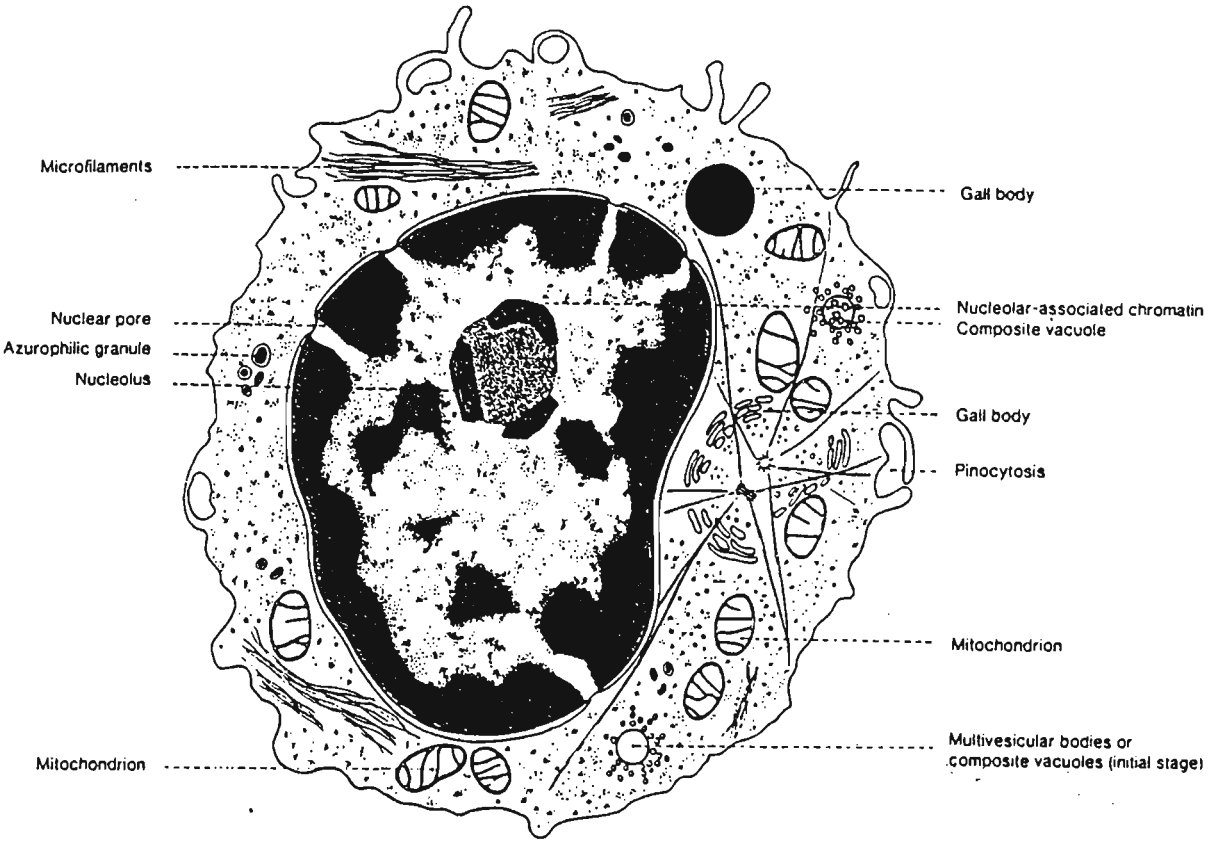

Figure 1.2 (Bessis, 1973) Diagrammatic representation of a section of a lymphoblast as seen with the electron microscope.

1.1.2 Mycotoxins

1.1.2.1 Mycotoxins in the environment

Mycotoxins are mould produced toxins and are contaminants of wide varieties of foods and feeds. Upon ingestion they cause a range of toxic responses from acute toxicity to long term or chronic health disorders (Coulombe, 1993). Mycotoxins are secondary metabolites, the natural products of a living organism. Primary metabolites comprise of amino acids, lipids, nucleic acids and intermediates of the citric acid cycle, among others. Secondary metabolites are produced at only one stage of the life cycle, have obscure physiological functions and are not essential to the growth of the producing organism. Secondary metabolites may have complex and even bizarre chemical structures, but they are all synthesised from a few simple precursors such as acetate or isoprene (Bennett, 1985). The selection of the ability of the fungus to produce toxins probably lies in the roots of protective or competitive advantages (Coulombe, 1993).

1.1.2.2 Taxonomy of the mycotoxin in this dissertation

The toxin of importance is produced by the fungi of the genus *Fusarium*. This species is associated with a growing number of the metabolites produced that are identified as a threat to human and animal life. The taxonomy of this genus was found to be difficult and complex to apply, but due to the toxicity of the metabolites produced, it is important to identify the strains that produce them. The toxigenic Fusaria are divided into sections that are further divided into species, and thereafter a number of strains. It is also possible to acquire mis-identified strains. Approximately 200 *Fusarium* strains are described as being toxigenic in literature. T-2 toxin is produced by *Fusarium sporotrichioides*, *equiseti* and *poae* (Marasas, *et al.*, 1985). T-2 toxin belongs to a range of toxins referred to as the trichothecenes, which are a group of sesquiterpenoids produced by *Fusarium* (Ueno and Ishii, 1985).

1.1.2.3 Biosynthesis and biotransformation of mycotoxins

The biosynthesis of most sesquiterpenoids begins with the cyclisation (aided by several sesquiterpene synthases) of farnesyl pyrophosphate (FPP) which may yield hundreds of different cyclic products and depending upon the producing organism may then be modified by oxygenation reactions (Hohn *et al.*, 1995).

The parent compound of trichothecenes (which are tetracyclic compounds) is called the trichodiene (Fig. 1.3). The oxygenation of the trichothecene analogue was found to be NADPH dependent, and inhibited by carbon monoxide, suggesting the participation of a cytochrome P_{450} (Hohn *et al.*, 1995). The *Tri4* gene of *Fusarium sporotrichioides* (isolated from a cloned DNA fragment carrying the *Tri5* gene by complementation of a *Tri4*- mutant) encodes a cytochrome P_{450} monooxygenase. This enzyme catalyses the first oxygenation step in the trichothecene pathway. Analysis of *Tri4* mRNA levels revealed that transcription reached maximum levels coincidentally with the onset of trichothecene biosynthesis, and then declined 20-fold over 8 hours. Gas chromatography (GC) revealed that the primary end product of the trichothecene pathway in F. *sporotrichioides* NRRL 3299 ($Tri4^+$) constituted 60-80% of the total trichothecenes produced (Hohn *et al.*, 1995).

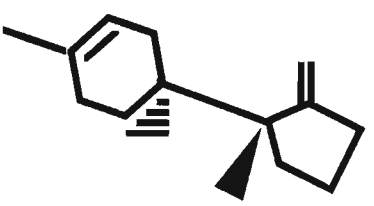

Figure 1.3 (Hohn *et al.*, 1995) The structure of trichodiene, the parent compound of trichothecenes.

The toxicants may be incorporated into the body through various pathways (including oral ingestion and respiration) and can thus be distributed to the various organs and tissues. The toxicants may then be biotransformed whilst in these organs and tissues. The main sites of metabolic transformation are the liver, lungs, stomach, intestine, skin and kidneys (Lu, 1985). Biotransformation involves the changing of parent compounds into metabolites that may form conjugates with various cellular macromolecules. The biotransformation of the compound may also include the detoxification of the compound, for example by binding to a detoxification enzyme such as glutathione-*S*-transferase.

Toxic conjugates that form as a result of biotransformation, often bind to cellular macromolecules, resulting in local tissue necrosis. Many of the conjugates formed are referred to as epoxides, due to the oxidative processes mediated by catalytic enzyme systems such as cytochrome P_{450}. The cytochrome linked monooxygenases are situated in the ER and these ER break down into small microsomes upon homogenisation. Due to the great variety of chemicals that they catalyse, these fractions are known as microsomal mixed function oxidases, while the non-microsomal oxidoreductases are found in the mitochondrial fraction or the 100 000xg supernatant of the tissue homogenates (Lu, 1985).

Due to their ubiquitous nature, mycotoxins have been implicated in a vast array of cellular and hence systemic abnormalities. Their complexity is exacerbated by the potential of each toxic compound to be biosynthesised into either reactive intermediates or toxic end products.

1.1.3 T-2 toxin and its effects

1.1.3.1 T-2 toxin

Trichothecenes such as T-2 toxin are acutely toxic. Naturally occurring trichothecenes have a tetracyclic structure with typically the olefinic bond at carbon groups (C-) 9 and 10 and the epoxy group at C-12 and C-13 (Fig. 1.4). These compounds may be characterised as 12,13-epoxytrichothecenes (Bieger and Dose, 1985). The approximately 60-70 derivatives of the trichothecenes can be divided into 3 groups: a) those having a functional group at C-8 other than a ketone, e.g. T-2 toxin; b) those derivatives with a ketone at the C-8, e.g. deoxynivalenol; c) macrolytic derivatives with a large ring between C-4 and C-15, e.g., verrucarin (Mirocha, *et al.*, 1985). T-2 toxin is a 4β, 15-diacetoxy 3α-hydroxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene (Pace and Watts, 1989). T-2 toxin ($C_{24}H_{34}O_9$) with a molecular weight of 466, is stable in the solid state, and is freely soluble in organic solvents such as ethyl alcohol, ethyl acetate, chloroform and dimethyl sulfoxide; slightly soluble in petroleum ether and is very slightly soluble in water (Yagen and Bialer, 1993).

The cytotoxicity of T-2 toxin can be attributed to its functional groups. Essential to its toxicity is the 12,13 epoxide. The double bond at C-9 and C-10, the acetyl groups at C-4 and C-15 and the isovaleroyl group at C-8 each contributes to different levels of toxicity (Fig. 1.4). The free α-hydroxyl group plays an important role in the toxicity of T-2 toxin by forming a hydrogen bond between C-3 and the cyclic oxygen in the ring of the trichothecene moiety, by intercalation of a water molecule (Fig. 1.4). The additional ring could protect the 12, 13 epoxide against rearside attack by nucleophiles prior to binding (Yagen and Bialer, 1993).

Bieger and Dose (1985) found that the epoxide groups in trichothecenes such as T-2 toxin and its derivative HT-2 toxin do not bind to the catalytic or regulatory centre of glutathione-*S* -transferase and that reduced glutathione does not react with these toxins. Thus the epoxide groups are resistant to metabolic degradation by rat liver enzymes.

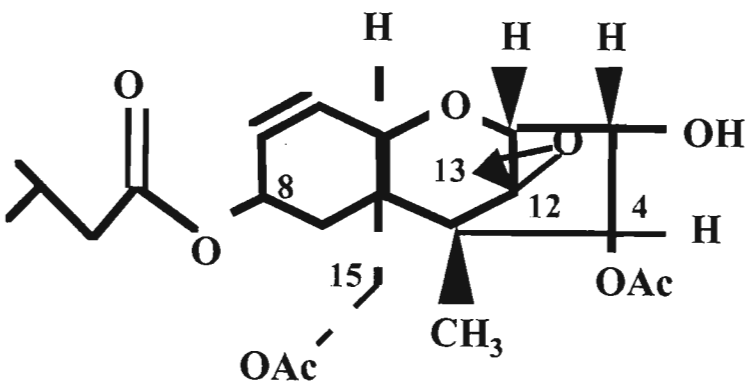

Figure 1.4 (Bieger and Dose, 1985) The molecular structure of T-2 toxin. Important to its toxicity are the epoxide group between C-12 and C-13, the isovaleroyl group at C-8 and the acetyl groups (OAc) at C-4 and C-15.

1.1.3.2 The excretion and detoxification of T-2 toxin

Experiments with oral administration of tritiated T-2 toxin (1 mg/kg) in mice showed that about 80% of the total radioactivity was excreted in the faeces, and that the remaining amount was excreted in the urine. In swine the free (unconjugated) metabolites accounted for

20% and 30% of the total urinary and biliary metabolites, with 3'-OH-HT-2 toxin and T-2 triol being the major metabolites. In cattle as in swine, less than 0,1% of the T-2 toxin dose was excreted intact. Following a 1 M administration of tritiated T-2 Toxin (0,5 mg/kg) to three cynomolgus monkeys, urine was collected for 10 days following dosing. A high level of radioactivity was found in the urine and no radioactivity was noted after five days following dosing. Of the urinary metabolites 30% to 50% were in the form of glucuronide conjugates. It is not clear how much of the toxin remains in the body, although there are indications that this is little to none (Yagen and Bialer, 1993).

The metabolism of T-2 toxin and its elimination from the body involves glucuronidation. It is likely that T-2 toxin is rapidly hydrolysed at the C-4 position to HT-2 toxin by hepatic microsomes, and that this is followed by conjugation to glucuronic acid and excreted into the bile (Fig. 1.5). Glucuronide conjugates are more water soluble than their aglycones and are easily eliminated via the bile into the faeces and urine. Consequently it is possible that the metabolism, conjugation and elimination of T-2 toxin may play a significant role in its detoxification (Yagen and Bialer, 1993).

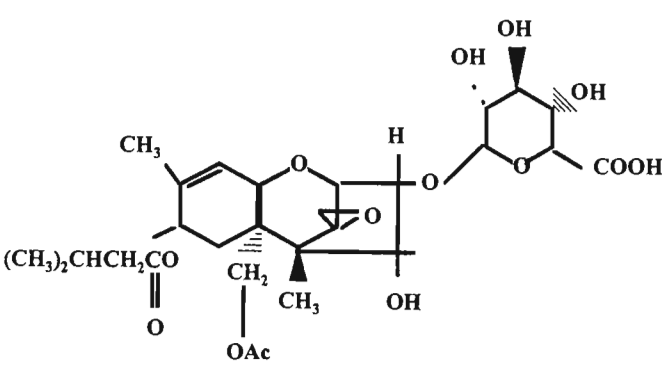

Figure 1.5 (Yagen and Bialer, 1993) The structure of HT-2 toxin glucuronide, which is eliminated from the body via the bile into the faeces and urine.

1.1.3.3 The effects of T-2 toxin on the body systems

T-2 toxin plays a destructive role in almost all of the various body systems. Along with AFB_1, it causes haematomas and haemorrhages in the cardiovascular system, caustic effects on mucous membranes and intestinal haemorrhages in the digestive system and inflammatory or necrotic change in the cutaneous system. It affects the reproductive system of pigs, resulting in small litters and weak pigs (Morehouse, 1985). High concentrations of T-2 toxin in mouldy corn have been suggested in the causal relationship of extensive haemorrhagic lesions in dairy cattle (Smalley, 1973). Alimentary toxic aleukia in Russia was the first implication of the role of trichothecenes in human disease (Bunner *et al.*, 1985).

The T-2 toxin ingestion patterns resulted in human and animal morbidity and mortality, such as severe bone marrow suppression, anaemia, bleeding and immune suppression with infectious complications leading to death after several weeks or even within days (Bunner *et al.*, 1985). Further findings show that continuous sublethal T-2 toxin intake by rabbits alters ovarian activity (e.g., underdeveloped, atrophied luteal formations). It also alters the activity of some blood enzymes, and serum creatinine concentrations (Fekete and Huszenicza, 1993). The biochemical, haematological and coagulation data generated by Bunner *et al.* (1985) studying cynomolgous monkeys showed that the 50% lethal dose (LD_{50}) of 8 monkeys given intramuscular T-2 toxin was 0,8 mg/kg and the minimum lethal dose calculated was 0,31 mg/kg.

Although the mechanism for the reported haemorrhagic syndrome in trichothecene toxicosis is not clearly understood, it is known that in the rat, clotting and prothrombin time are delayed and vascular permeability is increased with resultant widespread injuries (Morehouse, 1985).

1.1.3.4 Tests on animal models

T-2 toxin is well documented in the disruption of the immune response and immune related lesions in domestic animals (Table 1.1). T-2 toxin, diacetoxysirpenol and

stachybotrytoxin have been named as the major immunosuppressive agents in the trichothecene group. They have been implicated in the alteration in serum proteins and Ig profiles, reduced antibody formation, thymic aplasia and generally reduced cell mediated immunity (CMI) responsiveness. However, it induces enhanced delayed cutaneous hypersensitivity and impairment of bacterial clearance and acquired immunity (Pier and McLoughlin, 1985) (Table 1.2).

Seven week old pigs fed with T-2 toxin daily showed that diets containing 2 and 3 mg of T-2 toxin per kg caused significant decreases in the red blood cell count, the mean corpuscular volume, and the haemoglobin concentration. Decreases were also observed in the leukocyte counts and in the proportion of T-lymphocytes. Furthermore, there were also dose dependent, significant decreases in antibody formation and in the blastogenic transformation of lymphocytes (Rafai *et al.*, 1995).

Mice (3H/HeN) fed with a dose of T-2 toxin (1 mg/kg) every other day for 10 days, and thereafter challenged with the pathogen *Salmonella typhimurium* every other day for 10 days, showed markedly larger and more bacterial related lesions in the spleen, kidneys and liver. Animals exposed to *S. typimurium* alone showed normal post-infection pathology. Thus T-2 toxin compromised murine resistance to the pathogen infection and ultimately caused death in animals challenged with a sublethal dose of the organism (Tai and Pestka, 1990).

Table 1.1 (adapted from Pier and McLoughlin, 1985) Immune related lesions for T-2 toxin.

Animal	Dose	Response to toxin
Chicken	16 mg/kg in feed	Smaller size of spleen and bursa
Cat	0,06-0,10 mg/kg body weight (1,2-2,0 mg/kg in feed)	Thymus reduced in size, bone marrow hypoplasia.
Chicken	1,25 mg/kg body weight (18 mg/kg in feed)	Thymus and bursa had atrophy of cortex.
Mouse	1/4-1/2 LD_{50} (*Fusarium* extract)	Reduced thymic weight at 1-7 days; lymphoid depletion in spleen and thymus.
Cattle	0,3-0,6 mg/kg body weight (10-20 mg/kg in feed)	Dose-related decrease in thymic weight; cortical atrophy.

Table 1.2 (adapted from Pier and McLoughlin, 1985) Effects of T-2 toxin on mechanisms of immunity and resistance.

Animal	Effect
Turkey	Thymic aplasia (*P. multocida* antibody normal).
Mouse	Thymic aplasia (anti sheep red blood cells (SRBC) and graft versus host reduced).
Mouse	Reduced clearance (*Listeria*); rise in tetanus antitoxin.
Mouse	Enhanced delayed cutaneous hypersensitivity; T-suppressor cell reduction.
Mouse	Transplacental thymic aplasia; reduced lymphoblastogenesis.
Cattle	Antibody (*A. marginale*) normal; IgA, IgM reduced; IgG normal; C_3 reduced; lymphoblastogenesis and leukocytic migration reduced.
Mouse	antibody (SRBC) reversibly reduced; thymic aplasia, reduced graft versus host.

1.1.3.5 T-2 toxin compared to AFB_1

The toxic actions of AFB_1 and T-2 toxin is comparable because the immunosuppressive action of both toxins appears to be associated with the inhibition of protein synthesis. Mechanistically however, they are different. Aflatoxins inhibit protein synthesis, by impeding transcription whilst T-2 toxin affects initiation of translation, thus resulting in different lesions. T-2 toxin reduces complement fixation, not unlike aflatoxins, but primarily C_3. It diminishes serum Ig levels (IgA and IgM but not IgG) like aflatoxins and diminishes antibody production. Furthermore, T-2 toxin is associated with graft rejection, reducing lymphoblastogenic responsiveness and the inhibition of neutrophil migration and like aflatoxins are capable of transfer across the placenta thus affecting the foetus *in utero* (Pier and McLoughlin, 1985).

1.1.3.6 Threshold levels and lethal doses of T-2 toxin in lymphocytes

T-2 toxin is rapidly taken up during the first 10 minutes of incubation, and thereafter the uptake levels off and the amount of label associated with the lymphocytes remains unchanged for up to 200 minutes. Analysis of the kinetics of uptake of [^{3}H]T-2 toxin was done on several dose-response experiments and a mean affinity constant (K_a), $K_a = 1{,}6 \times 10^7$ M^{-1} was derived. T-2 toxin uptake and release can be influenced by temperature. This is indicative of either an energy dependent process, or one that involves membrane fluidity or both (Gyongyossy-Issa and Khachatourians, 1984).

Energy inhibitors such as sodium azide, 2-deoxyglucose, 2,4-dinitrophenol and ouabain were incubated simultaneously with the lymphocytes for a duration of 60 minutes. The total uptake was reported as being the same in all cases and this was attributed to the possibility that both energy depletion and [^{3}H]T-2 toxin uptake, take place in less than 10 to 20 minutes. Furthermore, labelled T-2 toxin yielded a number of binding sites per cell of the order of 10^5, which would correspond to about 0,5 ng T-2 toxin per 5×10^6 cells.

500-fold excess of unlabelled T-2 toxin decreased the saturation of T-2 toxin uptake by 40%, and this was attributed to the possibility that two uptake mechanisms are in operation

viz., a) the presence of few (approximately 10^5) 'high affinity' binding sites and, b) several 'low affinity' binding sites (Gyongyossy and Khachatourians, 1984).

Few high energy sites may be dependent on energy, while the rest of the binding may be influenced by certain unknown factors (possibly involved in the low affinity binding sites) and thus dependent on the physical property of the molecule rather than the physiological state or activity of the lymphocyte. T-2 toxin is an amphipathic molecule and may thus tend to partition into the "organic phase" present, which is the cell membrane. This differential solubility may be subject to the constraints of parameters influencing the partition coefficient as well as factors influencing the state of the solvent (the cell membrane), thus possibly explaining the rapid uptake and release of the toxin from lymphocytes (Gyongyossy-Issa and Khachatourians, 1984).

In murine lymphocytes, RNA, DNA and protein synthesis are inhibited and the concentration of T-2 toxin required for the inhibition of nucleic acid and protein synthesis is approximately 1 x 10^5 molecules of T-2 toxin per cell (Gyongyossy-Issa and Khachatourians, 1985). T-2 toxin acts on both mitogen-stimulated and non-stimulated lymphocytes in culture (Gyongyossy-Issa and Khachatourians, 1984). DNA synthesis is not readily discernible in lymphocytes until after the first cell cycle, and thereafter, there is a dose-dependent inhibition of DNA synthesis. Inhibition of RNA synthesis in both non-stimulated and stimulated lymphocytes is seen after 4 hours. These results were established by the incorporation of radiolabelled nucleosides into acid-insoluble material, and the cultures were pulsed with radiolabel at 0, 24, 48 and 72 hours. However, the decrease of radiolabelled nucleoside incorporation may also be interpreted as the inhibition of nucleoside transport (Gyongyossy-Issa and Khachatourians, 1985).

The effectiveness level for T-2 toxin (the threshold dose) was calculated to be approximately 1100 +/- 100 pg/ml or (5,7 +/- 0,5) x 10^5 molecules of toxin per cell (Fig. 1.6) (Gyongyossy-Issa and Khachatourians, 1985). This value is important when one takes into account the critical number of T-2 toxin-lymphocyte receptor interactions at approximately 1,1 x 10^5 molecules per cell (Gyongyossy-Issa and Khachatourians, 1984).

Below the threshold level there is very little or no effect, while above this dose the time-dose constant applies, i.e., $K_a = 1,6 \times 10^7 M^{-1}$ (as mentioned previously). This implies that full receptor occupancy is possibly indicative of the first step towards the expression of T-2 toxin toxicity (Gyongyossy-Issa and Khachatourians, 1985). This situation is similar to the interaction of lymphocytes and antigens. When the receptor on the lymphocyte becomes occupied by an antigen, a signal is transmitted from the membrane to the nucleus to induce a response from the cell (Gutowski and Cohen, 1983). The association of the toxin with membrane-nucleus communication may also elicit a cellular response and this response is probably related to the toxicity of T-2 toxin (Gyongyossy-Issa and Khachatourians, 1985).

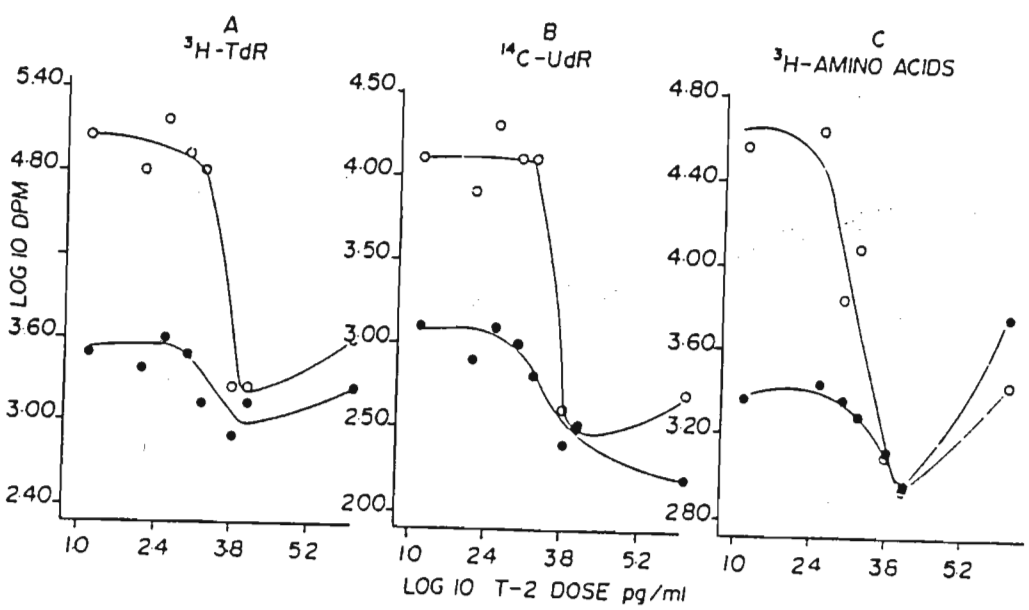

Figure 1.6 (Gyongyossy-Issa and Khachatourians, 1985) The dose response of DNA, RNA and protein synthesis to T-2 toxin. Concanavalin-A-stimulated (O) and non-stimulated (●) lymphocytes were tested for the levels of T-2 toxin required to inhibit DNA (A), RNA (B) and protein synthesis. Log 10 of T-2 toxin dose (pg/ml) is plotted against log 10 label DPM. The threshold dose was approximately 1100 +/- 100 pg/ml or 5,7 +/- 0,5 x 10^5 molecules of toxin per cell. (TdR) Thymidine; (UdR) Uridine; (DPM) dose per minute of radioactivity incorporated.

1.1.3.7 The immunosuppressive action of T-2 toxin

Fusarium crude extracts including T-2 toxin cause significant reductions in thymus weight and inhibits responsiveness to sheep red blood cells at 1/32 LD_{50} and 1/16 LD_{50} respectively. These were established by haemagglutinin tests. The crude extracts were injected intra-peritoneal into male Swiss, inbred IC and C57B1/6 mice (4-6 weeks old). The dose used, corresponded to the LD_{50} (the volume of crude extract killing half the population of 12 mice) within one day and this dose was usually the extract of approximately 35 ml of a *Fusarium* culture (Rosenstein *et al.*, 1979).

However when mice were treated daily with purified extracts of T-2 toxin (0,75 mg/kg/24 hours for 7 days) and thereafter the treatment was stopped, the effect of T-2 toxin diminished. The immune response was comparable to the control at 6 days after the cessation of treatment. Furthermore, the immunosuppressive activity of T-2 toxin was observed in prolonging the period required for skin graft rejection. In the control mice, necrosis occurred rapidly on the 7th and 8th day and unequivocal graft rejection occurred during the next two or three days. However, in the T-2 toxin treated mice the skin grafts remained soft and did not undergo necrosis until the 12th or 13th day, after which rejection occurred within three to five days.

Delayed hypersensitivity, depressed anti-SRBC response as well as prolongation of allograft survival possibly resulted from the selective effects of the mycotoxins on the subpopulations of the T-suppressor cells or their precursors. This is suggested by the depletion in the T-cell population and the decrease in PHA response found in the spleens of T-2 toxin-treated animals. There was also an adoptive transfer of the suppressor effect upon SRBC response with spleen cells from the toxin-treated animals (Rosenstein *et al.*, 1981).

T-2 toxin and its analogs, inhibit the incorporation of [^{3}H]thymidine in mitogen-stimulated human lymphocytes (Fig. 1.7). Species differences do not affect the toxicity of T-2 toxin in lymphocyte culture systems, nor do different mitogen stimulations show any difference in the inhibition of T- and B-cells, i.e., other workers show that the lymphocytes stimulated by the various mitogens, are inhibited by similar levels of the toxin. There was also

a slight stimulation of lymphoblastogenesis when low T-2 toxin concentrations (5 x 10^{-4} to 5 x 10^{-3} ng/ml) were applied (Forsell *et al.*, 1985).

Lymphocyte progenitor cells represent highly sensitive targets of T-2 toxin exposure, responsible for thymic atrophy. This was established by the treatment of experimental animals with T-2 toxin, resulting in the marked decrease of thymic cellularity as well as the suppression of cell mediated immunity (Holladay *et al.*, 1993). T-2 toxin readily crosses the placenta thus resulting in significant foetal thymic atrophy in mice. Reduction of foetal liver B lymphocytic cells exposed to T-2 toxin were also noted thus altering humoral mediated immunity (Holladay *et al.*, 1995).

T-2 toxin among other natural mycotoxins, induces apoptotic changes in promyelocytic leukaemia cells (Ueno *et al.*, 1995). Apoptosis refers to the morphological changes in the nuclei characterised by the DNA fragmentation and an apoptotic nuclear body. The cytotoxicity results indicated that the viability of the cells was depressed in a dose dependent manner (Fig. 1.8), and the IC_{50} (50% inhibition concentration) value for T-2 toxin was estimated to be 0,004 μg/ml with DNA fragmentation at a minimum effective dose of 0,01 μg/ml. The extent of DNA fragmentation increased with the concentration of the trichothecenes, whilst no fragmentation was noted in the control cells in culture.

The electron microscopic observations made, indicated that when the cells were treated with 0,1 μg/ml of T-2 toxin for 1 hour, they were morphologically altered. This was observed as the disappearance of cell surface microvilli, marked chromatin condensation and karyopicnosis (apoptotic body), karyorrhexis and cytoplasmic vacuolation (Ueno *et al.*, 1995). Karyorrhexis refers to the fragmentation of the nucleus into several (usually four to five) small spheres (Bessis, 1973). One of the causes of the T-2 toxin-induced apoptosis in HL-60 human promyelocytic leukaemia cells, might be the suppression of Bc1-2 protein (Ueno *et al.*, 1995). In all the examined cases Bcl-2 was shown to block a relatively early event associated with apoptotic cell death, in that none of the characteristic morphological changes such as cell shrinkage, chromatin condensation, and nuclear fragmentation occurred, and DNA degradation into oligonucleosomal fragments was reduced or prevented (Reed, 1994).

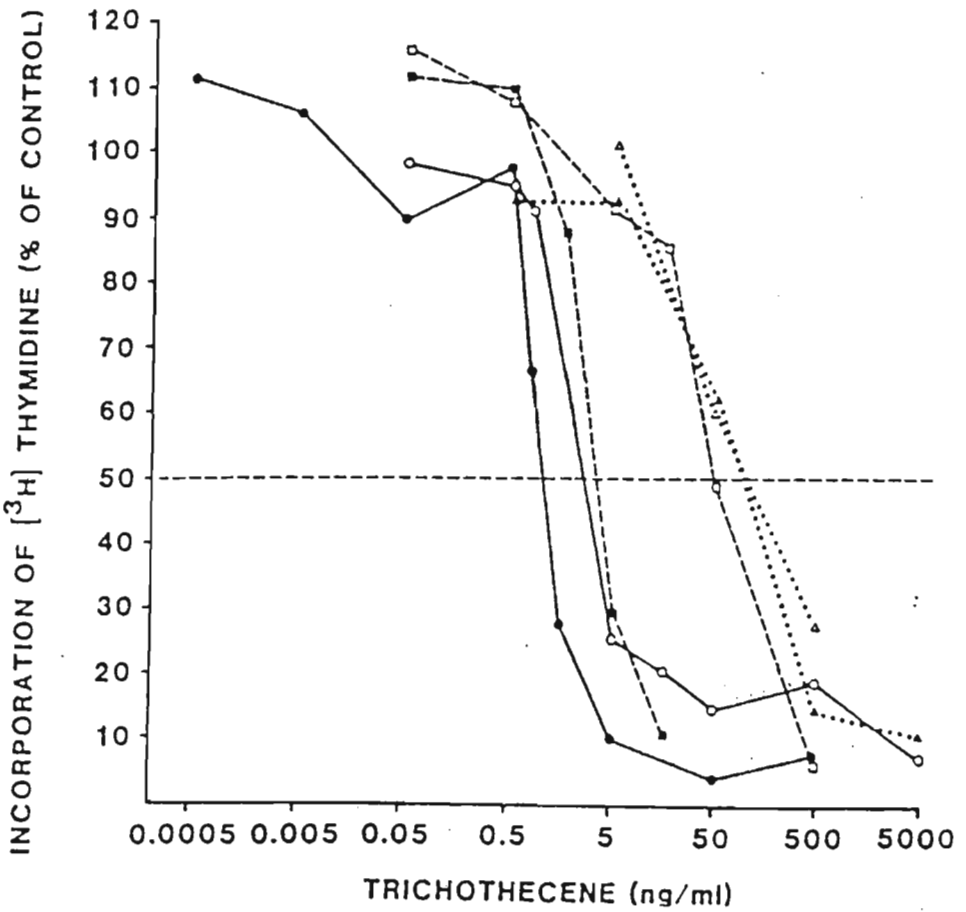

Figure 1.7 (Forsell *et al.*, 1985) Inhibition of [^{3}H]TdR incorporation in mitogen-stimulated human lymphocytes by T-2 toxin and its analogs. Each point represents the mean inhibition in lymphocytes stimulated by leukoagglutinin (LA), pokeweed mitogen (PWM) and concanavalin A (ConA) at two concentrations and each in triplicate (a total of 18 determinations). Symbols: ●, T-2 toxin; ○, HT-2 toxin; ■, 3'-OH T-2 toxin; □, 3'-OH HT-2 toxin; ▲, T-2 triol toxin; and the clear triangles refer to T-2 tetraol toxin.

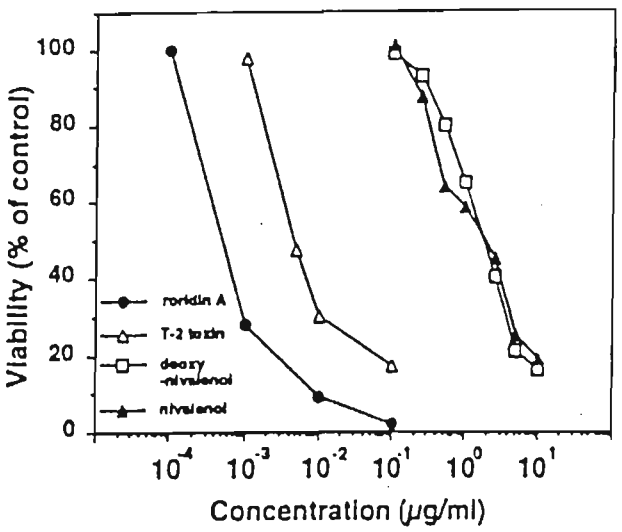

Figure 1.8 (Ueno *et al.*, 1995) The cytotoxicity of trichothecenes. The cells were incubated for 24 hours and the values were expressed as a percentage of the control. (●) represents Roridin A, (▲) nivalenol, (□) deoxynivalenol, and the clear triangle represents T-2 toxin.

1.1.3.8 The association of T-2 toxin with protein synthesis

Thompson and Wannemacher Jr. (1986) describe 12, 13-epoxytrichothecenes as powerful protein synthesis inhibitors. T-2 toxin can affect protein synthesis in different cell lines and some cases are more drastic than others. An example is the effect of T-2 toxin on Chinese hamster ovary cell line (CHO) and a bovine kidney cell line (MDBK) where the percentage control response of the MDBK line decreases with the increase in toxin concentration (Holt and DeLoach, 1988). This was established by pulsing the treated cultures with tritiated L-leucine and reading on a liquid scintillation counter.

A tetrazolium salt assay for mitochondrial activity also verified a decrease in percentage control response with an increase in toxin concentrations. The studies indicated that the MDBK cell line was lethally affected at lower doses than CHO, and significant doses were noted, that is the $TCID_{50}$ values (tissue culture 50% inhibitory dose) was 1 ng/ml and 10 ng/ml for MDBK and CHO respectively (Holt and DeLoach, 1988).

Thompson and Wannemacher Jr. (1984) found with the use of a rapid sensitive bioassay, that although shutdown of protein and DNA synthesis occurred in a dose response fashion with T-2 toxin incubation, the effect on RNA synthesis was minimal. Further studies using the protein synthesis inhibition assay (PSI assay) showed that human cervical

carcinoma cell line (HeLa) required 17,3 (+/- 0,61) ng/mg of T-2 toxin for 50% PSI after 30 minutes of exposure, whilst human lung carcinoma (A-549) required 12,5 (+/- 1,76) ng/mg and human lung fibroblast (MRC-S) required 12,6 (+/- 1,14) ng/mg of the toxin.

In 1986, Trusal and O'Brien attempted to study the correlation between the ultrastructural effects of T-2 toxin on cultured hepatocytes and the release of lactate dehydrogenase (LDH) and inhibition of protein synthesis. Hepatocytes, under experimental parameters, are not killed by T-2 toxin. This was assessed in terms of cell viability, which was established as the measure of LDH release in cells exposed to 1,0 μg/ml of T-2 toxin for 12 hours. However, after a 12 hour recovery period there was a significant increase in the release of LDH. This finding was supported with the observation of mitochondrial lesions. The lesions might be related to the presence of T-2 toxin, however it was also stated that this might be non-specific as had been observed in other pathological conditions.

Following the exposure of hepatocytes to 0,01 μg/ml T-2 toxin for 1 hour there was a net decrease of 75% in protein synthesis, but when the toxic insult was removed allowing cells to recover for 12 hours there was an overall increase of 87% in protein synthesis. Protein synthesis was totally inhibited at 1,0 μg/ml after 1 hour of incubation, with recovery only after the toxin was removed for 12 hours. No recovery was noted even after 12 hours by cells that were incubated with 1,0 μg/ml of T-2 toxin for 12 hours, thus protein synthesis was irreversibly inhibited (Trusal and O'Brien, 1986).

Lowered protein synthesis inhibition response is seen by the addition of acetyl groups at the C-3 position or an epoxide between C-9 and C-10, which involves size and conformational changes (Thompson and Wannemacher Jr., 1986).

1.1.3.9 The association of T-2 toxin with mitochondria and endoplasmic reticulum

T-2 toxin inhibits mitochondrial protein synthesis in isolated mitochondria, i.e., it is capable of decreasing the rate of protein synthesis by 50% in a 0,02 μg/ml dose. This was established by evaluating amino acid incorporation into the mitochondria whilst

cyclohexamide was used to inhibit cytoplasmic protein synthesis, in order to properly assess mitochondrial protein synthesis inhibition. Mitochondrial protein synthesis is inhibited by chloramphenicol and is unaffected by cyclohexamide (Pace *et al.*, 1988). Pace and Watts (1989) found that [^{3}H]T-2 toxin after 5 minutes of incubation was found to be associated with soluble proteins, smooth endoplasmic reticulum (sER) and plasma membranes of the liver cells of Fischer rats. Perfusion after 120 minutes showed label in the sER, rough endoplasmic reticulum (rER) and mitochondria. T-2 toxin is also rapidly metabolised by microsomal enzymes, because the radiolabelled polar forms remained in the liver and were seen after 5 minutes of incubation bound to the cellular membranes. The metabolites found were HT-2 toxin, T-2 tetraol, 4-deacetylneosolaniol and glucuronide conjugates.

The most salient feature observed, was that at every point in the experimental time, the radiolabel in the hepatic mitochondria was that of T-2 toxin. This could be as a result of the inability of the organelle to metabolise the toxin or form a selectivity in its toxin/metabolite uptake mechanism. This suggests that the effect of T-2 toxin on mitochondrial respiration and protein synthesis, is a result of direct sites of action of the parent toxin (Pace and Watts, 1989).

Longer exposures to T-2 toxin (1,0 μg/ml for 12 hours) overwhelmed the hepatocytes ability to detoxify T-2 toxin (Trusal and O'Brien, 1986). However the hepatocytes can rapidly detoxify the toxin to less reactive metabolites such as T-2 triol and T-2 tetraol, providing that the concentration is small and the exposure time short.

Hepatocytes that were treated with T-2 toxin at 1,0 μg/ml for 12 hours show mitochondria with translucent non-membrane bound foci containing electron dense cores. The foci were not accompanied by increases in matrical density, compartmental swelling or disruption of the outer membrane. The ER in these hepatocytes contained attached ribosomes, when incubated with 1,0 μg/ml T-2 for 1 hour with a 12 hour recovery period. The majority of cells exposed for 12 hours to the same concentration did not have attached ribosomes. The degranulation is consistent with observations made on CHO and African green monkey kidney (VERO) cells. In CHO and VERO cells, T-2 toxin induces total polysomal breakdown and degranulation of the limited amount of rER (Trusal and O'Brien, 1986).

T-2 toxin (2,2 mM) caused a 40% reduction (Fig. 1.9) in the rate of oxygen consumption by state 3 mitochondria when succinate was the substrate. T-2 toxin was shown to resemble rotenone a known inhibitor of site I of the electron transport chain, that is it is an inhibitor of the oxidation of pyruvate plus malate. The effect of T-2 toxin on NAD-dependent substrates was overcome by the addition of menadione, and it is in this respect that T-2 toxin resemble rotenone. T-2 toxin also partially blocked electron flow between cytochrome c_1 or c and O_2 (Pace, 1983).

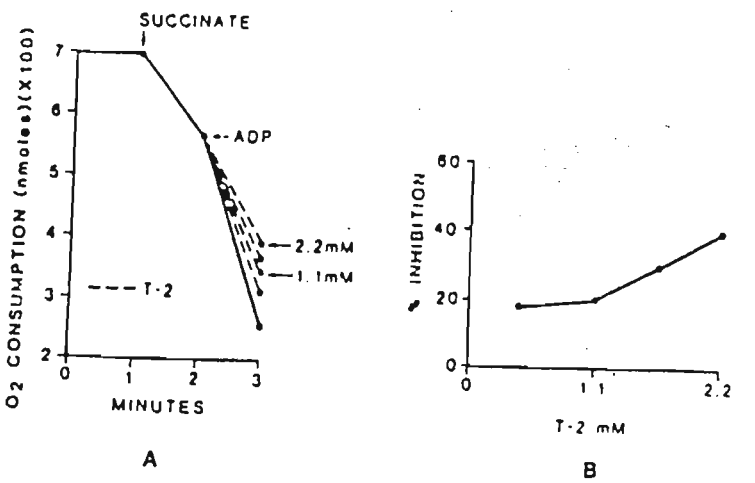

Figure 1.9 (Pace, 1983) *In vitro* T-2 toxin inhibition of state 3 respiration in the presence of 3 mg mitochondrial protein and 5,0 mM succinate. (A) T-2 was added in methanol to final concentrations ranging from 0,005 mM to 2,2 mM. (B) shows the percentage inhibition of state 3 respiratory rates as a function of T-2 concentration in the presence of 3 mg mitochondrial protein.

1.1.4 Cell death

1.1.4.1 The patterns of cell death

Cell death plays a variety of important roles in Biology, both physiological and pathological. Physiological cell death is an important part of development, cell turnover and various aspects of immunological defences. Pathological cell death is an element of some of the most important diseases plaguing modern society. Recent studies on the mechanisms of calcium mediated cell death, thought to be important for both physiological and pathological cell death, have indicated that it is a multistep process in which cells are active participants, triggering their own self-destruction. Such mechanisms suggest the potential for pharmacological intervention, either to prevent disease-induced tissue death or to remove unwanted tissues (Shier, 1988).

Developmental systems seem to form the best measures of cell death, for example, the most important of which is lymphocyte deletion in the development of self-nonself recognition. Physiological cell death includes immune mediated cell death, phagocytosis, cell turnover and senescence (Shier, 1988). Evolution has conserved a form of cell suicide (apoptosis) that causes physiologic cell death (Thompson, 1995). Pathological cell death includes ischaemic cell death, toxic cell death, genotoxicity, trauma, viral infection and possibly neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer's diseases (Shier, 1988).

1.1.4.2 Classification of cell death

The existence of two different pathways for cell death have been postulated: the passive and traumatic process leading to necrosis and the active program characterised by organelle integrity called apoptosis (Cossarizza *et al.*, 1995). Other schools of thought, with particular reference to Majno and Joris (1995), maintain that necrosis does not indicate a form of cell death but refers to changes "secondary" to cell death by any mechanism including apoptosis.

Disease therapy demands a knowledge of the difference between apoptosis and necrosis. Whether cell death in pathological disorders is apoptotic or necrotic, the prevention is of medical importance. This requires an elucidation of the mechanisms of the particular cell death associated with various disorders (Uchiyama, 1995).

Accidental cell death, namely ischaemic cell death, is named oncosis, as a result of swelling, due to the failure of ionic pumps of the plasma membrane. The term oncosis comes from the Greek word "ónkos", meaning swelling. Oncosis leads to necrosis with karyolysis, in contrast to apoptosis, which leads to karyorrhexis and cell shrinkage (Majno and Joris, 1995).

1.1.4.3 Necrosis

Necrosis is described as a degenerative phenomenon that follows irreversible injury (Walker *et al.*, 1988). It is the period that occurs after cell death and is characterised by the autodigestion of different portions of the cell caused by enzymes activated or liberated by the destruction of the lysosomes (Bessis, 1973). Necrosis may be referred to as the progressive degradation of cell structure that occurs after cell death caused by severe, injurious changes in environmental conditions.

Figure 1.10, displays the evolution of necrosis, beginning with the swelling of mitochondria with granular matrices, followed by the disintegration of ribosomes and discontinuities in the plasma, organelle and nuclear membranes. Also shown is the irregular clumping of chromatin which eventually disappears to leave ghost-like nuclei.

Light microscopic evaluation of necrotic cells, define them as swollen, with eosinophilic cytoplasm and indistinct cell boundaries. The nuclei may initially appear normal, but eventually show pyknosis or uniformly condensed chromatin, or dispersed chromatin masses (karyorrhexis), which correspond to the irregular clumps of chromatin seen ultrastructurally. The later stage karyolysis refers to the dissolution or disappearance of the nuclei or the presence of faintly staining nuclear ghosts (Walker *et al.*, 1988).

Necrosis *in vivo* typically affects groups of contiguous cells and an inflammatory reaction usually develops in the adjacent viable tissue. The necrotic cells are finally removed by mononuclear phagocytes (Walker *et al.*, 1988). According to Majno and Joris (1995) cell death and necrosis are two very different occurrences. Cells die long before necrotic changes can be viewed under a light microscope. Necrosis (features of a cell's cadaver) is signalled by irreversible changes in the nucleus (karyolysis, pyknosis and karyorrhexis) and in the cytoplasm (condensation, intense eosinophilia, loss of structure and fragmentation). The mechanisms of cell death, therefore, could be ischaemia, heat, toxins, mechanical trauma, or apoptosis (Majno and Joris, 1995).

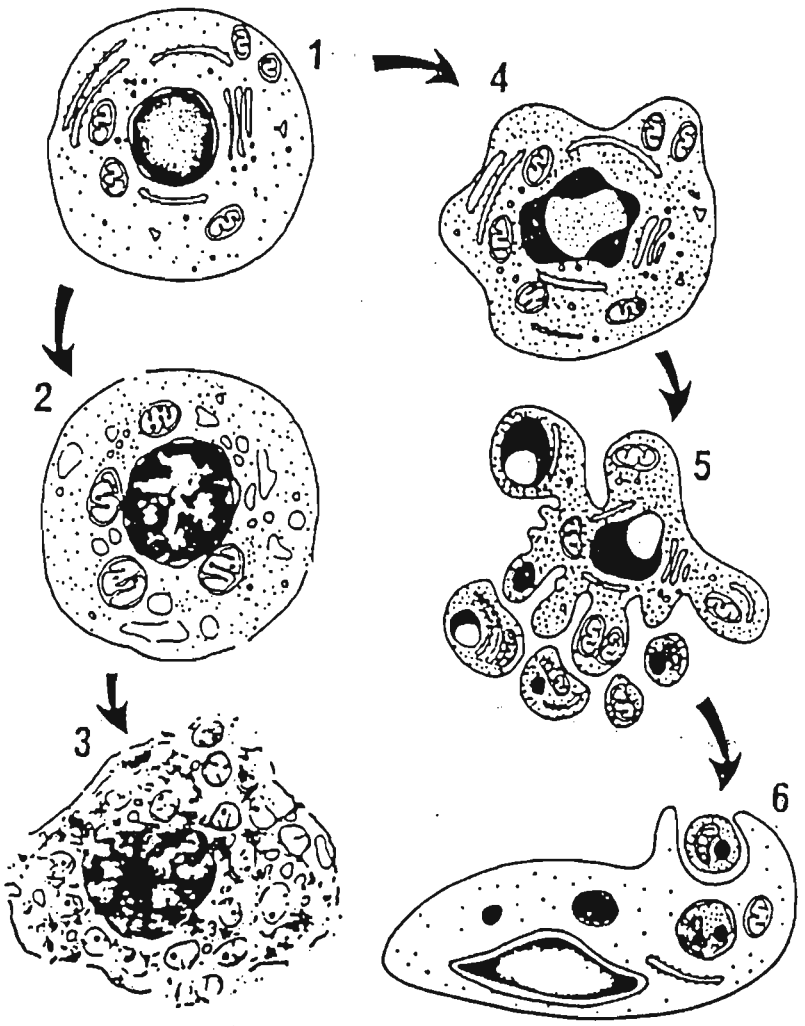

Figure 1.10: (Walker *et al.*, 1988) Diagram illustrating the change in morphology in necrosis (left) and apoptosis (right). Irreversible injury to a normal cell (1) is followed by (2) by irregular clumping of chromatin, gross swelling of cytoplasmic compartments, the appearance of matrical densities in mitochondria and the development of discontinuities in membranes. At a latter stage of necrosis (3), chromatin disappears and organelles disintegrate. Early apoptosis (4) is characterised by compaction and margination of nuclear chromatin to form sharply circumscribed masses, condensation of cytoplasm and convolution of nuclear and cell outlines. This culminates in nuclear fragmentation and separation of protuberances that develop on the cell surface (5) to produce membrane-bound apoptotic bodies, which are phagocytosed and digested (6) by nearby cells.

1.1.4.4 Apoptosis

The Greek name apoptosis is most felicitous, suggesting as it does the discrete image of leaves dropping off here and there from a tree (apó, meaning from and ptósis, meaning a fall) as opposed to the massive cell death of an infarct (Majno and Joris, 1995).

The key features of apoptosis as discussed by other authors is as follows:

1) It is characterised by morphological as well as biochemical criteria and can be considered as a counterpart of mitosis.

2) The cell shrinks and becomes denser, and was described as shrinkage necrosis. The chromatin becomes pyknotic and packed into smooth masses, in the shape of half-moons or crescents. The nucleus also breaks up and this is referred to as karyorrhexis, and the cell emits processes (budding) that often contain pyknotic nuclear fragments. These processes eventually break off and are then referred to as apoptotic bodies, and these may then be phagocytosed by macrophages and neighbouring cells or remain free (Fig. 1.10).

3) There is little or no swelling of the mitochondria and other organelles.

4) The DNA is broken down into segments that are multiples of approximately 185 base pairs, due to specific cleavage between nucleosomes.

5) The process is under genetic control and can be initialised by an internal clock, or by extracellular agents such as hormones, cytokines, killer cells and a variety of chemical, physical and viral agents.

6) Apoptosis runs its course in a matter of minutes. In the routine determinations, the best ultrastructural marker of apoptosis is karyorrhexis.

(Majno and Joris, 1995)

Apoptosis requires that the dying cells be metabolically active, and this is usually dependent on RNA and protein synthesis (Cohen and Duke, 1984 and Hockenbery, 1995). Apoptosis includes both cell death and necrosis (the secondary break-up into a cluster of apoptotic bodies), thus implying that apoptosis can not be opposed to necrosis, if it produces classic images of necrosis. The process may therefore then be referred to as "apoptotic necrosis" (Majno and Joris, 1995).

The margination of chromatin is also a feature of apoptotic changes, and this may be caused by events such as proteolysis, DNA damage and disruption of the cytoskeleton (Payne *et al.*, 1995). During apoptosis, microfilaments play a role in determining the distribution of components of the plasma membrane as well as the cell shape. The microfilaments were visualised using immunofluorescence using smooth muscle antibodies in cells undergoing apoptosis. The patterns that were observed, indicated that redistribution of microfilaments is associated with apoptosis, and may be related to the budding process (Clouston and Kerr, 1979).

1.1.4.5 Apoptosis and programmed cell death

There may be two different programs that are involved in apoptosis: 1) a program to carry out suicide, and 2) another program to trigger the suicidal program. Programmed cell death is a phenomenon that refers to the situations in which cells are programmed to die at a fixed time by a genetic clock, for example the structuring of the chick embryonic plate. Eventually, a different program must dictate to these cells how to engineer suicide (e.g. apoptosis). The terms "programmed cell death" and "apoptosis" may not be used interchangeably for the above reasons (Majno and Joris, 1995).

The contributors to apoptotic cell death are, physical (ionising radiation, hyperthermia) and toxic exposures (azide, hydrogen peroxide), cellular effects of cytokines (tumour necrosis factor, transforming growth factor-β), viral infection, and immunologically relevant mechanisms (cytotoxic T lymphocyte-mediated killing, anti-Ig and T-cell receptor signalling) (Hockenbery, 1995).

The process of cell death is important in the shaping of the developing organism by eliminating transient structures and supernumerary cells. Controlled cell death is also necessary for the generation of a number of structures in the adult, such as skin, hair, nail and bone. Furthermore programmed cell death is also responsible for the correct formation of the spinal cord as well as the sympathetic and cranial ganglia (Soares *et al.*, 1994).

On the other hand, the specific agents that trigger apoptosis may not be necessarily distinct from those that trigger necrosis. It has now become evident that it is not necessarily the type of agent (DNA damaging, ionophore, reactive oxygen metabolite), but the concentration and/or duration of application that is responsible for triggering the particular type of cell death (Payne *et al.*, 1995).

1.1.4.6 Internucleosomal DNA cleavage in apoptosis

Along with evidence at the electron microscope level, the study of DNA fragmentation patterns remains the gold standard for the visualisation of apoptotic cell death. However certain studies have shown that the fragmentation is associated with certain types of cells, or with cells characteristically undergoing necrosis and this indicates that fragmentation itself cannot alone determine apoptosis in cells.

The cleavage of the DNA occurs at internucleosomal linker regions, resulting in a "ladder" of 180-200 base pair fragments and multiples thereof, and this can be determined on agarose gel electrophoresis. The cause of the ladder type DNA fragmentation has been attributed to the activity of a calcium and magnesium ion (Ca^{2+} /Mg^{2+}) -activated endonuclease, DNAse I or DNAse II, all of which are capable of generating internucleosomal cleavage of DNA (Payne *et al.*, 1995). There is evidence that this enzyme is a neutral endonuclease dependent upon the coincident presence of Ca^{2+} and Mg^{2+} ions and that alteration of the ionic environment in the nucleus may be sufficient to activate this enzyme (Arends *et al.*, 1990).

It is the mechanism of action and activation of the endonuclease that is of importance. Identification of the enzyme specifically involved with programmed cell death remains a mystery, and it is not certain whether apoptosis involves a new intranuclear nuclease activity (by synthesis or activation) or rearrangement of chromatin proteins to permit DNA cleavage by pre-existing enzymes (Arends *et al.*, 1990).

In studies conducted by Wyllie *et al.* (1984) the Ca^{2+}/Mg^{2+} ionophore A23187 induced apoptosis and chromatin cleavage in thymocytes. The data confirms that the condensed

chromatin which characterises apoptosis morphologically consists of endogenously digested chromatin fragments.

All cells in the body are bathed in fluid very rich in Ca^{2+} (10^{-3} M), while the intracellular Ca^{2+} concentrations are much lower (10^{-6} M). The electrical potential of these cells drives the Ca^{2+} into them. Damage to the plasma membrane by any of a number of different mechanisms would disrupt this permeability barrier with a resultant influx of Ca^{2+}. Calcium ions are biologically active and are capable of metabolic destruction. In order to fully understand the role of Ca^{2+} in toxic cell death Schanne *et al.* (1979) evaluated the dependence on extracellular Ca^{2+} on primary cultures of adult rat hepatocytes treated with a range of toxins that interact with cellular membranes. Half the cells were killed in 1 to 6 hours in the presence but not in the absence of extracellular Ca^{2+} (Schanne *et al.*, 1979).

1.1.4.7 The cellular regulation of apoptosis

Apoptosis is viewed as a gene-directed cellular activity, in which cellular products result in self-destruction of the cell (Hockenbery, 1995). One of the protein mediators essential to cell cycle regulation is p53. It is a tumour suppressor gene that has the function of initiating apoptosis, which is thought to occur when cells cannot cope with DNA damage and thus avoid their own survival while suffering from an excessive mutational load (Kruyt *et al.*, 1996). The gene was first thought to be an oncogene, but it was subsequently realised that the allele initially cloned and studied was, in fact, a mutant allele, and the wild-type gene functions as an oncosuppressor (Hooper, 1994).

The protein (53 kDa) is a transcription factor that is a conditional regulator of the G_1 to S stage of the cell cycle (Knudson, 1993) and is phosphorylated in a cell cycle-dependent manner. Following DNA damage, the protein levels rise as a result of post-translational stabilisation, leading to the growth arrest of the G_1 stage of the cell cycle (Hooper, 1994).

The p53 gene is expressed as either a wild-type p53 or a mutant p53. The p53 tumour suppressor gene is the most widely mutated gene in human tumourigenesis (Lowe *et al.*, 1993). Mutations in the p53 cluster predominantly within the DNA binding domain of p53,

leading to a loss-of-function of both the DNA binding and normal biological activity of the protein (Fan *et al.*, 1994). Lowe *et al.* (1993) isolated thymocytes from p53 homozygous mutant, heterozygous and wild type animals. The thymocytes were treated with a) 10 nM phorbol ester and 500 nM calcium ionophore, b) 1 μM dexamethasone and c) 500 centiGreys (cGy) ionising radiation (Fig. 1.11). The results indicated that there was a dramatic increase in the p53 levels with the irradiation treatment and little or no increase in p53 levels with the phorbolester/calcium ionophore and dexamethasone treatments. Taking into consideration the apparent requirement of p53 function in radiation-induced apoptosis of thymocytes, the workers found that irradiation of thymocytes caused an increase in p53 levels, and that the accumulation of p53 protein was apparent within 1 hour before significant DNA degradation, thus establishing the involvement of p53 in specifically radiation-induced apoptosis.

Moreover, the p53 deficient thymocytes remained viable up to treatments of 200 cGy, and wild type cells were susceptible to treatments of 100 cGy. The heterozygous cells displayed intermediate viability (Fig. 1.11). The same was true for reductions in thymus cell surface markers $CD4^+$ and $CD8^+$, with the wild type thymuses yielding low percentages of the markers whilst the mutant thymuses yielded higher numbers after γ-irradiation. The p53 protein has been implicated in controlling the checkpoint at the G1 phase of the cell. Thus the cells are checked for DNA damage before they enter the S phase of the cycle (Lowe *et al.*, 1993).

The retention of wild-type p53 leads to G1 arrest and it is at this point that the DNA damage is assessed and is either corrected and the cell proceeds as normal or undergoes apoptosis, e.g., the loss of cell surface markers in the wild type thymuses in experiments conducted by Lowe *et al.* (1993). Activation of the p53 control system can therefore have two consequences for a cell, stable G1 arrest; and perhaps enhanced DNA repair or deletion of cells with DNA damage through the apoptotic pathways (Fan *et al.*, 1994). The mutant p53 (the absence of p53 function) does not induce G1 arrest and this in turn can lead to inappropriate cell survival after damage. The failure to eliminate these cells that have incurred DNA damage could lead to a group of cells that have undergone neoplastic transformation (Lowe *et al.*, 1993).

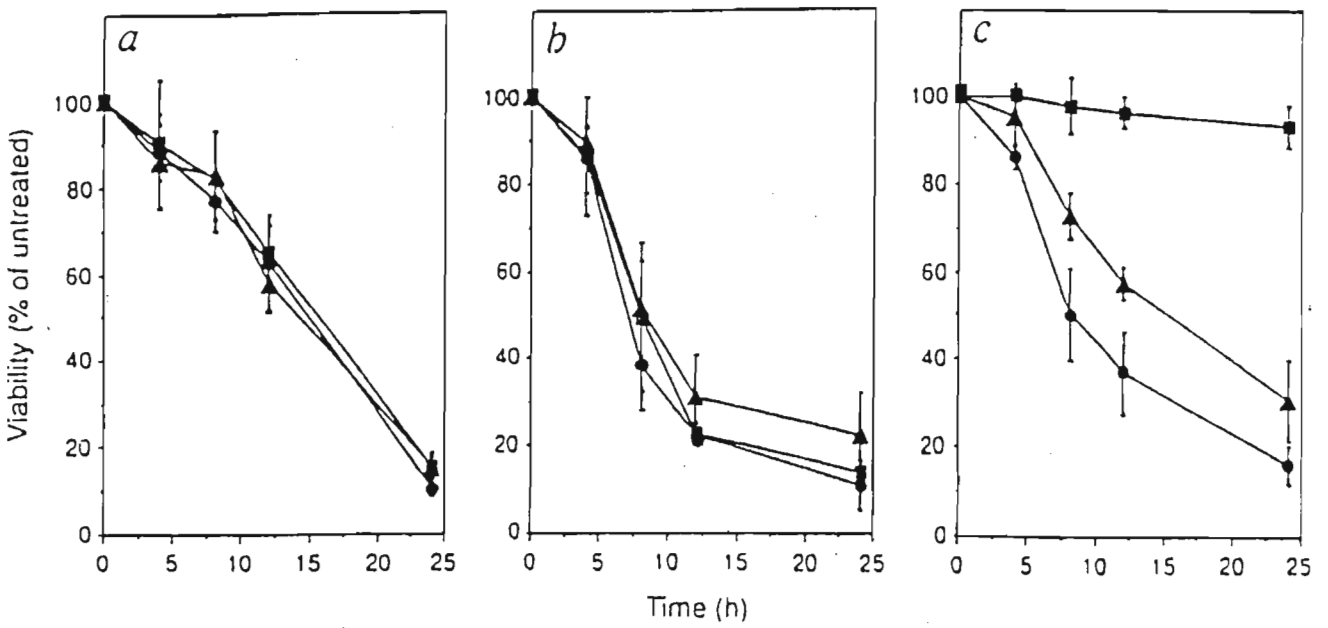

Figure 1.11: (Lowe *et al.*, 1993) a) 10 nM phorbol ester and 500 nM calcium ionophore; b) 1μM dexamethasone; c) 500 cGy ionising radiation. Thymocytes were derived from p53 homozygous mutant

(■), heterozygous (▲) and wild-type (●) animals.

p53 has recently been shown to induce the tumour growth suppressers, Waf 1 and CIP 1 genes (21 kDa) (Nasmyth and Hunt, 1993), which are potent inhibitors of cyclin-dependent kinases, particularly the inhibition of cyclin E/Cdk2 kinase activity in wild-type p53 cells (El-Deiry *et al.*, 1994). Cyclin-dependent protein kinases (CDKs) (Fig. 1.12), comprising a cyclin regulatory subunit and a CDC2-family kinase unit, trigger the onset of two key cell-cycle events, namely DNA replication and mitosis, which begins with the G1 phase (Fig. 1.12) (Nasmyth and Hunt, 1993).

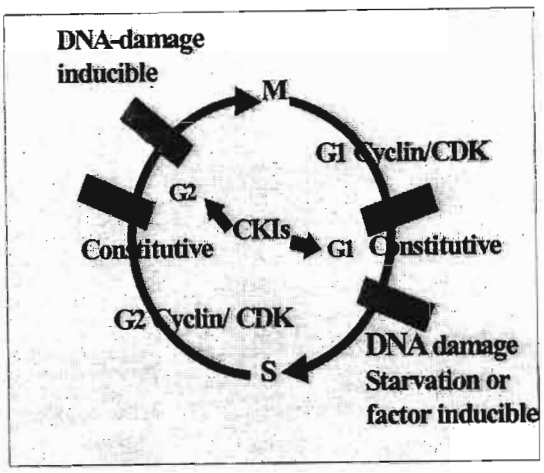

Figure 1.12 (Nasmyth and Hunt, 1993) A simplified cell with G1 and G2 cyclin kinase inhibitors (CKIs). The dark red blocks represent the constitutive CKIs, the paler ones are CKIs that can be induced under particular circumstances. These blocks to cell cycle progression may be removed by proteolysis or phosphorylation.

The ability of p53 to activate transcription from specific sequences, suggests that the genes induced by p53 may mediate its role as a tumour suppressor. El-Deiry *et al.* (1993), using a subtractive hybridisation technique identified a highly induced gene, and named it the wild-type p53-activated fragment 1 (Waf 1). Waf 1 is directly regulated by p53 and can itself suppress tumour cell growth in culture. p53 was found to bind to DNA in a sequence-specific manner and activate transcription from adjacent genes. Therefore genes whose expression is activated by p53, might be mediators of p53-dependent tumour growth suppression. Concurrently Harper *et al.* (1993) identified a gene called CIP 1 whose product binds to cyclin complexes and inhibits cyclin-dependent kinases, and that the sequence of CIP 1 is identical to Waf 1 (El-Deiry *et al.*, 1993).

Waf 1 expression was induced by p53, and this induction was observed in human, mouse and rat cell lines. At least one strong functionally active p53 binding site existed within the Waf 1 transcription regulatory region. In short DNA damage or cellular stress results in the stimulation of p53 expression and in turn p53 binds to Waf 1 regulatory elements and transcriptionally activates its expression. The Waf 1 protein is transported to the nucleus and subsequently binds to and inhibits CDK activity, preventing phosphorylation of critical

cyclin-dependent kinase substrates and blocking cell cycle progression (El-Deiry *et al.*, 1993 and El-Deiry et al., 1994). This means that inhibition of these kinases results in the failure of the cells to exit G1. It is possible for a cell to enter G1 arrest without the activation of Waf 1/CIP 1 (El-Deiry *et al.*, 1994).

The identification of Waf 1 and its regulatory region potentially provides a novel drug discovery approach, in that, compounds that activate expression of Waf 1 might bypass the p53 defect in tumours with endogenous p53 mutations (El-Deiry *et al.*, 1993).

Another cell cycle regulator of some importance is the Bcl-2 protein. Ueno *et al.* (1995) suggested that apoptosis could be caused as a result of the suppression of Bcl-2 proteins. Bcl-2 is the acronym for the B-cell lymphoma/leukaemia-2 gene (Reed, 1994). This gene was first discovered due to its involvement with B-cell malignancies, where t(14;18) translocation activates the gene in the majority of follicular non-Hodgkin's B-cell lymphomas (Hockenbery, 1994 and Reed, 1994). It is more commonly associated with the inhibition of programmed cell death.

Bcl-2 over-production accords the prevention of cell death without necessarily affecting cellular proliferation, and this implies that the Bcl-2 gene defines a new category of oncogenes. Classical oncogenes, primarily exert their effect by accelerating the rate of cellular proliferation. The cancer problem could be likened to an equation where:

Rate of cell accumulation = Rate of cell of proliferation - Rate of cell death

then, one can derive excess cells by either increasing the rate at which cells divide or by decreasing the frequency with which they die. In addition to this Bcl-2 can prevent or markedly reduce cell killing induced by a wide variety of stimuli, among these are p53, gamma and UV-radiation, free radicals, lipid peroxidation, chemotheraputic drugs and glucose.

Bcl-2 seems to act by preventing the presence of damaged DNA from being translated into a signal for activation of the genes involved in apoptosis, or by blocking the action of the products of those genes once induced.

The Bcl-2 oncoprotein (amino acid sequence of 25-26 kDa) is located within the nuclear envelope, parts of the ER, and outer mitochondrial membrane (Reed, 1994). It was concluded from extraction methods that Bcl-2 is an inner mitochondrial membrane protein, and that its association with the outer membrane is as a result of the junctional complexes where the inner and outer mitochondrial membranes abut (Reed, 1994).

The ability of Bcl-2 to protect cells from a wide variety of pathological, as well as physiological, stimuli indicates that many triggers can serve to activate the same suicide pathway, even some thought to cause necrosis, and not physiological cell death (Vaux, 1993).

1.1.4.8 The disposal of apoptotic cells

Apoptotic cells are phagocytosed by neighbouring cells and macrophages, although the fate of these phagocytic cells remains unknown (Uchiyama, 1995). Most apoptotic cells *in vivo* appear to undergo phagocytosis and secondary degeneration within phagolysosomes (Hockenbery, 1995). It is usually stated that apoptosis does not induce an inflammatory response, whereas ischaemic cell death does (Majno and Joris, 1995). When macrophages have made contact with the target apoptotic cell, they stick to the cell via vitronectin receptors (Savill *et al.*, 1990). However, the macrophages must, in some way, have been attracted to the target, albeit over a short distance, and this sequence is certainly typical of inflammation (Majno and Joris, 1995).

Apoptotic cells do not appear to attract neutrophils or lymphocytes, and this could reflect two possibilities: 1) a qualitative difference that exists in apoptotic cells, and 2) that cells dying singly (such as apoptotic cells) release such small quantities of chemoattractants, that not all the molecular species reach the vascular endothelium (responsible for initiating the sequence for leukocyte emigration) in effective concentrations (Majno and Joris, 1995).

When apoptosis occurs on a large scale, phagocytes appear, and in these situations the entire mass of dead apoptotic cells is replaced by a crowd of mononuclear phagocytes. However one inflammatory process of apoptosis does seem unusual, and that is the fact that

the cell debris (apoptotic bodies) are often phagocytosed by neighbouring cells such as epithelial cells, which are not professional phagocytes. This might be cellular cannibalism, but question whether it is specific to apoptosis, or whether it represents a general tendency of cells to devour their disabled neighbours, regardless of the mechanism of cell death (Majno and Joris, 1995).

1.1.5 Conclusion

T-2 toxin has been shown to have many cytotoxic effects, often resulting in death within days from acute exposure. Mechanisms of action (including cell death) of T-2 toxin action have been proposed for many cell lines. Therefore a link needs to be established between the cytotoxic effects of T-2 toxin and the mechanisms of cell death in normal human lymphocytes. Irreversible damage to lymphocytes results in immuno-compromised organisms. Thus the toxic effect of T-2 toxin on an organism is both direct and indirect.

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 The Preparation of the Lymphocyte Sample Material

Mammalian cells are used as alternatives to animals in toxicology (Benford and Hubbard, 1987) and the culture of human and animal cells is common practice in modern research laboratories. Peripheral venous blood is easily obtainable with negligible discomfort to the patient. Peripheral venous blood was used in all experiments. The primary reason was because of the accessibility of peripheral venous blood and secondly in attempt to keep the sample type constant. The latter arises from the known difference in pH of the peripheral arterial and peripheral venous bloods due to the absence and presence of CO_2 respectively.

In vitro cultures allow for a more controlled and manipulated environment, thus also allowing the biochemical mechanisms of toxicity to be analysed more specifically. Furthermore this type of experimentation involves a reduction of animal studies that are often in the public protest limelight. This in turn reduces the financial costs incurred by animal studies (Benford and Hubbard, 1987).

2.1.1 The collection of blood

Whole human peripheral venous blood (60 ml) was collected for each experiment. Institutional ethical approval was obtained for this study. The source of the blood was a healthy 24 year old female donor. The blood was collected in 10 ml sterile lithium heparin tubes. All further work was conducted in a sterile environment (Appendix 1).

2.1.2 The isolation of lymphocytes

Histopaque-1077 (Sigma) was used to separate the whole blood. Histopaque-1077 is a solution of polysucrose and sodium diazoate adjusted to a density of 1077 g/ml (Sigma Catalogue, 1997). Histopaque-1077 facilitates the rapid recovery of viable mononuclear cells from small volumes of blood (Sigma Diagnostics, 1997). The Histopaque was stored at 4°C and prior to use was warmed to 37°C in a sterile incubator. The whole blood was layered in

equal volumes of 5 ml onto Histopaque (5 ml) in sterile 10 ml heparin tubes and were not shaken or inverted, because mixing would prevent sedimentation of the different cells. The tubes were centrifuged at 400xg for 30 minutes at room temperature.

The whole blood was separated into 4 layers (Fig. 2.1) with the lymphocytes in the buffy coat (mononuclear layer). The upper layer, the plasma, was aspirated with a sterile Pasteur pipette to within 0,5 cm of the second opaque layer (the mononuclear cells) (Fig. 2.1). The plasma was discarded. The mononuclear layers, containing the lymphocytes, were aspirated and transferred to sterile test tubes and resuspended in 10 ml Hank's Balanced Salt Solution (HBSS) (Appendix 2) warmed to 37°C. The test tubes were centrifuged in a bench top centrifuge at 250xg for 10 minutes at room temperature. The HBSS served as a wash in order to remove extraneous platelets. Erythrocyte contamination was negligible.

The supernatants were discarded and the pellets were resuspended in 5 ml HBSS and centrifuged twice more at 250xg for 5 minutes each at room temperature. The pellets were finally suspended in 20-30 ml of complete culture medium (CCM) warmed to 37°C. The CCM comprised of 82,66% RPMI-1640 (Appendix 2); 10,22% foetal calf serum and 7,12% penicillin/streptomycin (Appendix 3). RPMI-1640 medium was developed at Roswell Park Memorial Institute hence the acronym RPMI (Sigma Catalogue, 1997). The use of 10% foetal calf serum was suggested by Reubel *et al.*, 1987 and Ueno *et al.*, 1995.

The lymphocytes were counted at room temperature using a trypan blue haemocytometer method (Appendix 4). The lymphocytes were adjusted to approximately 1,8 x 10^6 cells/ml. The suspension was stored at 37°C in preparation for the experimentation.

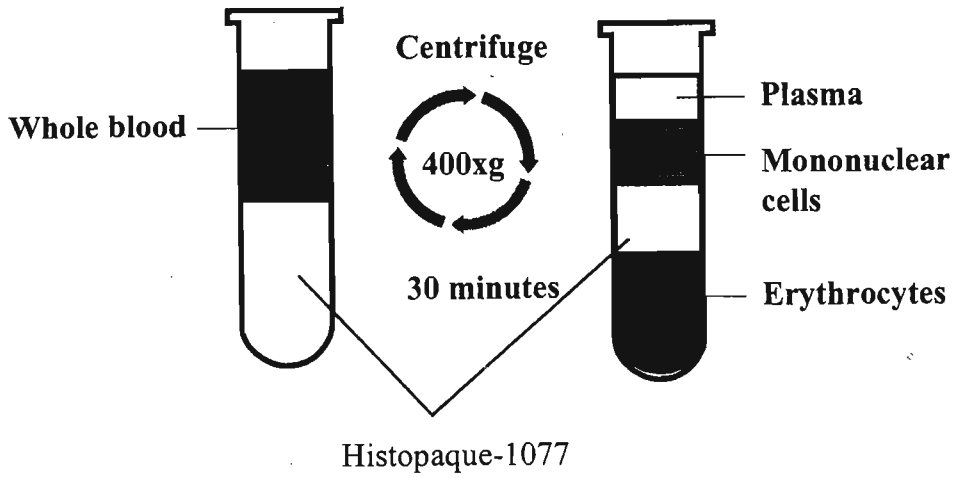

Figure 2.1 The isolation of lymphocytes from normal human blood using Histopaque-1077.

2.2 The Cytotoxicity Assay

Scientific research constantly strives for methods that are accurate and efficient. The easiest route in the assessment of the viability of cells or the cytotoxicity of a mycotoxin, is via a colorimetric assay.

This study involved the use of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a yellow tetrazolium salt. The assay was found to be rapid (24 hours) and useful in testing a large variety of sample materials. The bioassay can be carried out in microtitre plates and allows for fast and simultaneous testing of various parameters, such as different mycotoxins, concentrations and cell types (Hanelt *et al.*, 1994).

The assay works by means of the conversion of the yellow tetrazolium salt (MTT) by mitochondrial dehydrogenases of metabolically active cells to an insoluble purple formazan product, which is then solubilised with dimethylsulfoxide (DMSO) (Hanelt *et al.*, 1994). According to Reubel et al. (1987) the addition of DMSO to the residue achieved a homogeneous solution of the formazan product, which is necessary for an exact photometric measurement. This was in contrast to Mosmann's method of using 0.04 N HCl in isopropanol, in which case the formazan was not totally dissolved and non-reduced MTT still remained in the wells (Reubel *et al.*, 1987). The tetrazolium salts are cleaved to formazan by the succinate-

tetrazolium reductase system that belongs to the respiratory chain of the mitochondria and is active only in viable cells (Slater *et al.*, 1963 and Richard *et al.* 1994). The assay is evaluated spectrophotometrically, and is a measure of absorbance values in individual wells, allowing the replicate testing of several drugs at multiple concentrations on various cell types (Carmichael *et al.*, 1985). The main advantage of the MTT assay, is its simple applicability and the independence of radiolabelling (Reubel *et al.*, 1987).

The optical density of the solubilised formazan product is directly proportional to the number of cells per well. This makes the assay directly comparable with assays counting the total number of cells in the culture before and after treatment (Carmichael *et al.*, 1985).

2.2.1 The preparation of T-2 toxin serial dilutions

A stock amount of 250 µg of T-2 toxin (Appendix 5) was solubilised in 100 µl DMSO and 200 µl of absolute ethanol (EtOH) (Pillay, 1997). T-2 toxin is highly soluble in DMSO and ethyl alcohols (Yagen and Bialer, 1993). To this solution, 2,2 ml of CCM was added to yield a stock solution of 100 µg/ml. A control stock solution was prepared identically, without the addition of T-2 toxin. A set of serial dilutions of T-2 toxin and controls were prepared from 25 µg/ml to 1,56 µg/ml, as outlined in Table 2.1.

Table 2.1 The preparation of serial dilutions of T-2 toxin.

Concentration of T-2 toxin (µg/ ml)	**Toxin added (ml)**	**CCM added (ml)**	**Total volume (ml)**
25	1,25	3,75	5
12,5	0,625	4,375	5
6,25	0,3125	4,6875	5
3,125	0,156	4,844	5
1,56	0,078	4,922	5

2.2.2 T-2 toxin treatment of the lymphocytes

The lymphocytes were removed from the incubator and dispensed into 96-well microtitre plates (100 µl per well). The plating follows the format indicated in figure 2.2. T-2 toxin serial dilutions and control serial dilutions were dispensed in a column-wise pattern into the wells at 100 µl per well. Each concentration was placed in a separate column of five replicates. The total volume of each well, therefore, was 200 µl. The plates were incubated at 37°C for 1, 3, 6, 12 and 24 hours.

2.2.3 The MTT assay

The MTT salt (5 mg) was dissolved in HBSS (1 ml). The solution was filtered through a 0,45 µm filter (Millipore). According to Reubel *et al.* (1987), this sterilises and separates the insoluble yellow powder from the solution. Subsequent to each incubation, the plates were centrifuged at 1 600xg at 25°C for 20 minutes (Dombrink-Kurtzman *et al.*, 1993). This pelleted the lymphocytes to the floor of each well and thereby prevented the removal of the cells together with the supernatant. The supernatants were removed and discarded (Appendix 5) with a micropipette.

The MTT solution was added to each well at a concentration of 10 µl of MTT in every 100 µl of fresh CCM added to each well. In accordance with work done by Reubel *et al.* (1987) and Dombrink-Kurtzman *et al.* (1993), the plates containing the MTT were incubated at 37°C for 4 hours. The optical density (OD) was read on a Biorad (model 3550) microplate reader at 595 nm with a reference wavelength of 655 nm.

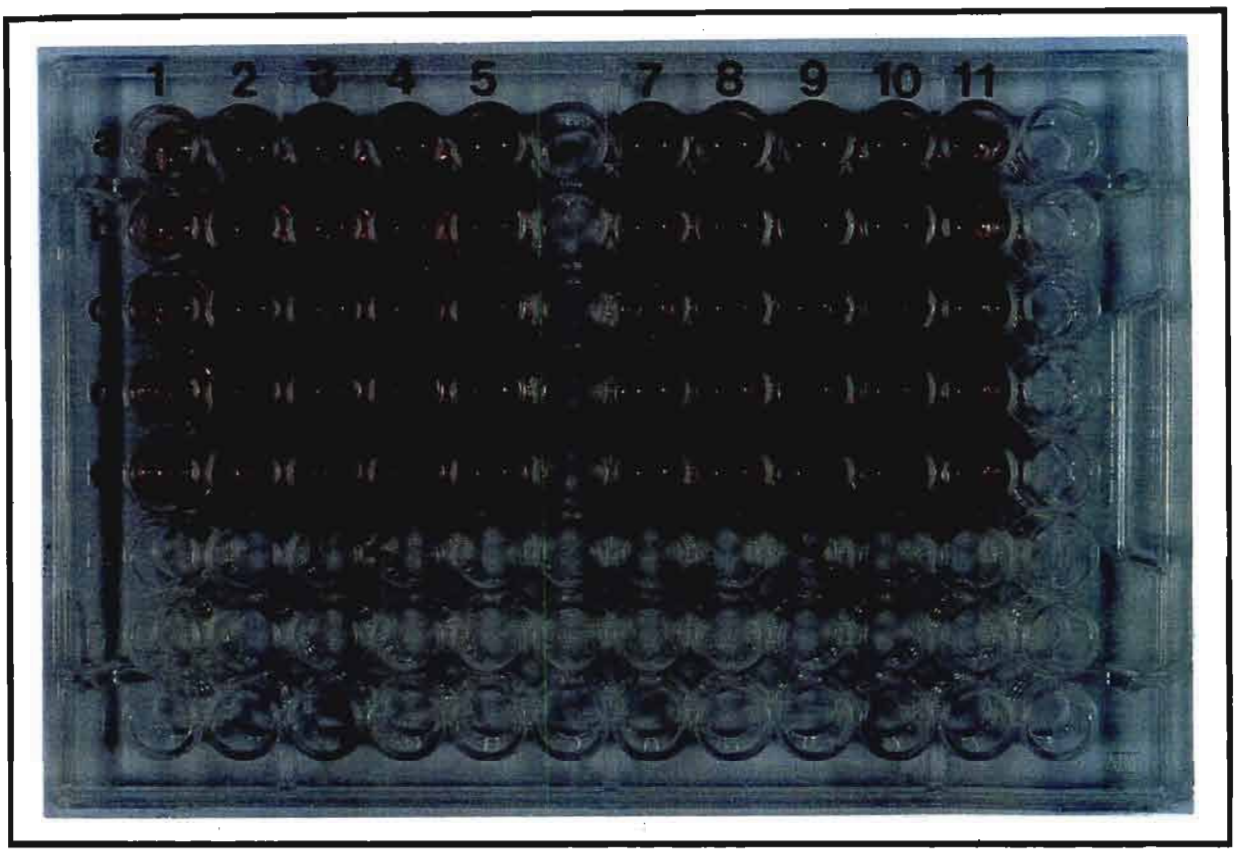

Figure 2.2 The incubation of lymphocytes with T-2 toxin and controls were arranged as above, with (T-2) representing the wells containing T-2 toxin and (C) representing the wells containing the control samples. The toxic concentrations were placed in separate columns, for example 1a to 1e contained 25 µg/ml of T-2 toxin. This plate was prepared as a demonstration of dispensing techniques. The actual experimental plates were always kept in a sterile environment.

2.2.4 Statistical analyses

Means and standard deviation values (the latter represented as error bars) of the five replicates of each toxin concentration were compared with the corresponding controls. The significant difference between the means of the 12 and 24 hour study was calculated by the students t-test at $p < 0,05$ (Appendix 6). The viability of the 1, 3 and 6 hour samples was calculated as the mean OD expressed as a percentage of cleavage activity in comparison to the controls (100%):

$$\% \text{ Cell viability} = mT\text{-}2 / mCon * 100$$

where : mT-2 = mean OD of T-2 toxin treated cells, and

mCon = mean OD of control cells

2.3 Transmission Electron Microscopy (TEM) and Immunocytochemistry (ICC)

Much of our current knowledge concerning the fine structure of cells, viruses and even large molecules comes from electron microscopy (Mathews and van Holde, 1990). The use of electron microscopy is necessary for the examination of morphological changes at subcellular levels. Cytotoxicity assays provide a keen insight into the quantitative damage caused by the toxic agents. However, it is of equal importance to assess the ultrastructural damage induced by toxic insult in order to pinpoint areas of toxic stress, in the hopes that these observations may help to form an organelle-specific diagnosis which may lead to possible treatment.

Immunocytochemical staining involves the visualisation and localisation of antigens in cells and tissues. In this technique the tissues are fixed, dehydrated and embedded in resin. Ultrathin sections are mounted on coated or uncoated electron microscopic grids and these are incubated with a series of immune reagents, washed, contrasted, dried and viewed (De Mey, 1986).

2.3.1 Preparation of lymphocyte samples for testing

A lymphocyte suspension (10^6 cells/ml CCM) was dispensed into 10 wells of a 12-well culture plate (Sterilin) at 1 ml of cells per well. Five wells represented the five incubation times for the T-2 toxin treated samples and the remaining five wells were allocated for the five control incubation time studies. Separate, but identical plates, were prepared for transmission electron microscopy (TEM) and immunocytochemistry (ICC). The incubation times used were 1, 3, 6, 12 and 24 hours. A T-2 toxin concentration of 1,56 µg/ml was prepared from a stock solution of 100 µg/ml as described in table 2.1. The control solution was prepared similarly but without the addition of T-2 toxin. The T-2 toxin and control

solutions were dispensed at 1 ml/well into the appropriate wells, such that each well contained 2 ml in total. The plates were incubated at 37°C for the appropriate incubation times.

2.3.2 The processing, sectioning and staining of samples for TEM and ICC

After each incubation time, the associated T-2 toxin treated and control cell suspensions were aspirated separately from their wells with sterile Pasteur pipettes and placed in separate clean and labelled eppendorfs. The samples for ICC were placed in separate, labelled eppendorfs. Care was taken to keep the treated and control samples apart and identifiable. These were centrifuged at 250xg for 2 minutes at room temperature. The supernatants were discarded (Appendix 5) and the pellet were resuspended in HBSS (1 ml) for washing. After two washes, followed by centrifugation at 250xg for 2 minutes at room temperature, the lymphocytes were processed for TEM as outlined in table 2.2. Eppendorfs were used because the samples were in suspension. The lymphocytes do not attach to the culture flask surfaces and had to be pelleted out. This prevented the loss of cells. The eppendorfs were found to be most effective for this process. The eppendorfs were also convenient for moulding the embedding resin to a tip which contained the pelleted lymphocytes, thus making sectioning easier. The plastic eppendorf was removed prior to sectioning.

Table 2.2 The method used for processing experimental and control cells for transmission electron microscopy.

Materials (Appendix 7)	**Volume (ml)**	**Time**	**Temperature (°C)**
Fixation: 1% Glutaraldehyde in HBSS	0,5	30 min	37
Buffer rinse: PBS (pH 7,3-7,4) (x2)	0,5	10 min	25
Post fixation: 1% OsO_4 in cacodylate	0,5	1 hour	25
Buffer rinse: PBS (x2)	0,5	15 min	25
Dehydration: 70%, 90%, 100% (x2) in EtOH	0,5	15 min each	25
Resin incorporation: 50:50 (araldite: 100% EtOH)	0,5	30 min	25
Resin incorporation: 100% araldite resin	1	1 hour	60
Embedding medium: 100% araldite resin	1	48 hours	60

Each step of the processing procedure except for the embedding medium (48 hours) required the centrifugation of the samples at 250xg for 2 minutes. This was necessary because the lymphocytes were in suspension. Pelleting then allowed for only the supernatant to be discarded. The embedded samples were removed from the eppendorfs and sectioned with glass knives in a Reichert-Jung ultramicrotome. The sections were cut to 60 nm thick (gold sections) and were stretched with xylene fumes. This was done by holding a piece of filter paper soaked with xylene immediately over the sections until stretching was viewed.

The sections were then transferred to copper viewing grids for TEM. The TEM sample grids were counterstained by first placing the grids on a droplet (10 µl) of uranyl acetate (Appendix 7) for 3 minutes. When the time elapsed the grids were transferred with fine forceps to a droplet of distilled water. This was followed by jet washing the grids. This was done by picking up the grids (perpendicular to the work surface) and running a gentle stream of distilled water (10 ml) along the curve of the forceps, so that the grids were gently but

thoroughly washed. The grids were then blotted on fibre free paper and transferred to a droplet (10 µl) of lead citrate (Appendix 7) for 3 minutes. This was followed by another droplet (10 µl) of distilled water and a jet wash with 10 ml of distilled water. The grids were blotted on fibre-free paper and stored in sealed plastic petri dishes until required.

The samples were processed for ICC using procedures outlined in table 2.3. A smaller percentage of glutaraldehyde (0,5%) was used in order to prevent leaching of antigen out of the sample during processing whilst not damaging the internal morphology of the cells (Snyman, 1993).

The embedded samples were sectioned to 60 nm thick sections and transferred to nickel viewing grids. Copper grids are not compatible with immunolabelling since some of the chemicals may react with the metal. This results in contaminated grids and specimens (Snyman, 1993).

Table 2.3 Method for the processing of experimental and control samples for immunocytochemistry.

Materials (Appendix 7)	**Volume (ml)**	**Time**	**Temperature (°C)**
Fixation: 0,5% Glutaraldehyde in HBSS	0,5	30 min	37
Buffer rinse: PBS (pH 7,3-7,4) (x2)	0,5	2 x 5 min	25
Dehydration: 70, 90, 100%(x2) in EtOH	0,5	15 min each	25
Resin incorporation: 50:50 (EtOH: araldite)	0,5	30 min	25
Resin incorporation: 100% araldite resin	0,5	1 hour	60
Embedding medium: 100% araldite resin	1	48 hours	60

2.3.3 Immunolabelling of T-2 toxin

The sections to be treated for immunocytochemistry were already air-fixed on nickel grids. The entire method was carried out at room temperature. The sections were etched by placing the grids (with a pair of fine curved forceps) in droplets (5-10 µl) of 5% hydrogen peroxide for 5 minutes. This renders the resin more permeable to reagents and exposes masked antigenic sites (Snyman, 1993). Etching also increases hydrophilicity of the resin (Varndell and Polak, 1986).

The grids were then placed in droplets of distilled water. Each grid was then jet washed with distilled water (10 ml). The grids were placed on droplets of normal goat serum (NGS) in Tris-HCl buffer, pH 7,2 (Appendix 8) at a dilution of 1:40, for 30 minutes. The NGS serves to block non-specific background staining caused by ionic irregularities, specific but unwanted antigen recognition and cross-reactivity (Snyman, 1993). The effectiveness of the blocking agent depends on the source of the secondary antibody (Snyman, 1993). In this case the secondary antibody was raised in goat.

The grids were then drained on fibre free paper and immediately placed on a droplet (10 µl) of primary antiserum diluted in 0,2% bovine serum albumin (BSA) in Tris (Hydroxymethyl) aminomethane (Tris) buffer (Appendix 8) for three hours. The primary antiserum was polyclonal rabbit anti-T-2 toxin (Sigma) at a dilution of 1 µl in 100 µl of Tris-BSA buffer (0,2%). The method control was placed on a droplet of phosphate buffered saline (PBS) (Appendix 7) for three hours. The method control was necessary to verify the technical success of the experiment. In the absence of primary antibody, an observation of label would signify a failed method due to background staining.

The grids were first placed on a droplet and then jet washed with 20 ml of 50 mM Tris buffer (pH 7,2) (Appendix 8) per grid. The grids were blotted on fibre free paper. This was followed by placing the grids on droplets of 0,2% Tris-BSA buffer (pH 7,2) and immediately jet washed with 5 ml of this same buffer per grid. The grids were blotted and placed on droplets of 1% Tris-BSA buffer (pH 8,2) (Appendix 8) for five minutes.

The 50 mM Tris-HCl buffer is used to wash the preparations between incubations to remove unwanted proteins. The buffers containing BSA were necessary to block "sticky" sites for all proteins before and during application of immunological agents in order to prevent non-specific labelling. The BSA competes with the antibody for these non-specific sites (Snyman, 1993).

The grids were placed for an hour on droplets (10 µl) of secondary antibody. The secondary antibody was goat anti-rabbit IgG conjugated to a 5-10 nm gold probe (Sigma) and diluted to 1 µl: 15 µl in Tris-BSA buffer (pH 8,2) (Appendix 8). The gold probe (label) is the label that identifies the sites of T-2 toxin binding within the cells. This step was followed by alternately placing the grids in droplets and then jet washing with the same solution of 0,2% BSA in 50 mM Tris buffer (pH 7,2), 50 mM Tris buffer (pH 7,2) and eventually distilled water. The grids were counterstained with uranyl acetate and lead citrate as described previously (section 2.3.2).

2.4 DNA Fragmentation Analysis

This technique followed work done by Ueno *et al.* (1995) with minor modifications. Electrophoretic separation of compounds is the most widely used method in biochemistry. Various ions and macroions vary in charge and molecular dimensions, and their behaviour in an electric field provides a powerful way of separating them. A charged molecule, when placed in the electric field that exists between two electrodes immersed in a solution, migrates toward one electrode or the other. DNA molecules which are strong polyelectrolytes, carry multiples of negative charges (one unit charge on each residue), by virtue of the strongly acidic phosphate groups on the periphery of the DNA molecule. The molecules therefore migrate towards the positive anode and in a mixture of fragment sizes, each fragment will have a charge proportional to its molecular length. The molecular sieving effect of the agarose gel will determine the relative mobilities of such molecules at any given gel concentration (Mathews and van Holde, 1990).

2.4.1 The preparation of the lymphocyte samples

The lymphocytes suspensions (10^6 cells/ml) were treated in a sterile environment as in section 2.3.1 with 1,56 µg/ml (Table 2.1) of T-2 toxin and control media for incubation times of 1, 3, 6, 12 and 24 hours. Subsequent to each incubation time, the appropriate T-2 toxin treated and control lymphocytes were washed as described in section 2.3.2. The washed pellets were suspended in HBSS (1 ml) and stored at -70°C until required.

Preparation of the lymphocyte DNA for electrophoresis was preceded by thawing the pellets to room temperature. This did not require a sterile environment. The suspensions were centrifuged at 250xg for 2 minutes at room temperature. The supernatants were discarded and the lymphocytes were resuspended in 200 µl of a 1 x SDS gel loading buffer (Appendix 9). The suspensions were sequentially incubated with 50 µl RNAse (Boeringher Mannheim) and 100 µl proteinase K (Sigma), for one hour each in a 50°C water bath. The suspension thus contained the DNA for electrophoresis.

2.4.2 DNA electrophoresis

Electrophoresis of the DNA was carried out in 0,8% agarose gels (containing ethidium bromide) in TBE buffer (Tris-borate, 0,045 M; EDTA, 0,001 M; pH 8) (Appendix 10). Agarose, a polysaccharide, is a common gel-forming material used in the separation of DNA. Ethidium bromide is a florescent DNA-binding dye, and permits visualisation of the DNA under UV. Gloves were worn at all times during experimentation.

The gels were prepared by melting and boiling 0,9 g of low melting point agarose (SAARCHEM) and 50 ml of 1 x TBE buffer in a conical flask. The agarose was boiled in a microwave oven on high power. Whilst still hot, 2,5 µl of ethidium bromide (10 mg/ml) was added to the gel. Care was taken to ensure that the gel was sufficiently heated during this time to prevent it from setting. The gel was poured into a horizontal plastic plate with preset combs (10 wells/comb). There were two combs per plate which allowed for two gels to be run simultaneously. The gels were left to solidify at room temperature for approximately 30 minutes.

When the gels were ready to be used, the combs were carefully removed to expose the wells. The plate containing the gel was placed in a horizontal electrophoresis tank, such that the wells were closer to the cathode and the length of the gel faced the anode. The tank had been previously filled with 800 ml of 1x TBE buffer. The gel was completely submerged in the buffer, with a height of about 2-3 cm of buffer above the surface of the gel.

The samples were loaded into separate wells at 10 µl of sample per well. The samples were loaded by submerging a clean pipette tip of a micropipette about three quarters of the way into each well, but not touching the gel. The loaded gels were electrophoresed at 100 constant volts (V) for 45 minutes. Experiments were conducted to establish the optimal dilution of the DNA for electrophoresis. This was settled at 6 µl of the DNA suspension with 13 µl distilled water and 1 µl of the loading dye (0,02 % xylene/ cyanol, 0.02 % bromophenol blue, 50 % glycerol). The glycerol, in the dye as well as in the gel loading buffer, makes the sample solution dense and prevents mixing with the buffer solution in the upper electrode chamber (Mathews and van Holde, 1990). The anionic "tracking" dye migrates faster than most macroions, and this makes the progress of the experiment easy to follow (Mathews and van Holde, 1990). The DNA fragmentation patterns were examined under ultraviolet illumination and thermal prints and scanned images of the results were obtained.

CHAPTER 3

3.0 RESULTS AND DISCUSSION

3.1 The Cytotoxicity (MTT) Assay

At toxin concentrations of 25 µg/ml, there was an inhibition of MTT salt cleavage at all incubation times. However the 12 and 24 hour incubation times showed more significant inhibition with low percentage cell viabilities at 33,24% and 14,54% respectively (Table 3.1). The inhibition of MTT salt cleavage implies a reduced mitochondrial succinate dehydrogenase activity. This in turn means that due to damage to the mitochondria, cell viability is decreased. At the lowest concentration (1,56 µg/ml) the viabilities of the 1, 3 and 6 hour studies remain high, with significantly higher percentage viabilities in the 12 and 24 hour trials at 65,89% and 50,42% respectively (Table 3.1). This indicates that at lower concentrations of T-2 toxin there is less of an inhibition of MTT cleavage. However this was only evident at prolonged incubation times.

Table 3.1 Percentage cell viabilities of the T-2 toxin treated lymphocytes at respective incubation times

	Percentage (%) cell viability				
Concentration µg/ ml	**1 hour**	**3 hours**	**6 hours**	**12 hours**	**24 hours**
25	89,45	76,30	84,60	33,24	14,54
12,5	89,67	96,95	86,50	47,83	21,77
6,25	98,32	117,24	85,30	45,65	26,33
3,125	96,30	92,81	83,33	53,80	25,31
1,56	88,45	92,45	85,20	65,89	50,42

The trend line for the 1 hour incubation study is approximately a straight line (Fig. 3.1). This implies that there is a very small difference in the MTT cleavage at the different concentrations. This is probably because T-2 toxin is not effective at shorter incubation times,

and is possibly more effective at longer durations. The controls have a marginally higher viability than the toxin treated samples.

The 3 hour incubation study shows a trend line with a slope, revealing an increase in the percentage viability with a decrease in the concentration of T-2 toxin (Fig. 3.2). However, at 6,25 μg/ml, there seems to be a slight increase in the viability of the T-2 treated cells when compared with the controls. This implies that the toxin might have had a stimulatory effect on the cells at this point. At 3 hours of incubation with 25 μg/ml of T-2 toxin the lymphocytes exhibit a lower percentage viability than those exposed for 6 hours. It seems that at this concentration for 3 hours of exposure the lymphocytes readily assimilate the toxin and damage is induced, whilst at 6 hours some recovery could have occurred. The overall impression, however, is that very little cytotoxicity was induced by T-2 toxin at 3 hours at all concentrations when compared to the controls.

At 6 hours, T-2 toxin appears to have had some effect upon the viability of the lymphocytes. This is evident in the lower percentage viabilities at all concentrations, when compared to the same at the 1 and 3 hour incubation times (Table 3.1). The trend line for the 6 hour incubation trial is a straight line, indicating that there is not much of a difference in the percentage viabilities at different concentrations of T-2 toxin (Fig. 3.3).

The cytotoxic effect of T-2 toxin on normal human lymphocytes can be appreciated after a 12 hour exposure period. The trend line is represented by a slope, and indicates that there is an increase in the percentage viability of the lymphocytes with a decrease in the concentration of T-2 toxin administered to the cells (Fig. 3.4). T-2 toxin seems to have a slightly stimulatory effect on the lymphocytes at 12,5 μg/ml when compared to the percentage viability at 6,25 μg/ml. There is a greater inhibition of the cleavage of MTT at all concentrations in the treated samples than those of the controls (Fig. 3.4). The graph indicates that at prolonged incubation times there is an increase in the potency of toxic insult by T-2 toxin. At 1,56 μg/ml the cells have a viability of approximately 66%. This figure was considered useful for further studies in which living lymphocytes, as well as those in various stages of cytotoxic stress and death could be assessed.

The 24 hour incubation time shows the most inhibition at all concentrations than when compared with the controls and the toxin treatments at other incubation times (Table 3.1). The trend line is represented as a slope, indicating that there is a decrease in the inhibition of the cleavage of MTT (thus an increase in viability) with a decrease in concentration of the toxin administered (Fig. 3.5). Both the 12 and the 24 hour incubation studies reveal that T-2 toxin has a time-dose dependent relationship with the lymphocytes.

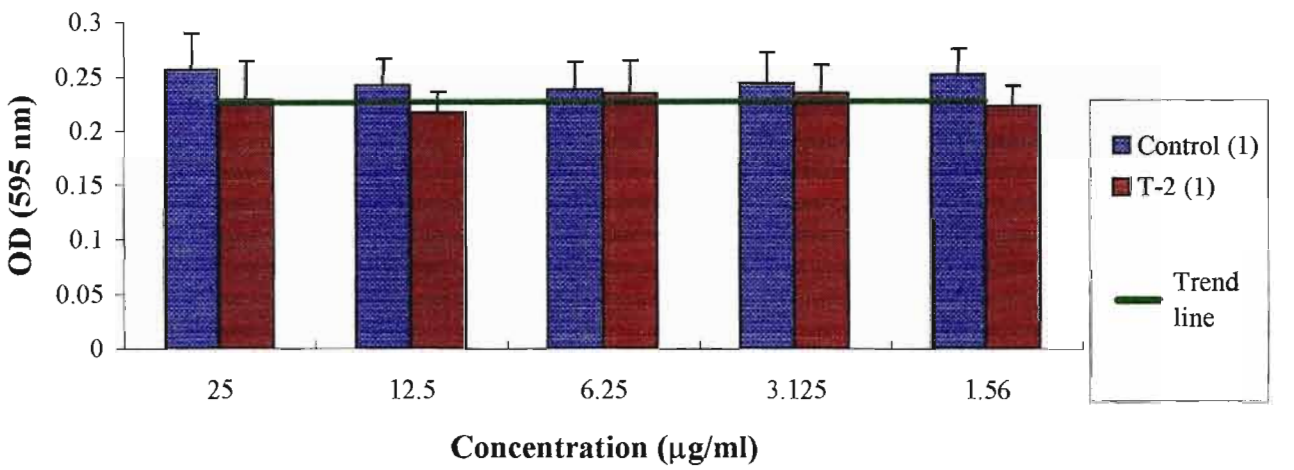

Figure 3.1 Bar graph showing the effect of T-2 toxin on normal human lymphocytes over an incubation period of 1 hour at serial concentrations. Cleavage activity of MTT is represented as absorbancy values (OD). Error bars represent standard deviations, sample number (n) = 5.

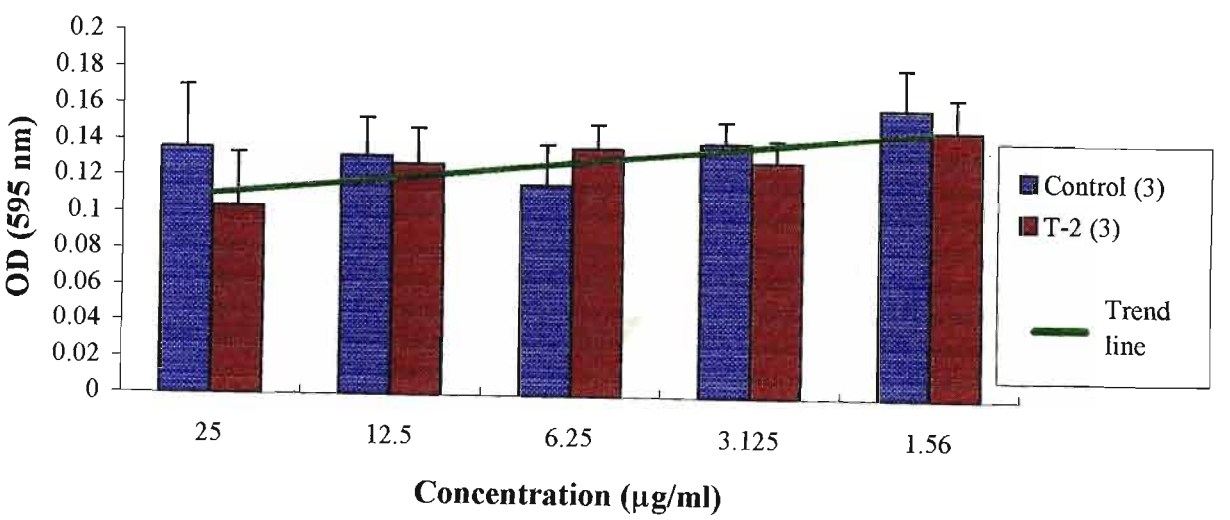

Figure 3.2 Bar graph showing the effects of T-2 toxin on normal human lymphocytes over an incubation period of 3 hours at serial concentrations. Error bars represent standard deviations, n = 5.

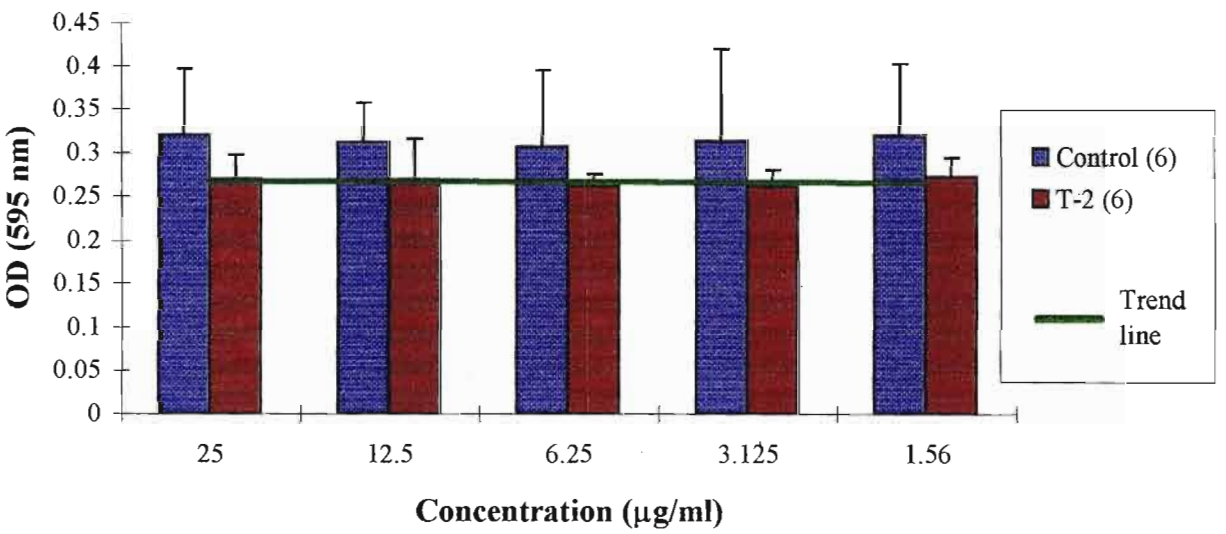

Figure 3.3 Bar graph showing the effect of T-2 toxin on normal human lymphocytes over an incubation period of 6 hours at serial concentrations. Error bars represent standard deviations, n = 5.

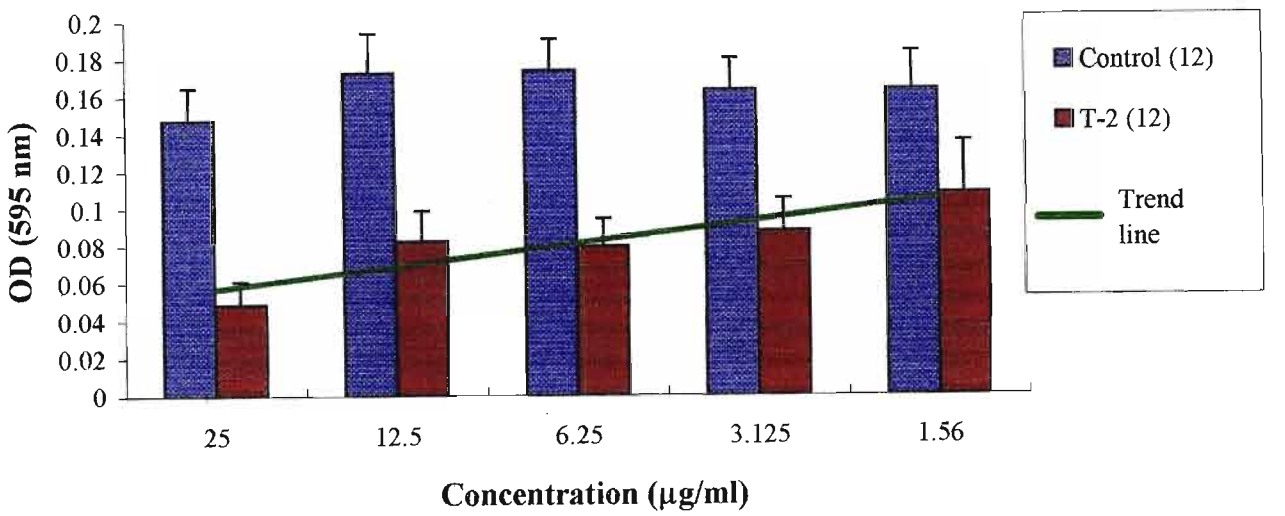

Figure 3.4 Bar graph showing the effect of T-2 toxin on normal human lymphocytes over an incubation period of 12 hours at serial concentrations. Error bars represent standard deviations, n = 5.

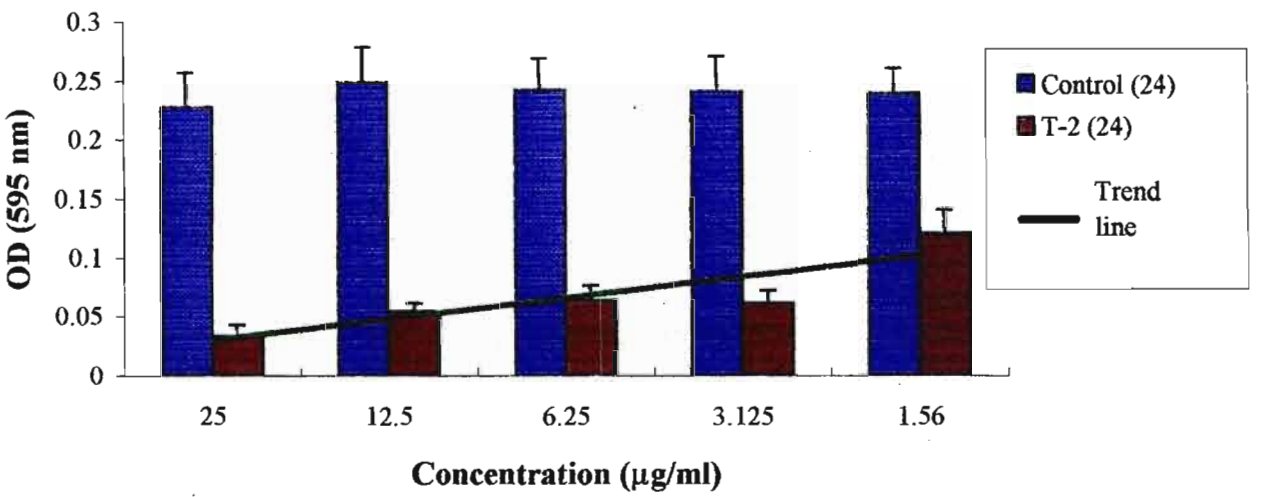

Figure 3.5 Bar graph showing the effect of T-2 toxin on normal human lymphocytes after an incubation period of 24 hours at serial concentrations. Error bars represent standard deviations, n = 5.

The higher rates of viability in the 1 and 3 hour studies implies that T-2 toxin might not have bound induced damage to the mitochondria in sufficient amounts to elicit a toxic response. The susceptibility of a cell to the adverse effects of an agent may be a reflection of its ability to bind the toxicant (Holt and DeLoach, 1988). However it is also possible that these incubation times were not sufficient for the uptake of the toxin into the cells. This might promote a decrease in the ability of the toxin to bind to cellular macromolecules or to impede mitochondrial succinate dehydrogenase activity, leading to an almost negligible cytotoxic effect. T-2 toxin was shown to be rapidly taken up by murine lymphocytes in 10-15 minutes in a saturable manner, and rapidly dissociated from the cells to leave approximately 10-15% of the original uptake in 1 hour. The possible rapid release of the toxin particularly during small incubation time frames may be explained by virtue of the fact that T-2 toxin is an amphipathic molecule and that it tends to partition into the cell membrane (the organic phase present). This differential solubility would be subject to constraints of parameters influencing the partition coefficient as well as factors influencing the state of the cell membrane (Gyongyossy-Issa and Khachatourians, 1984).

Full receptor occupancy is a "good candidate" as the first steps towards the expression of T-2 toxin toxicity, and that this idea is consistent with the suggestion that the cell membrane is involved in and may modulate T-2 toxin cytotoxicity (Gyongyossy-Issa and Khachatourians, 1985). It is possible that for the above reasons there is not much of a cytotoxic effect at 1 and 3 hours. That is, due to an insufficient amount of interaction between the toxin and the receptor sites, or to the rapid uptake and release of the toxin during this time, the cytotoxic effects of T-2 toxin were either little to none. In either event, there was no discernible evidence of increased cellular damage.

Although the 6 hour incubation study had only a marginally lower difference in cell viability, it seems that the threshold values and modulation of the cell membrane hold true. This is the first indication of the importance of time in the manifestation of the cytotoxic effect of T-2 toxin. It seems likely that even though the cell might possibly have taken up the toxin, the cytotoxic effects are not as evident in this time frame. That is, the toxin may have a subcellular effect on the cells, but this does not manifest in the total destruction of the cellular

mitochondria, implying that there would be no concurrent inhibition of MTT cleavage. The very small differences in cell viabilities at different concentrations at 1, 3 and 6 hours and the obvious dose-viability differences at 12 and 24 hours, reveals that the cytotoxic effect of T-2 toxin is primarily time dependent. However since the test is an indicator of mitochondrial viability (Holt and DeLoach, 1988) or an assessment of the mitochondrial damage to the cells, subcellular assessment, via electron microscopy is required to provide further information about cellular damage, other than death.

At $p < 0,05$ there is indeed a significant difference between the means of the 12 and 24 hour incubation time trials. This implies that over extended periods of time, T-2 toxin has a more toxic effect on the cells, thus increasing the percentage of mortality and thereby increasing the inhibition of MTT salt cleavage. However taking the time span of the toxin activity into consideration it is quite obvious that the cytotoxic effects of T-2 toxin are rather acute at 24 hours, and that prolonged exposure of the lymphocytes to the toxin would lead to near total mortality of the lymphocytes *in vitro*.

The diminished mitochondrial activity at the 12 and 24 hour incubation periods appears to be as a direct result of the effect of toxic insult. T-2 toxin has been demonstrated to inhibit the respiration in rat liver mitochondria. T-2 toxin inhibits the succinic dehydrogenase activity as well as site I of the mitochondrial electron transport chain (Pace, 1983). Furthermore T-2 toxin appears to have an inhibitory effect on mitochondrial protein synthesis (Pace *et al.*, 1988). It is for these reasons that the MTT assay is possibly the most relevant cytotoxicity assay for the study of T-2 toxin in normal unstimulated (resting) lymphocytes. The data provided by the MTT assay can be most accurate in terms of the actual damage induced by the toxin, as well as a perfunctory assessment of cell death, during lengthy incubation periods.

Thompson and Wannemacher Jr. (1986) attribute the variable potency of epoxytrichothecenes at the cell level to one or a combination of factors which are as follows: 1) the rate of transport into the cell; 2) metabolism of the mycotoxin by cytosolic enzymes; 3) changes in affinity for active binding sites; and 4) reduced capacity to interfere with protein

synthesis. Uptake, binding or interference effects could be due to either size, polarity or conformation of the mycotoxin. The increased sensitivity of the lymphocytes to T-2 toxin is due to the cell type sensitivity, i.e., lymphocytes have a slower rate of metabolism when compared to VERO cells and thus results in a more toxic situation (Thompson and Wannemacher Jr., 1986).

Gyongyossy-Issa and Khachatourians (1984) propose that the time-dose effect of T-2 toxin refers to the detectability of the toxic action at higher or lower doses. That is the cells reach a saturated state but cytotoxicity is evident faster in cells with higher toxin doses. In this study, it seems more likely that whilst the dose-threshold reasoning may be true, the detectability of cytotoxicity is time based. The cytotoxic effect of T-2 toxin as a whole is more evident at prolonged incubation times. Thus although the lymphocytes are saturated at 6 hours at any given concentration, the cytotoxic effect may not be detectable until much later. The differences in concentration are also more evident at prolonged incubation times.

Forsell *et al.* (1985) determined the cytotoxicity of T-2 toxin in human lymphocytes by considering the inhibition of mitogen induced blastogenesis in proportion to reduced [*methyl*-^{3}H]thymidine uptake. The use of radiolabel, although beneficial and precise, is an expensive an relatively unsafe mode of experimentation. These methods seem to be replaced by more rapid, inexpensive and safer techniques such as the MTT assay. The MTT assay is beneficial because it specifically outlines the percentage cell viability of the toxin treated samples in relation to controls.

3.2 Ultrastructural Evaluations Using Transmission Electron Microscopy (TEM) and Immunocytochemistry (ICC)

The control lymphocytes were typically rounded cells with large (sometimes slightly invaginated) nuclei and a thin ring of cytoplasm (Fig. 3.6) (Reubel *et al.*, 1987). There was a paucity of organelles within the lymphocytes. However the mitochondria were of ovoid shape and contained visible cristae. Golgi apparatus and endoplasmic reticulum (ER) were occasionally noticed. A normal dispersion of chromatin was noted (Fig. 3.6). Regular

distribution of heterochromatin aggregates adjacent to the nuclear envelope and between the nuclear pore is typical (Reubel *et al.*, 1987). Single nucleoli could be seen in some cells and cellular processes were evident (Fig. 3.6). The cytoplasm contained numerous polyribosomes. The controls at all incubation times, retained normal morphology and resembled healthy lymphocytes. Anti-T-2 toxin conjugated label was not found in the control cells and the method controls in the ICC experiments, thus verifying the success of the method.

In the T-2 toxin treated lymphocytes, the more obvious differences included indentation of the nucleus and increases in the number of cellular processes (Fig. 3.7). Workers described the first step towards the expression of T-2 toxicoses as full receptor occupancy (Gyongyossy-Issa and Khachatourians, 1985). The increase in cellular processes could be explained by inferring the need for a greater surface area for lymphocyte-toxin interaction thereby increasing the area of contact i.e. the cellular processes. Just as when the receptor on the lymphocytes become occupied by an antigen and a signal is transmitted from the membrane to the nucleus to elicit a response from the cell, so too might toxin interference with the membrane-nucleus communication be one of the causes of toxicity, by virtue of the binding. The lymphocytes treated for 1 and 3 hours, displayed increased amounts of cellular processes, as did slightly damaged lymphocytes at longer incubation times.

The nuclear indentation is described as abnormal in lymphocytes (Fig. 3.7). This was not viewed as a shrinkage artefact, because the mitochondria within these lymphocytes did not appear to be shrunken. This could implicate T-2 toxin in the folding of the nuclear membrane. The folding might be attributed to changes in the ionic concentration or increases in the area of contact between the nucleus and the cytoplasm. This could represent an increase in nucleocytoplasmic exchanges and an increase in metabolic activity (Ghadially, 1982). Lymphocytes incubated with T-2 toxin for 1 and 3 hours showed very little damage. Damage to the lymphocytes was more evident at the 6, 12 and 24 hour incubation stages. Normal dispersion of chromatin was still retained within the damaged lymphocytes (Fig. 3.7).

Concurrently, the ICC experiments showed anti-T-2 toxin conjugated label within the nuclear membrane (Fig. 3.8). Label was located within the nuclei and the cytoplasm of the

lymphocytes, thus placing T-2 at the site of damage. Label was also found around and within the cellular processes (Figs. 3.9 and 3.10). This was more evident in the lymphocytes treated for 1 and 3 hours. The cellular processes of undamaged and slightly damaged cells were larger and more numerous (Fig. 3.7). The label although in small amounts in these lymphocytes, was associated with the periphery of the cytoplasm (including the cellular processes) and the nucleus. Label was more dispersed in the lymphocytes treated with T-2 toxin for 6, 12 and 24 hours. Label was also located at the boundaries of adjacent (possibly communicating) cells (Fig. 3.11).

The cellular organelles such as mitochondria, ER and vacuoles were rarely seen. However they represented normal sizes in the controls, i.e., there were no signs of swelling (Fig. 3.12). The cytoplasm showed a normal dispersion of polyribosomes (Fig. 3.12). The treated cells contained fewer organelles, and some of these showed signs of swelling. The mitochondria showed the most obvious signs of swelling (Fig. 3.13). The swollen mitochondria also seemed to develop central lighter zones (Fig. 3.13), representing mitochondrial matrix lysis. The swelling of the mitochondria was most evident in the lymphocytes treated with T-2 toxin for 12 and 24 hours, with a few at 1, 3 and 6 hours. Figure 3.13 contains an example of a doughnut shaped mitochondrion. The centre resembles a state of vacuolation or cavulation (Ghadially, 1982). It also represents the most common form of swelling, i.e., the swelling of the inner chamber containing the mitochondrial matrix.

Literature corroborates this finding by also showing a displacement of cristae to the periphery of the cell as well as varying degrees of distortion. It is also stated that frank cavitation of the mitochondrial matrix occurs. The swelling is also termed "cloudy swelling" to describe the intracellular water accumulation in cells subject to toxic stress (Ghadially, 1982). Swollen mitochondria were also shown in lymphocytes treated with another trichothecene mycotoxin, namely deoxynivalenol. The internal architecture of the mitochondria were highly disintegrated, and the cristae were distorted, dilated and reduced in number (Reubel *et al.*, 1987) as shown in figure 3.13. Very few of the mitochondria were indeed damaged or swollen.

Label was isolated within relatively normal looking mitochondria (Fig. 3.14) as well as within doughnut shaped mitochondria with matrical lysis (Fig. 3.15). In some instances, label was associated with the cristae of normal-looking mitochondria (Fig. 3.16) and in other instances was present in the swollen cristae of treated lymphocytes (Fig. 3.17).

Vacuoles of various size and regularity were also present (Figs. 3.18 and 3.19). These were possibly transformed ER that hypertrophy and swell. Under pathologic conditions, the canaliculi of ER may become fragmented into smaller vesicles. Figure 3.18 depicts a gall body surrounded by azurophilic granules, found mainly in pathologic conditions, but also occasionally in normal patients (Ghadially, 1982). However these granules (which correspond in part to lysosomes) were not of relevant abundance to be associated with T-2 toxicosis.

Label was located within the vacuoles (Figure 3.20), normal and swollen ER (Figs. 3.21 and 3.22). Work done by Reubel *et al.* (1987) on porcine lymphocytes, show that deoxynivalenol induces endoplasmic reticulum fragmentation, dilatation and vesiculation of the cisternae. However Reubel *et al.* (1987) also reported a decrease in the cytoplasmic polyribosomes. This type of degradation did not occur in the lymphocytes treated with T-2 toxin as may be observed in any of the micrographs depicting treated cells, including many of the cells undergoing apoptosis. Label was also located within the cytoplasm (Fig. 3.22). The label was predictably more abundantly associated with the organelles after treatment with T-2 toxin for 6, 12 and 24 hours. Label was associated with secondary lysosomes (Fig. 3.23). The secondary lysosomes may result from degeneration of the cytoplasm (Bessis, 1973).

It was evident from TEM analysis, that T-2 toxin induced cell death. The most prominent sign of cell death was apoptosis, although a few necrotic cells were noted. The criteria used to discern the presence of necrotic cells were, extreme vacuolation, swelling, and the presence of secondary lysosomes (Figs. 3.24 and 3.25). Signs of necrosis were evident in lymphocytes treated with T-2 toxin for 6, 12 and 24 hours. However necrosis did not appear to be the predominant cell death mechanism. Label was located within the lymphocytes that were undergoing necrosis, more especially in the lysosomal compartments (Fig. 3.26) and vacuoles (Fig. 3.27).

Treatment of lymphocytes with 1,56 μg/ml T-2 toxin for 1 and 3 hours did not induce much cell death. However, among the very few dead cells, most of these were apoptotic. This is expected due to the low levels of cytotoxicity reported in the cytotoxicity assay (Figs. 3.1 and 3.2). However there was an observable increase in the number of apoptotic cells in the samples treated with T-2 toxin for 6 hours. Most of the cells in the 12 hour study seemed to have undergone apoptosis. This was also true for the 24 hour samples, however most of these cells were already dead or disintegrating (Fig. 3.28). The lymphocytes that were alive were abundantly labelled.

The gold standards for apoptotic cell morphology includes the fragmentation of the nucleus (karyorrhexis). In this situation the nucleus divides into two or more separate bodies within a single cell (Figs. 3.29 and 3.30). This is a clear indication of DNA damage. The chromatin is compact, dark and even (Fig. 3.29). In some of the lymphocytes, the nucleus seemed about to undergo karyorrhexis (Fig. 3.29) because the nuclear membrane can still just be discerned. In more advanced stages of karyorrhexis (Fig. 3.30) the nuclei are separated into distinct packages. The lack of cellular processes within the treated lymphocytes was also apparent. The presence of polyribosomes was also evident within apoptotic lymphocytes.

Chromatin condensation manifests as the crescent shaped dispersion of chromatin within the nuclei (Figs. 3.30 and 3.31). It appears that the condensation of the chromatin may occur in stages, due to the different patterns that are observed (Figs. 3.32 and 3.33). Chromatin condensation occurs when the dark chromatin forms one phase and the nuclear sap forms another. This may also be described as chromatin clumping and margination (Reubel *et al.*, 1987). The higher order chromatin structure which includes the DNA and histone association is supported by a nuclear matrix-intermediate filament scaffold. The protein complex of the nuclear matrix determines the basic morphological pattern of the nucleus. Disruption of this nuclear scaffold, alters the appearance of the chromatin (Payne *et al.*, 1995). Label was found at all incubation periods within the nucleus and often in association with the nucleolus in cells that were not undergoing apoptosis (Fig. 3.34). Label was also located within the condensed chromatin and nuclear sap of apoptotic cells (Fig. 3.35). In all cells

undergoing apoptosis, the cytoplasm still contains a good dispersion of polyribosomes (Figs. 3.30, 3.31, 3.32 and 3.33).

Damage to the nucleus of the cells resulted in disintegrating nucleoli, which were noted in apoptotic lymphocytes (Figs. 3.32 and 3.36). Label was located within the disintegrating nucleolus of the apoptotic cells (Fig. 3.37). Cytoplasmic blebbing was common in the lymphocytes treated for 6, 12 and 24 hours (Fig. 3.38). Label was found within the cytoplasmic blebs attached to the apoptotic cells (Fig. 3.39).

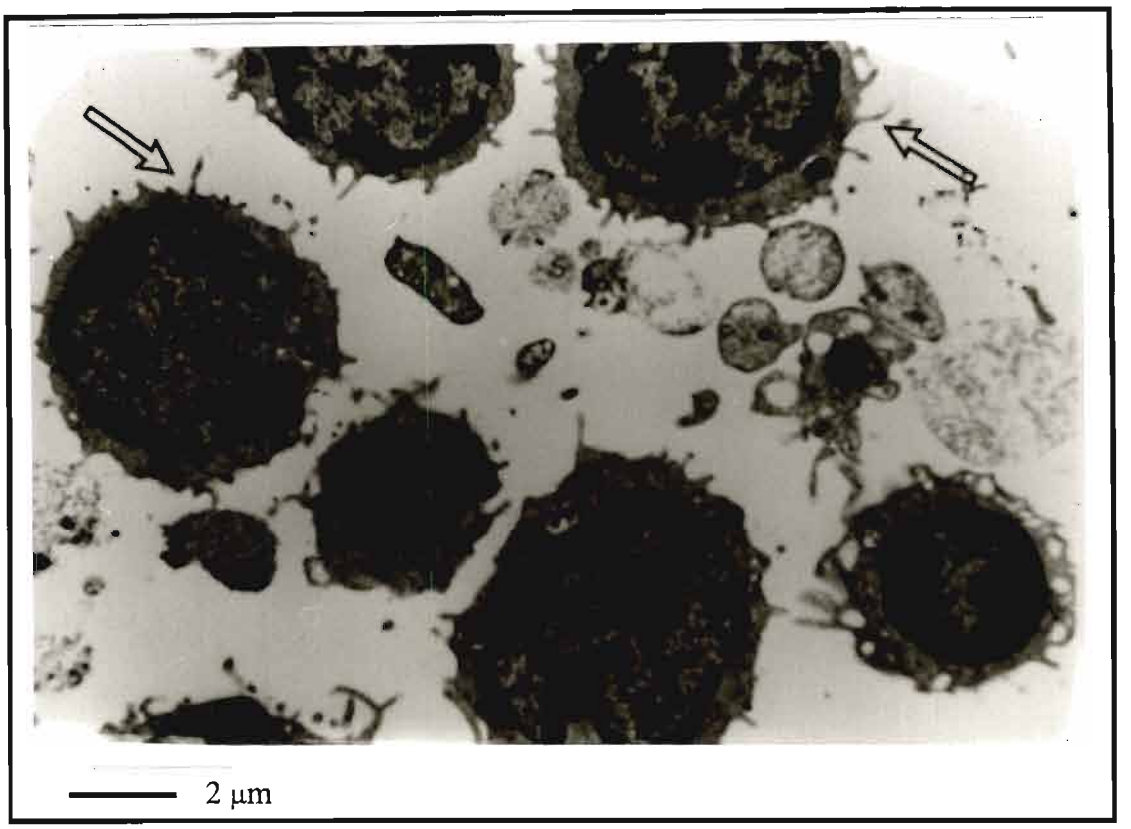

Figure 3.6 Control lymphocytes (3 hours). There is a paucity of organelles within the control lymphocytes. The structures are consistent, i.e., large rounded cells with a thin ring of cytoplasm (c) and large single nuclei (n). Nucleoli are visible in some (filled arrow). Cellular processes (clear arrows) are also visible. There is a normal dispersion of polyribosomes within the cytoplasm (c).

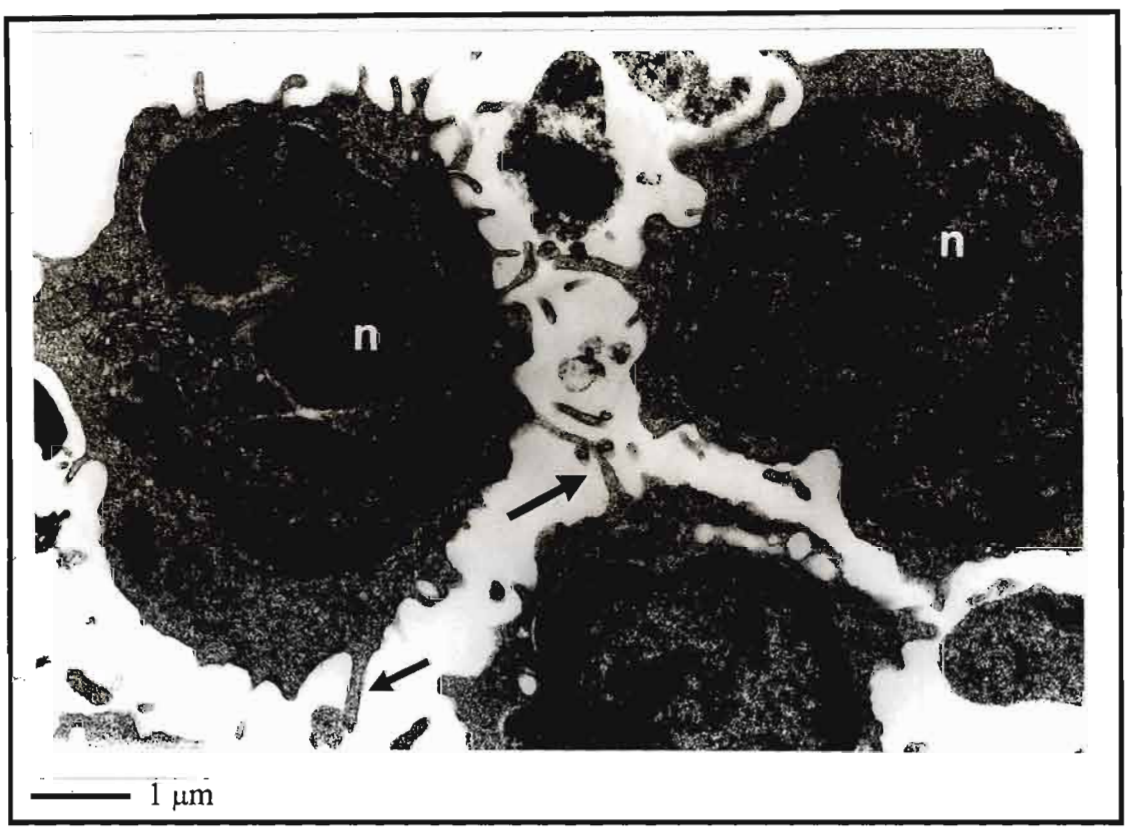

Figure 3.7 T-2 toxin treated lymphocyte (12 hours). Many of the treated cells show extensive damage to the nuclei (n) by way of indentations. The cellular processes (arrowed) seem larger and more numerous.

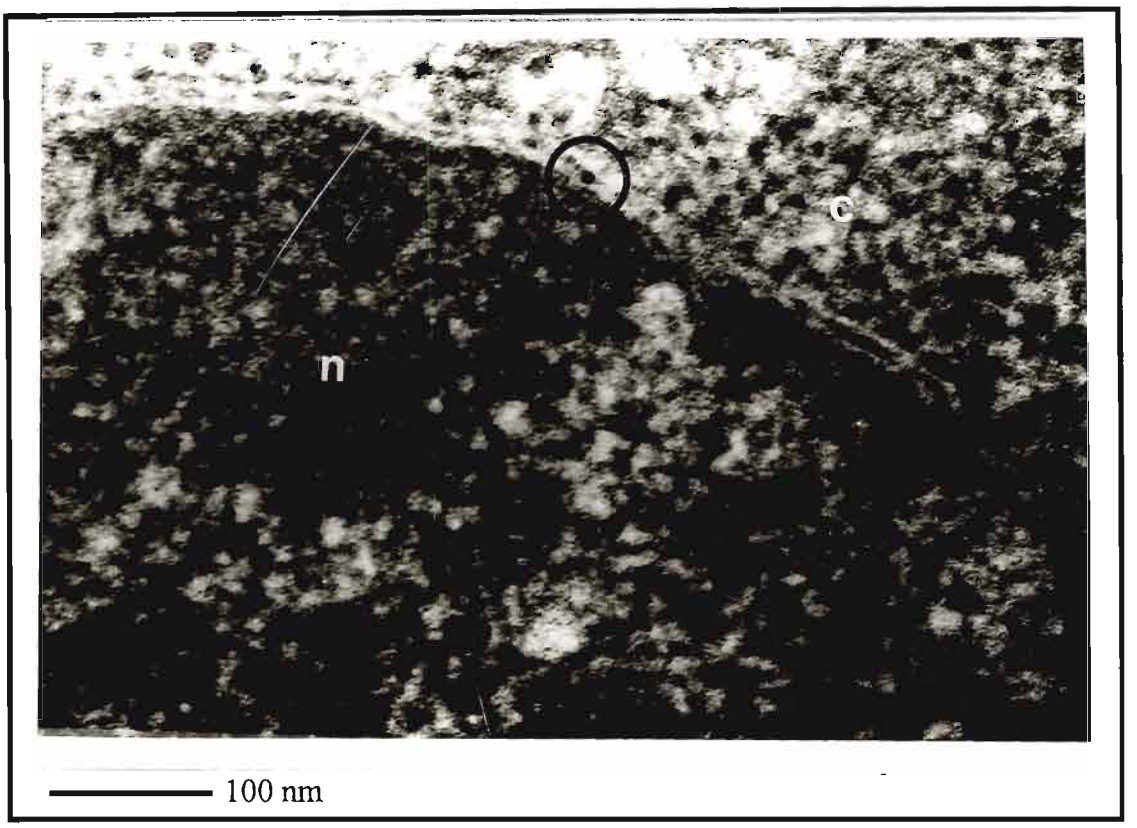

Figure 3.8 T-2 toxin treated lymphocytes (12 hours). Anti-T-2 toxin conjugated label was located within the nuclear membrane (circled). (n) Corresponds to the nucleus and (c) to the cytoplasm of the lymphocytes.

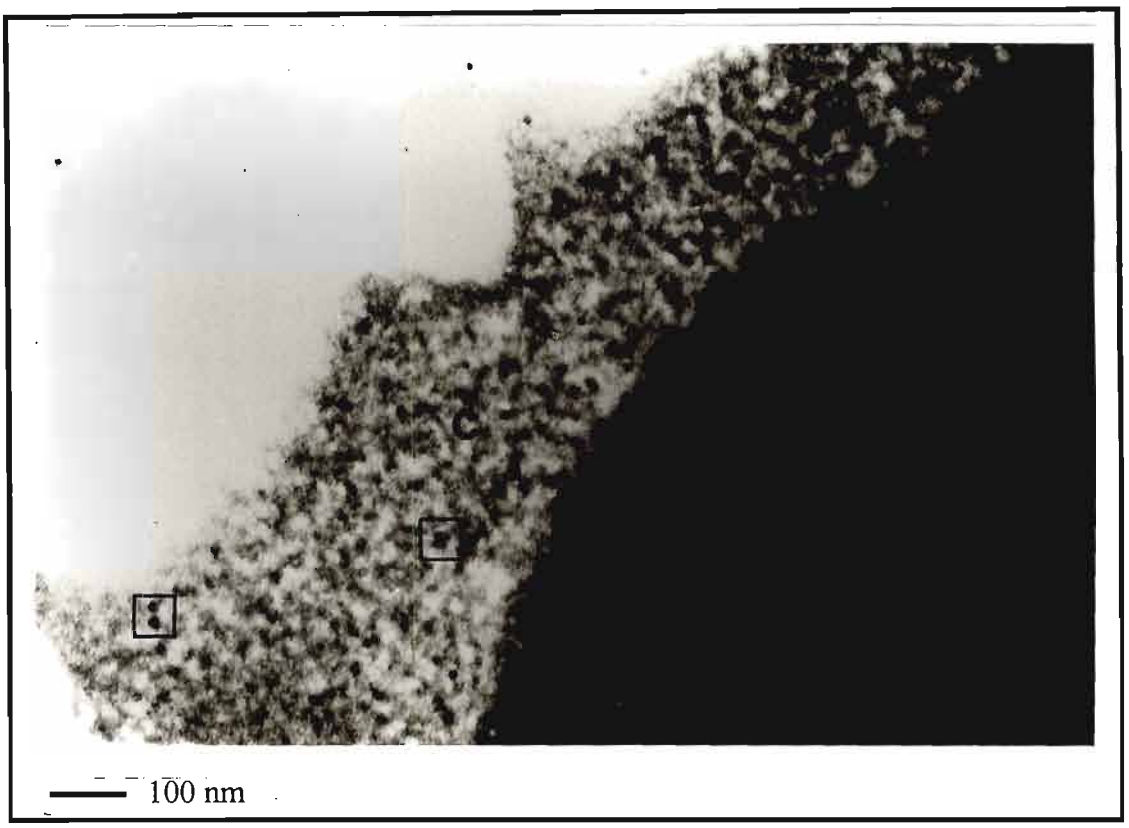

Figure 3.9 T-2 toxin treated lymphocytes (12 hours). Label (□) was associated with the cellular processes within the treated cells as well as within the cytoplasm (c).

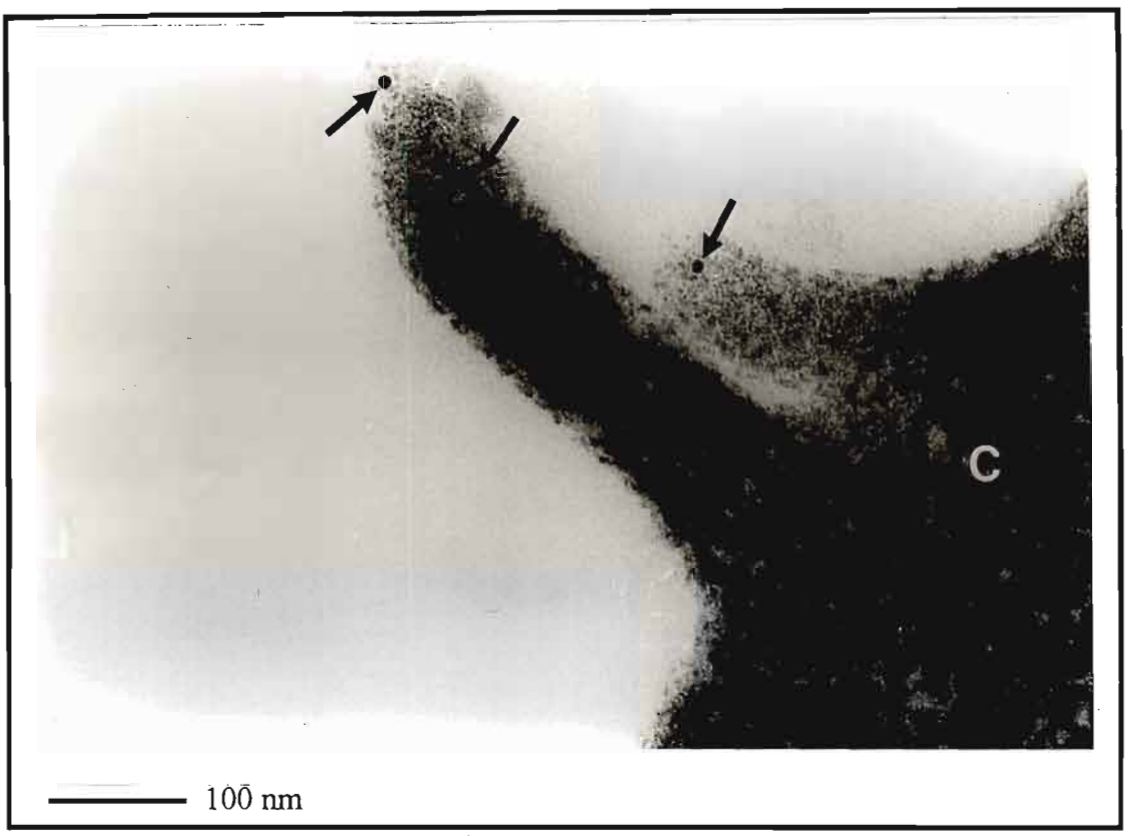

Figure 3.10 T-2 toxin treated lymphocyte (6 hours), clearly shows label (arrowed) within the cellular processes. The cytoplasm is represented by (c).

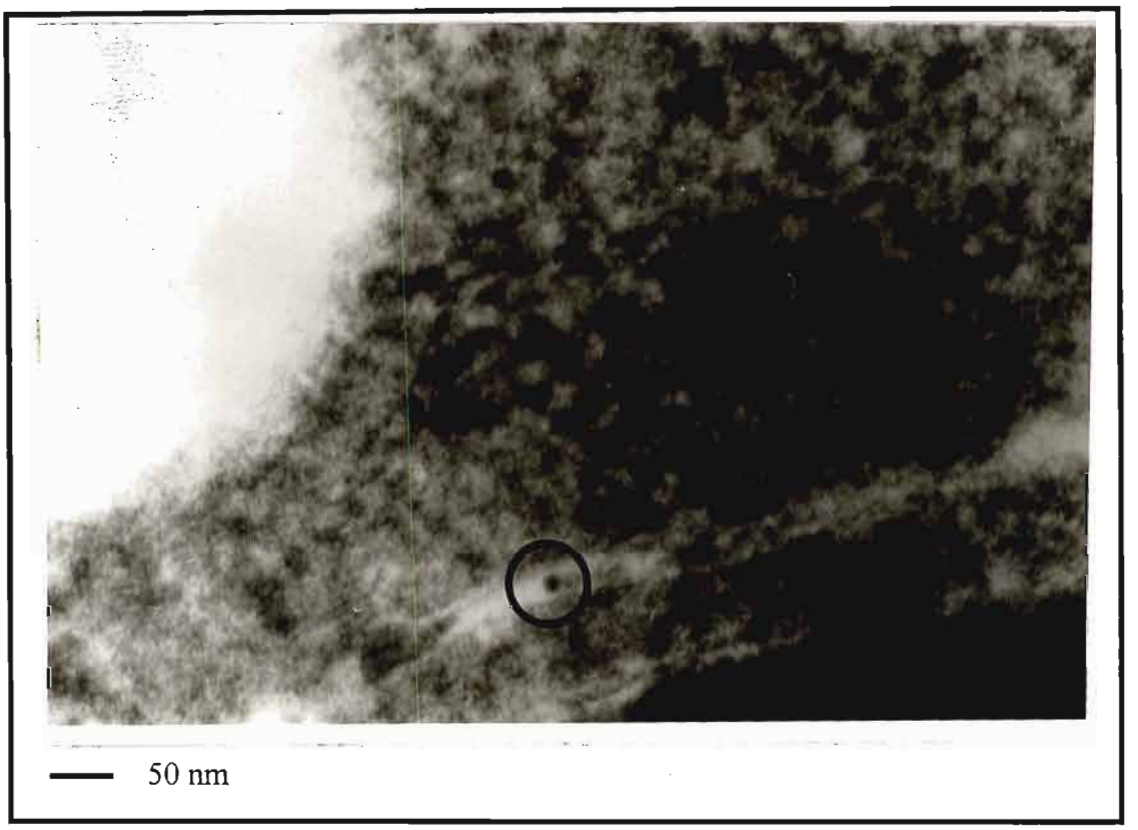

Figure 3.11 T-2 toxin treated lymphocytes (1 hour). Label (circled) at the boundary of two communicating cells. This was not viewed as a shrinkage artefact because the size of the label corresponds to 10 nm. Furthermore, other label of the same size were located within the cells depicted in this micrograph.

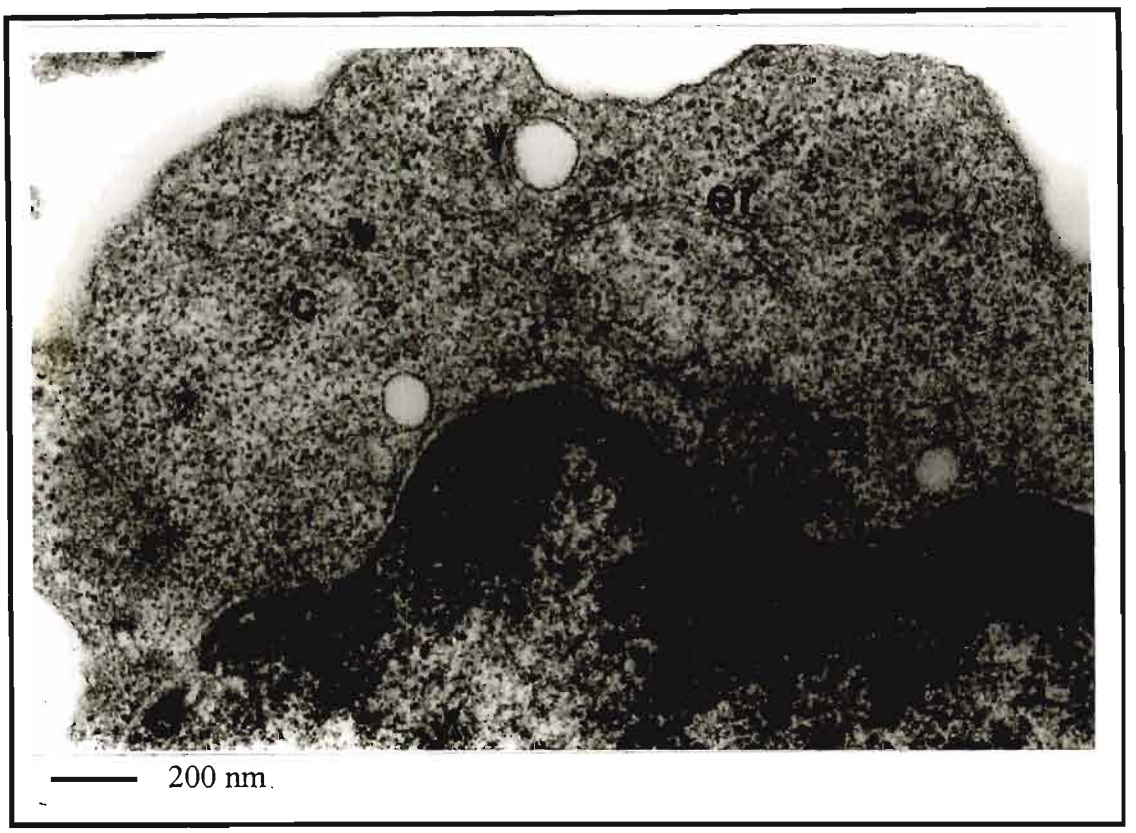

Figure 3.12 Control lymphocyte (12 hours). Organelles are rarely visible in lymphocytes. The controls contain seemingly healthy mitochondria (m), endoplasmic reticulum (er) and vacuoles (v). There is a normal dispersion of polyribosomes within the cytoplasm (c).

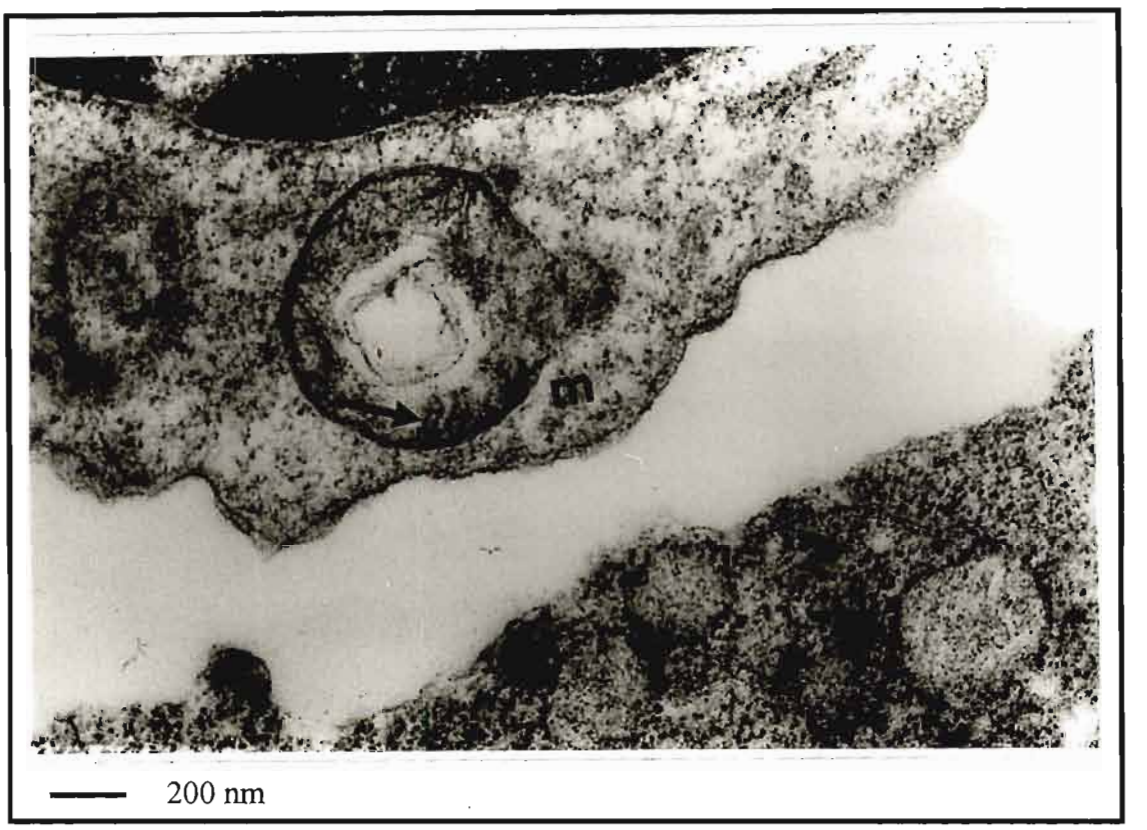

Figure 3.13 T-2 toxin treated lymphocyte (12 hours). This is a representation of a swollen mitochondrion (m) which resembles a doughnut shape. There is an apparent clearing (lysis) in the centre of the mitochondrion. Displacement of the cristae towards the periphery of the cell (arrowed) is also noticeable.

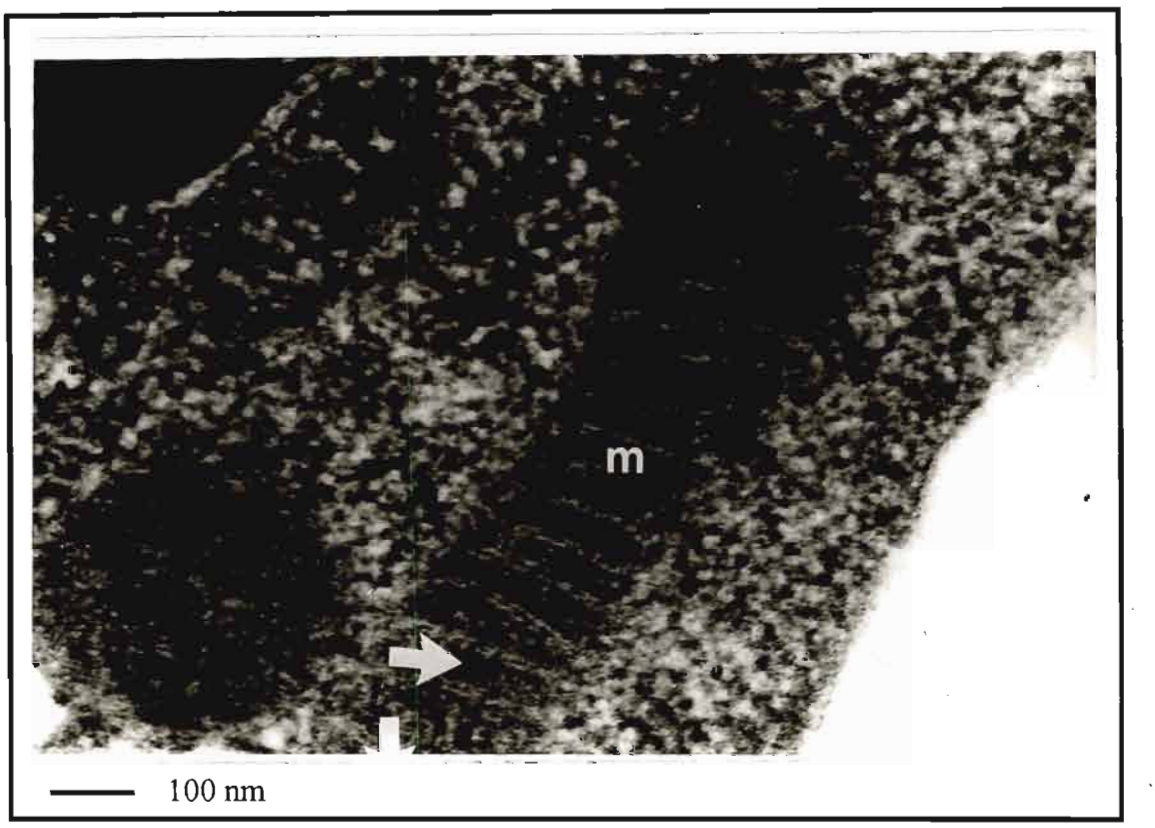

Figure 3.14 T-2 toxin treated lymphocyte (12 hours). Label (arrowed) was associated with normal looking mitochondria (m), with parallel arrays of cristae.

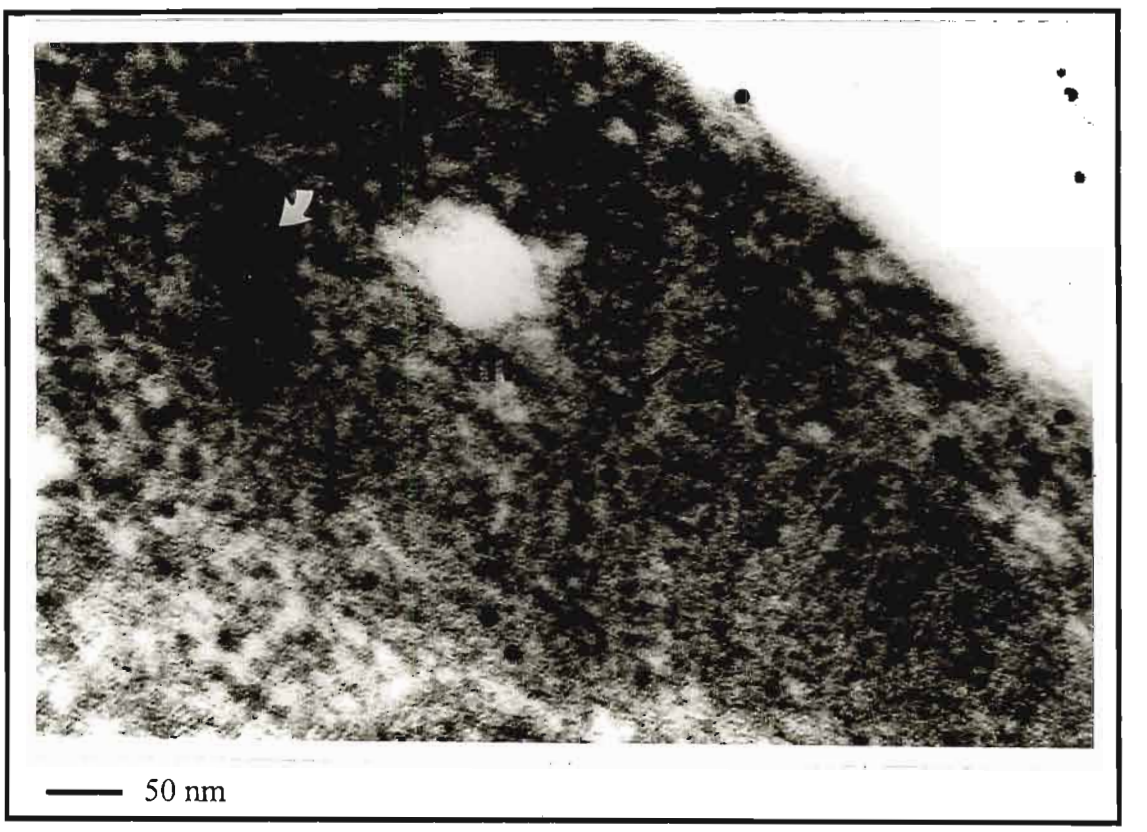

Figure 3.15 T-2 toxin treated lymphocyte (12 hours). Label (arrowed) was also located within the doughnut shaped mitochondria (m).

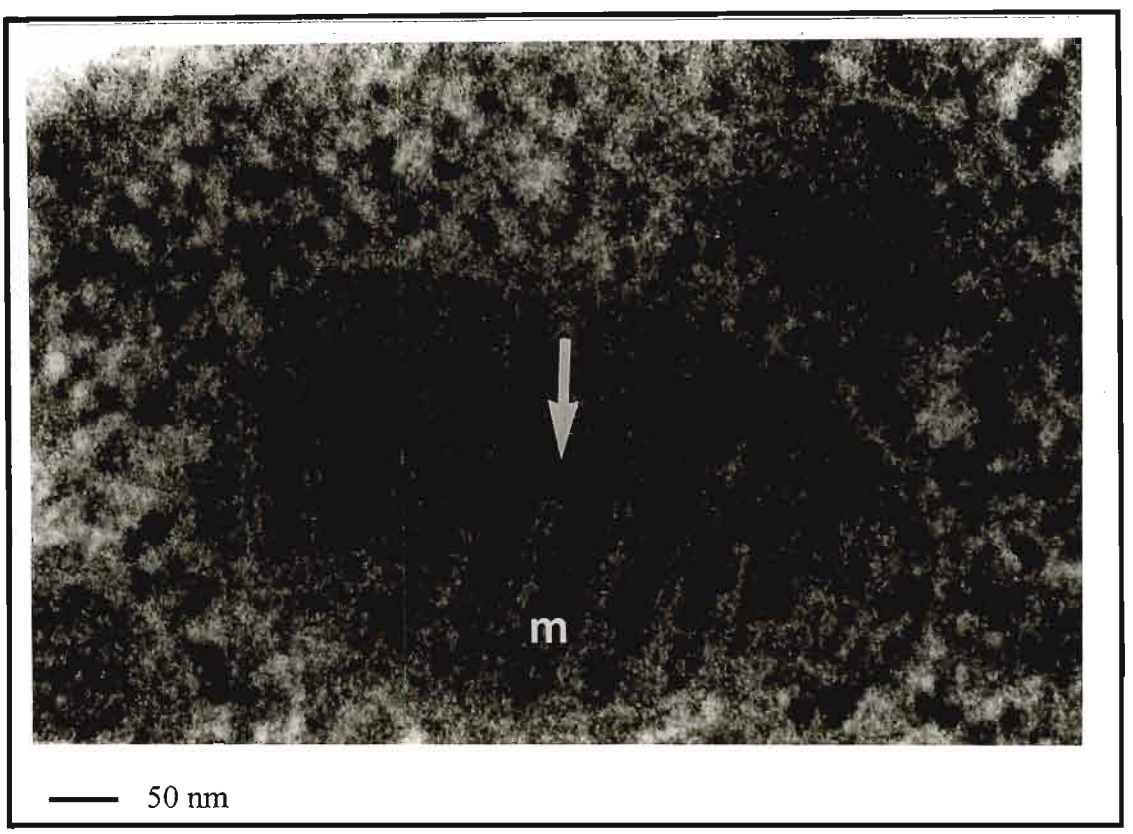

Figure 3.16 T-2 toxin treated lymphocyte (6 hours). Label (arrowed) was associated with undamaged mitochondria (m). The mitochondria contain parallel arrays of cristae which do not appear to be swollen.

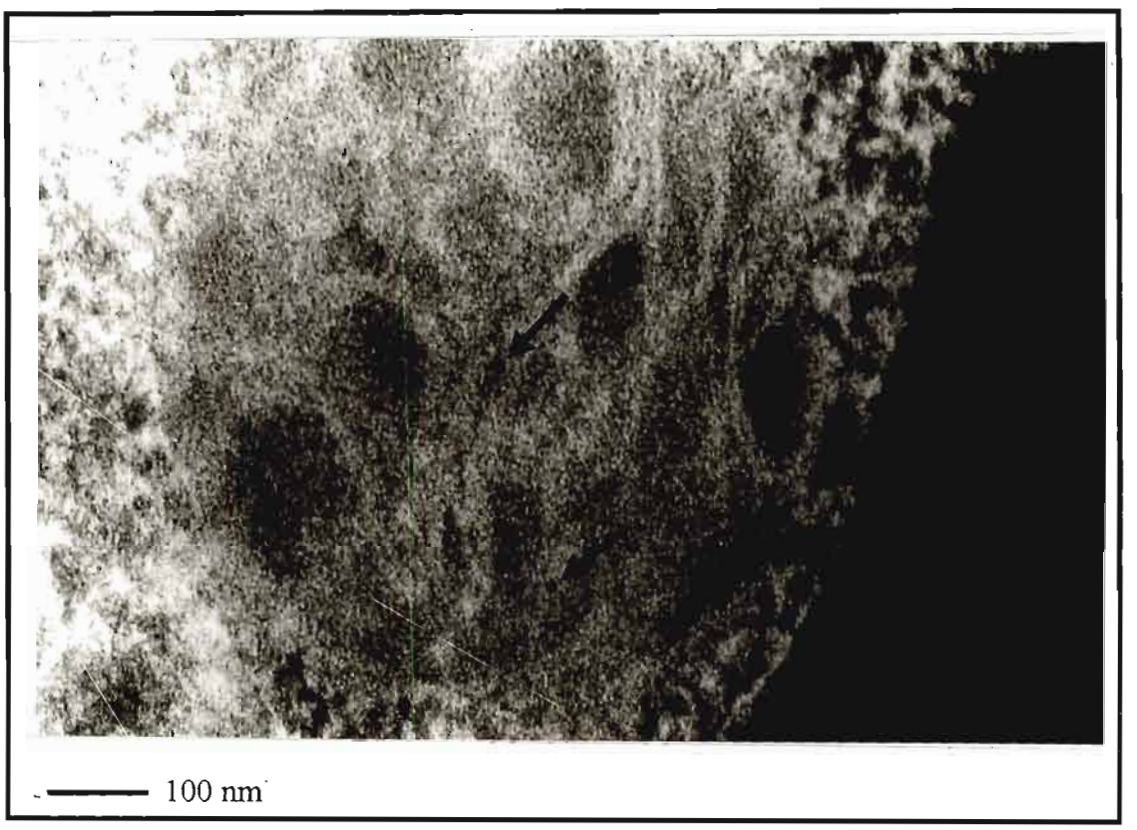

Figure 3.17 T-2 toxin treated lymphocyte (6 hours). Label (arrowed) within swollen mitochondria with swollen cristae.

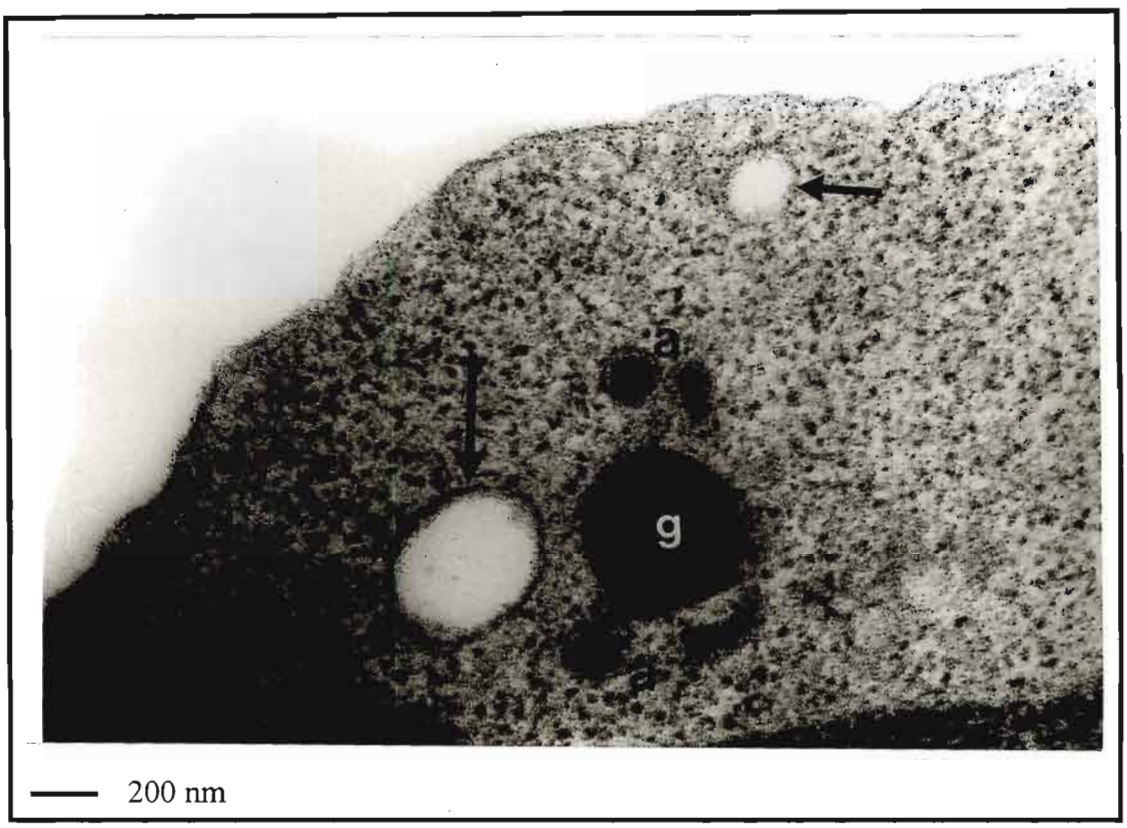

Figure 3.18 T-2 toxin treated lymphocyte (12 hours). Vacuoles or possibly fragmented and swollen ER were present in the treated cells (arrowed). A gall body (g) surrounded by azurophilic granules (a) in some instances refer to a pathologic condition.

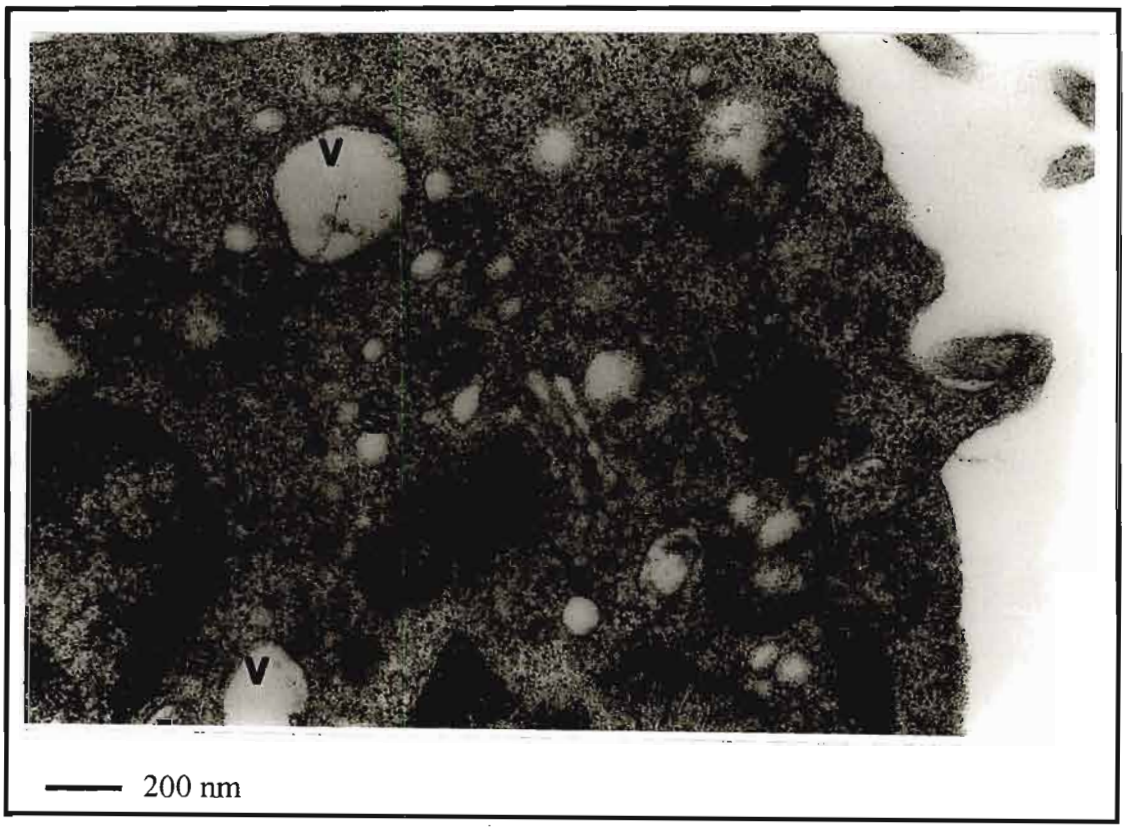

Figure 3.19 T-2 toxin treated lymphocyte (12 hours). Vacuoles (v) and smaller vesicles possibly swollen ER (s) were in abundance.

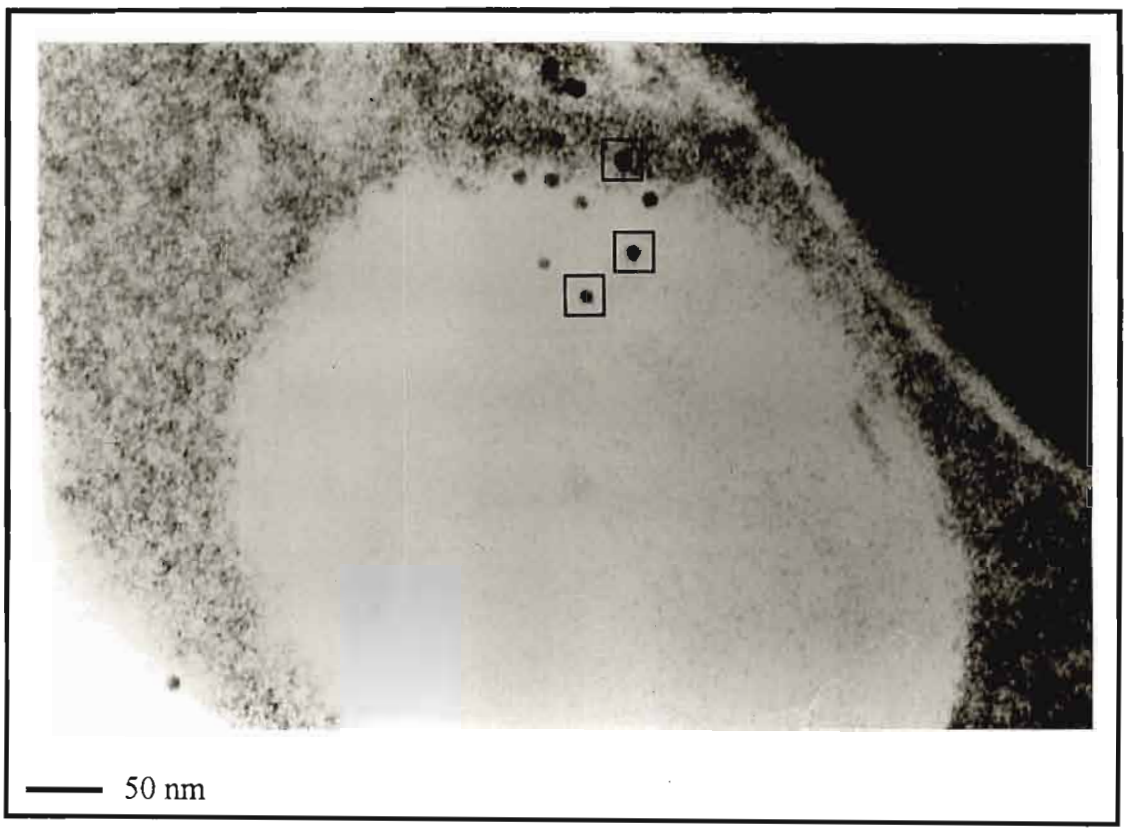

Figure 3.20 T-2 toxin treated lymphocyte (3 hours). Some of the gold label (□) within and around the vacuole of a treated cell.

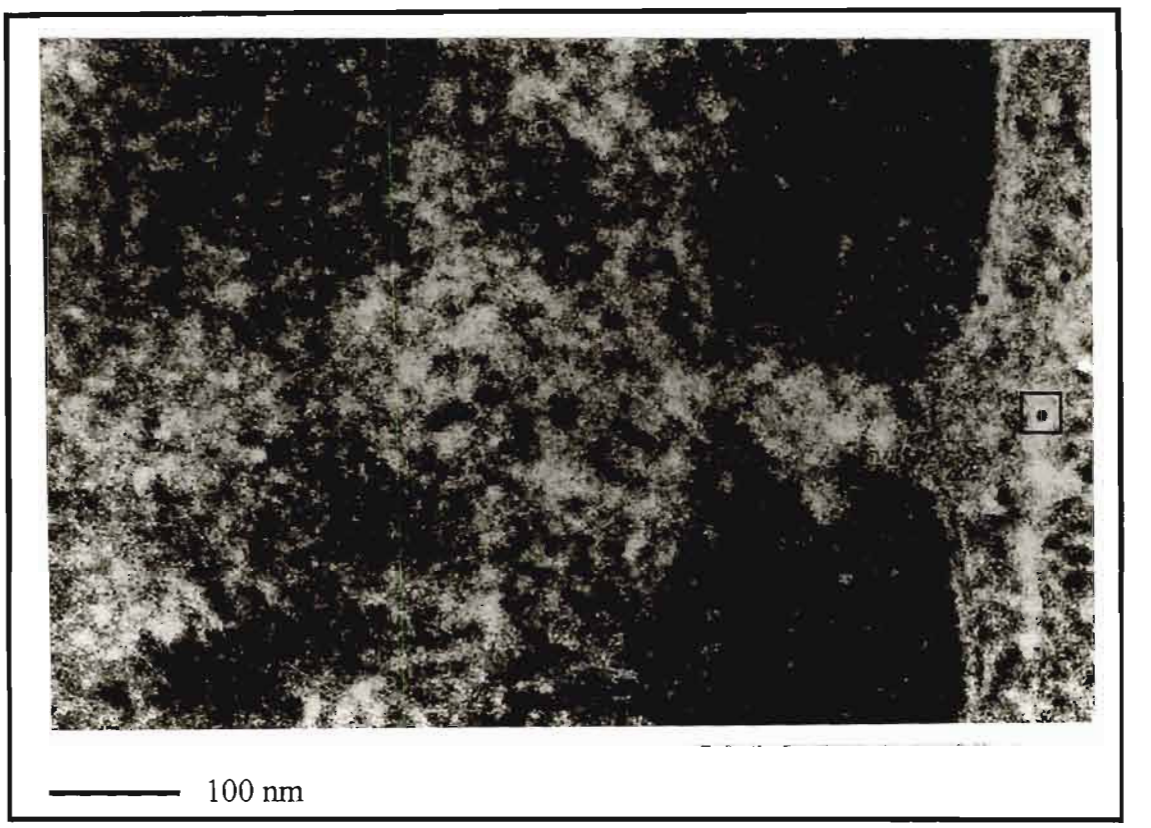

Figure 3.21 T-2 toxin treated lymphocyte (6 hours). Label (□) associated with the normal ER.

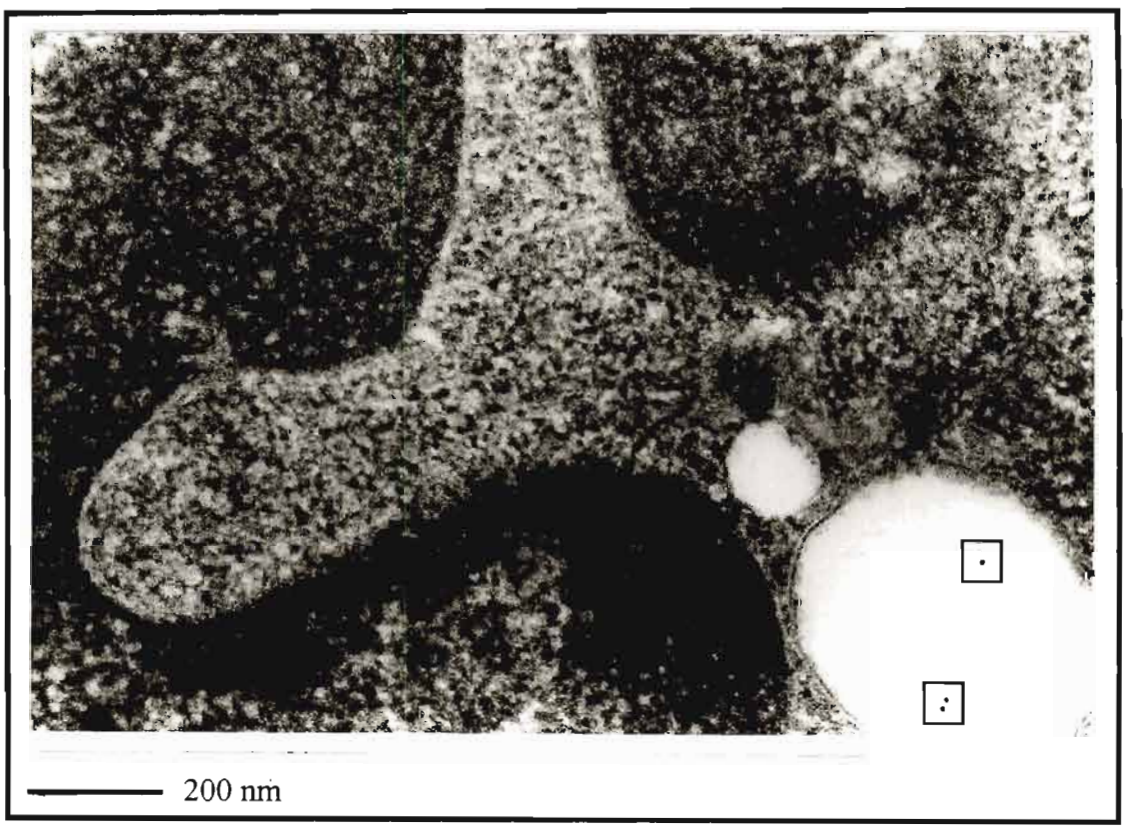

Figure 3.22 T-2 toxin treated lymphocyte (12 hours). Label associated with swollen ER (□), possibly transformed into vacuoles.

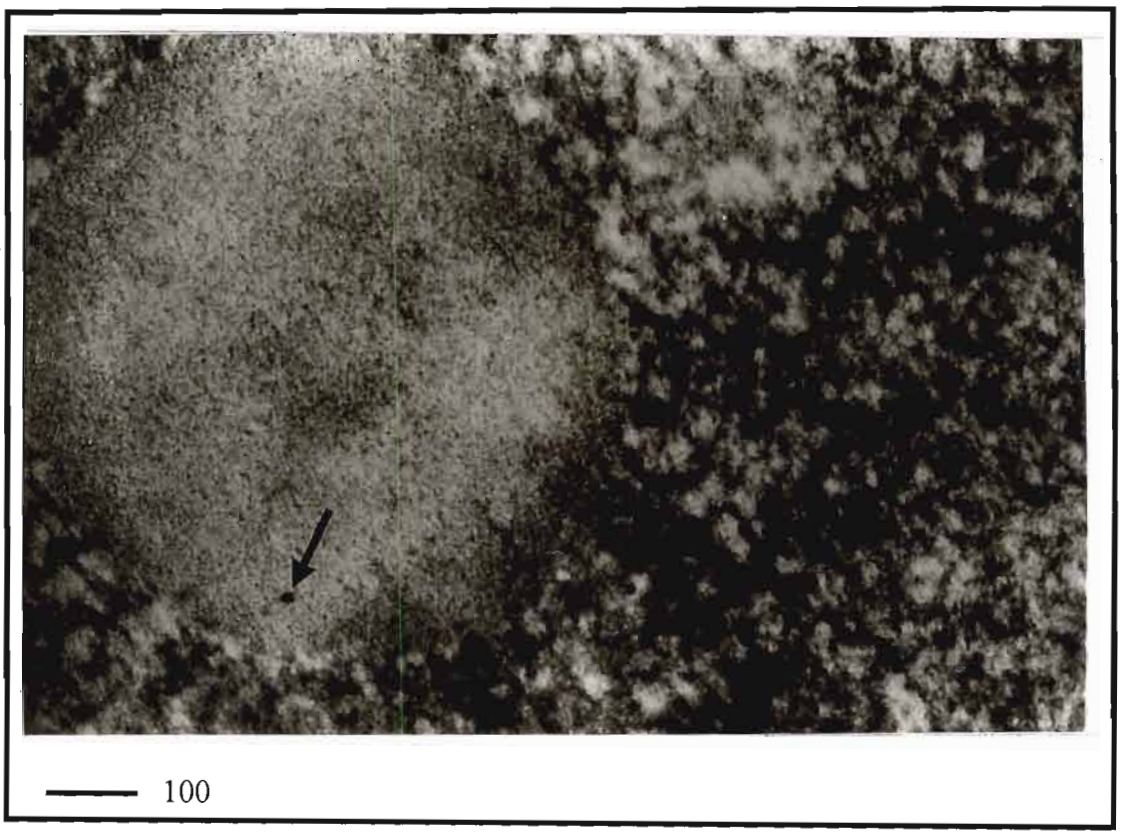

Figure 3.23 T-2 toxin treated lymphocyte (12 hours). Label (arrowed) in association with secondary lysosome.

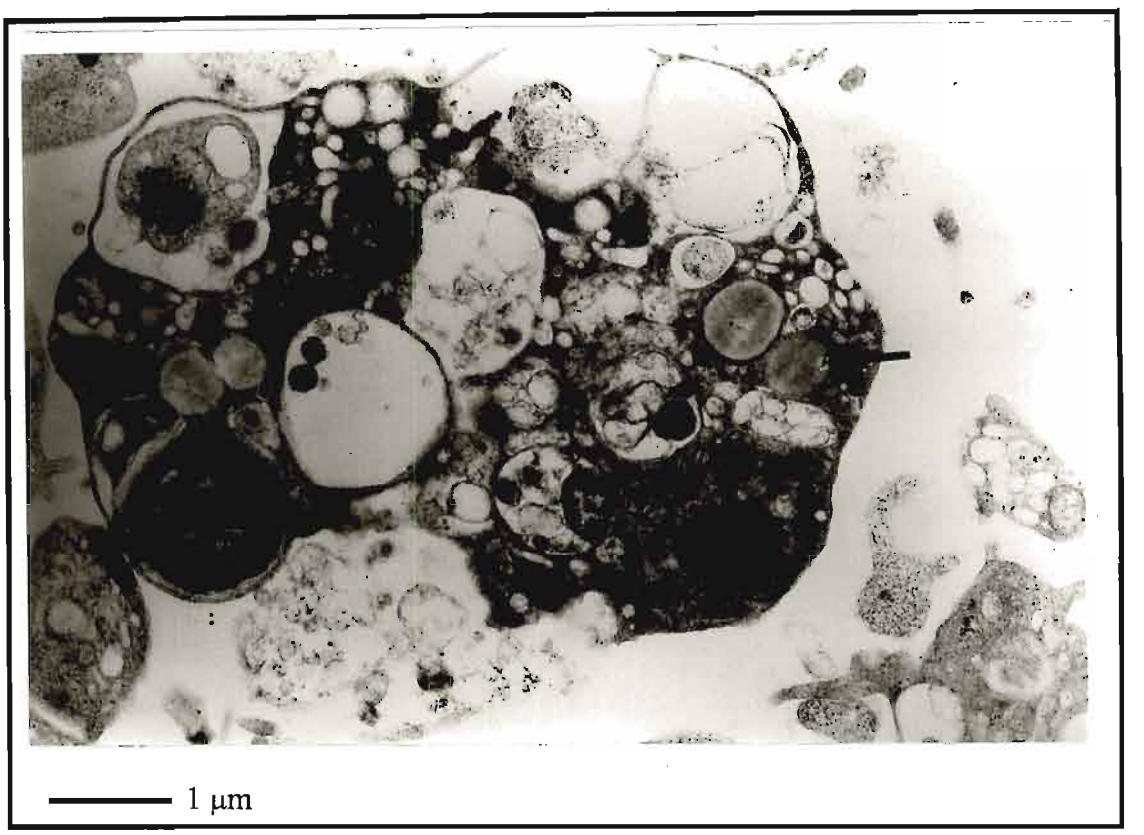

Figure 3.24 T-2 toxin treated lymphocyte (12 hours). A fully necrotic cell with just discernible signs of lysosomes (arrowed) but mainly extensive vacuolation and autolysis of cellular components.

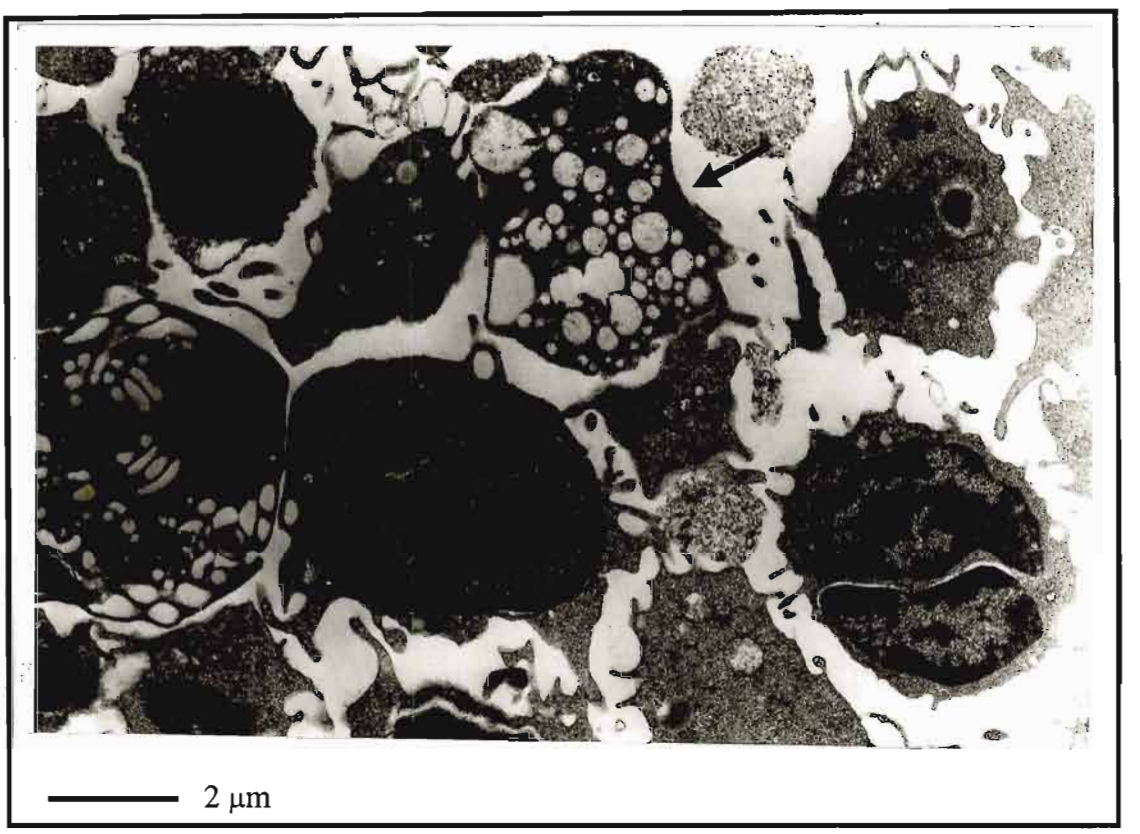

Figure 3.25 T-2 toxin treated lymphocytes (12 hours). Damaged lymphocytes undergoing necrosis (arrowed). Swelling of organelles was largely in evidence.

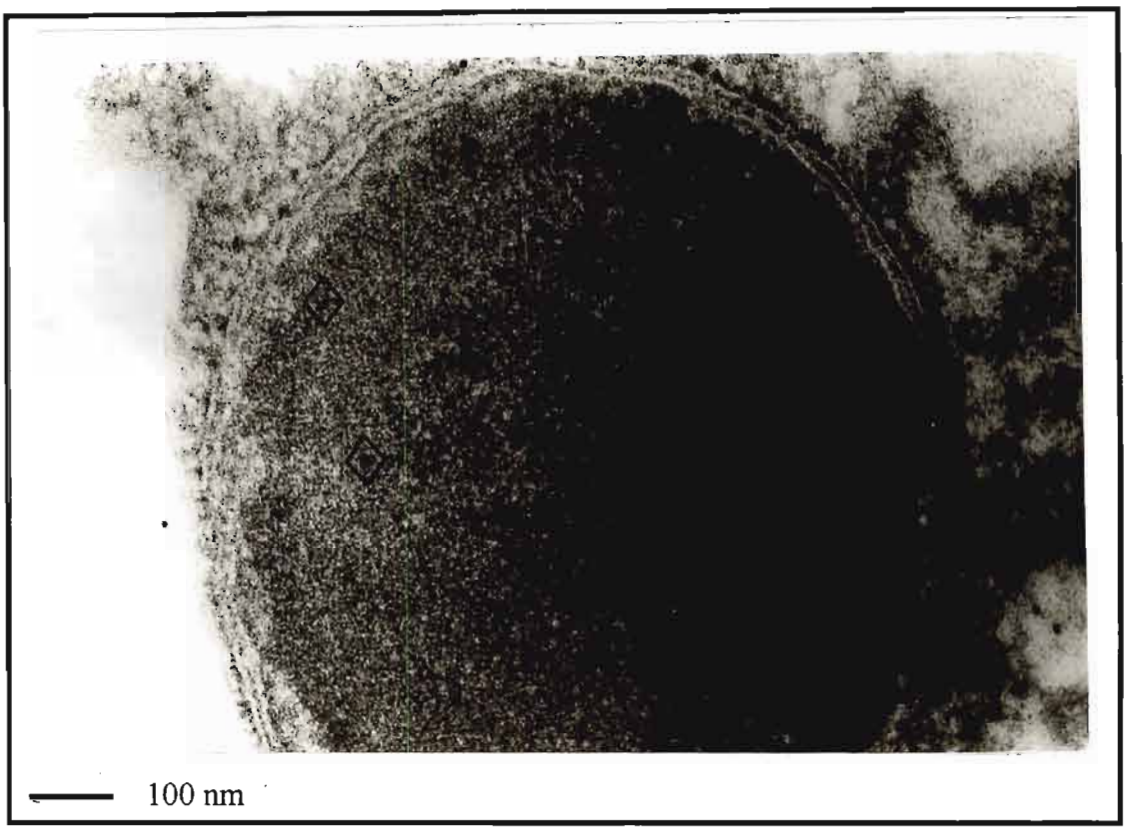

Figure 3.26 T-2 toxin treated lymphocyte (24 hours). Label (diamonds) within a possible lysosomal compartment within a necrotic lymphocyte.

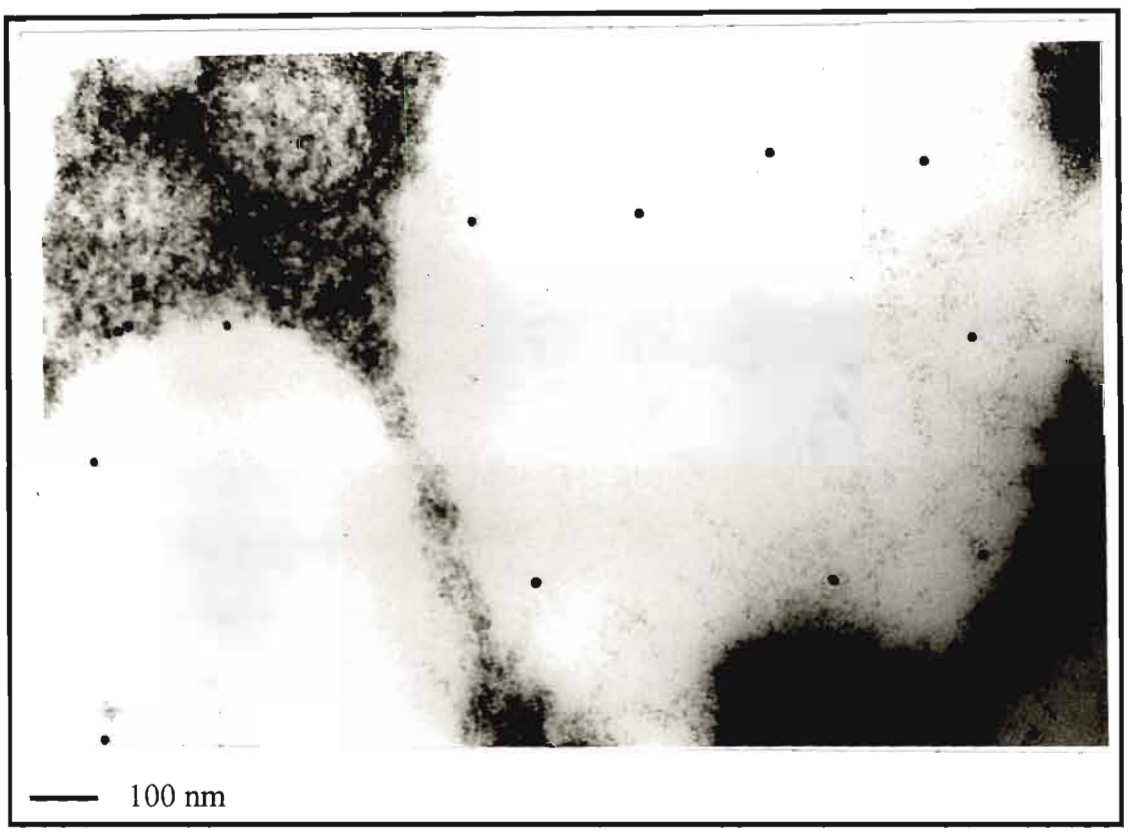

Figure 3.27 T-2 toxin treated lymphocyte (24 hours). The black dots are anti-T-2 toxin antibody conjugated to gold label. They are depicted within the vacuoles of a necrotic lymphocyte.

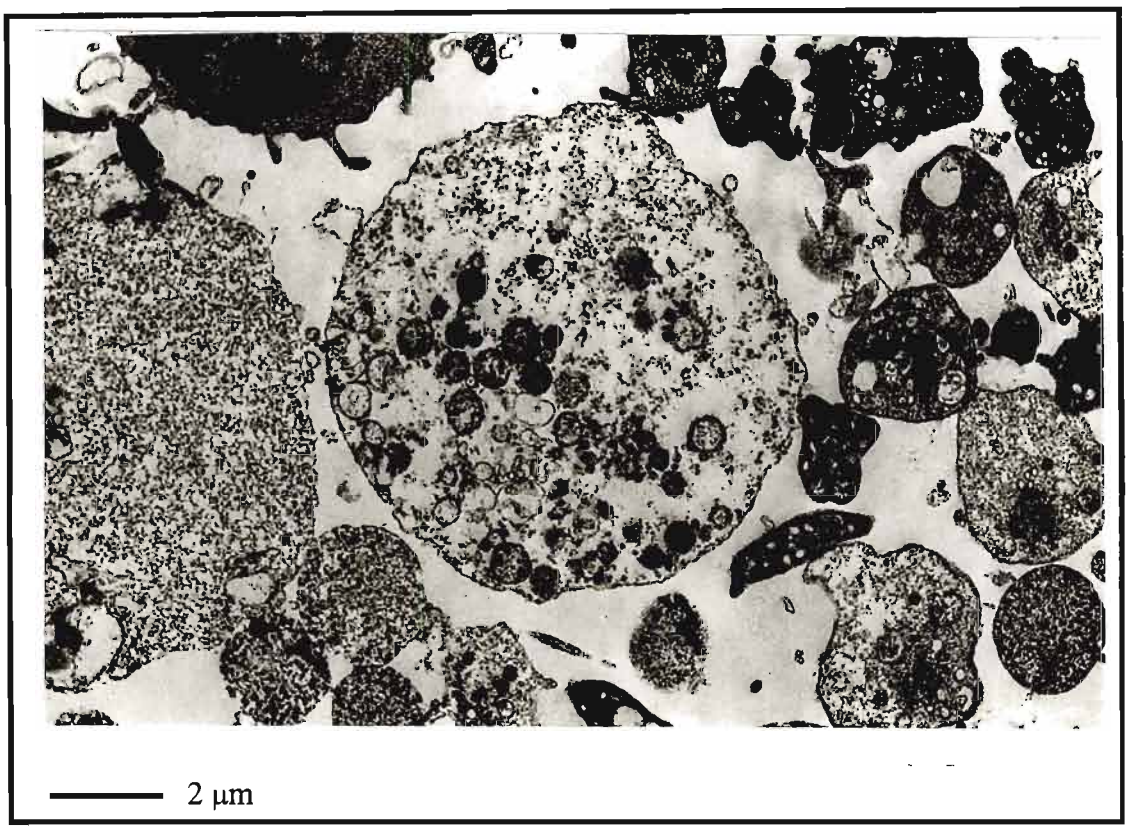

Figure 3.28 T-2 toxin treated lymphocyte (24 hours). The state of most of the cells after 24 hours of treatment with T-2 toxin shows most of the cells in a state of death and disintegration. The cells are surrounded by debris mainly consisting of blebs.

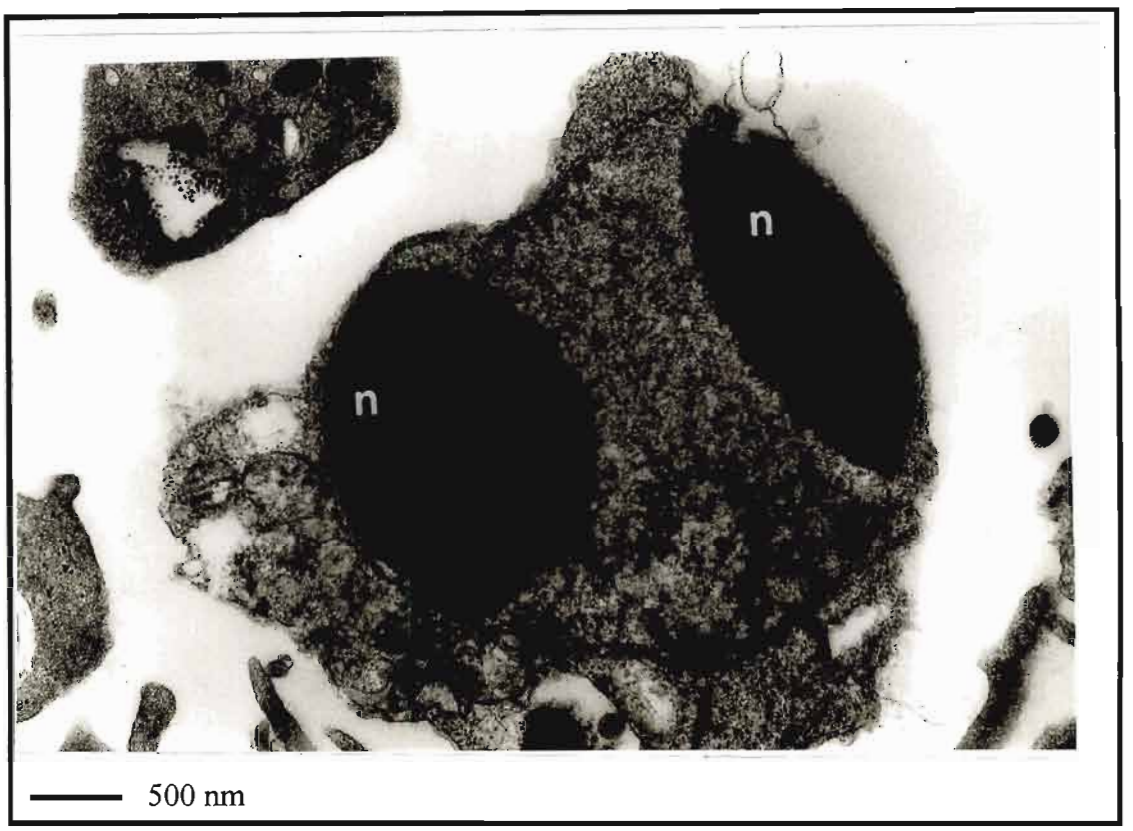

Figure 3.29 T-2 toxin treated lymphocyte (12 hours). Just as fragmentation of the nucleus (n) or karyorrhexis is one of the first signs of apoptosis, so too is the disappearance of cellular processes (microvilli).

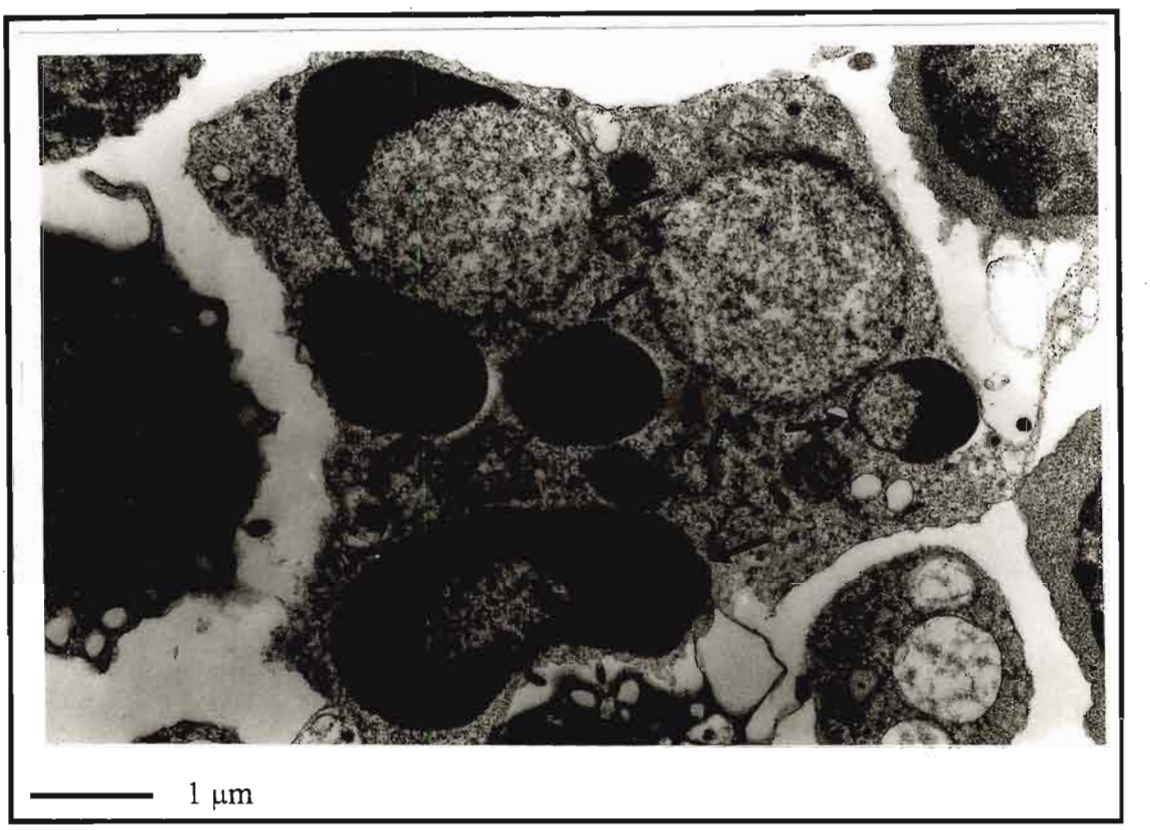

Figure 3.30 T-2 toxin treated lymphocyte (12 hours). The nucleus appears to be separated into 6 fragments in this lymphocyte. Note also the absence of cellular processes, as well as signs of chromatin condensation within the fragmented nucleus (arrowed). A regular dispersion of polyribosomes can still be discerned.

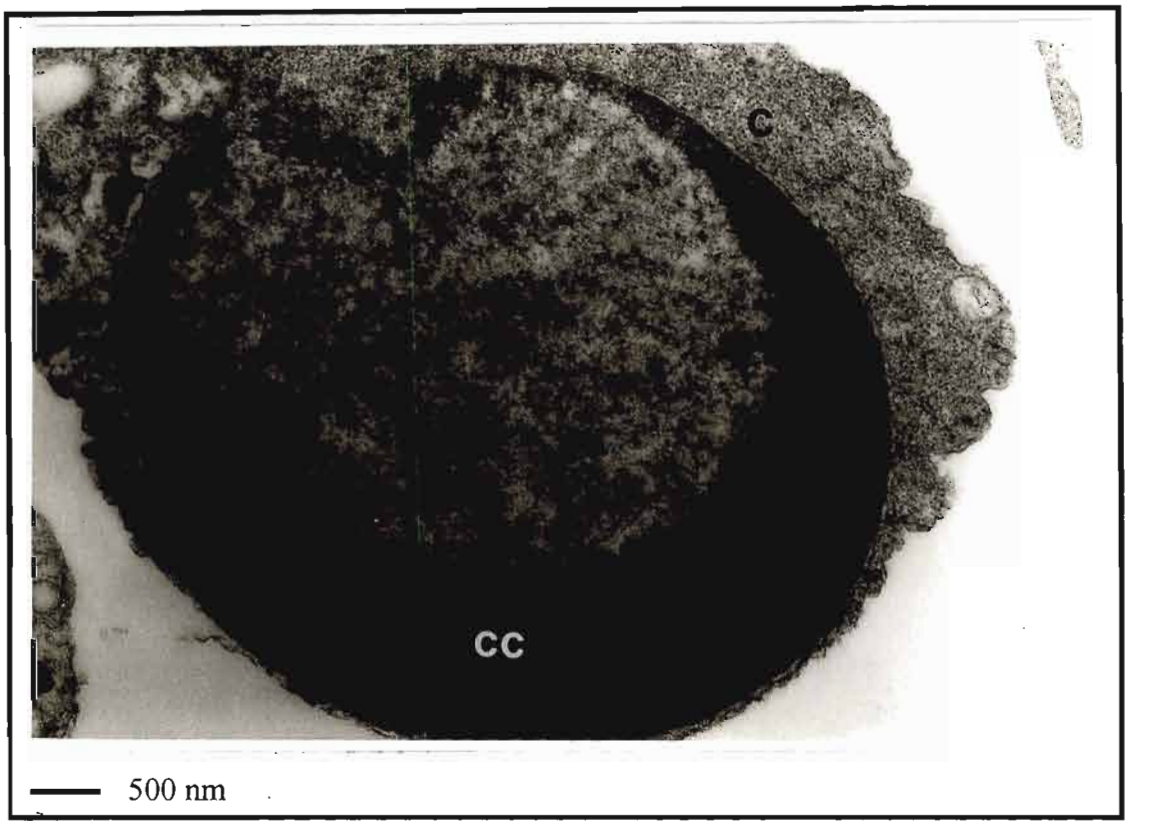

Figure 3.31 T-2 toxin treated lymphocyte (12 hours). Chromatin condensation (cc) within the nucleus is another manifestation of apoptosis. The chromatin forms a distinct crescent shape. In this case it seem as though the nuclear membrane is disintegrating (arrowed). Polyribosomes can still be distinguished within the cytoplasm (c).

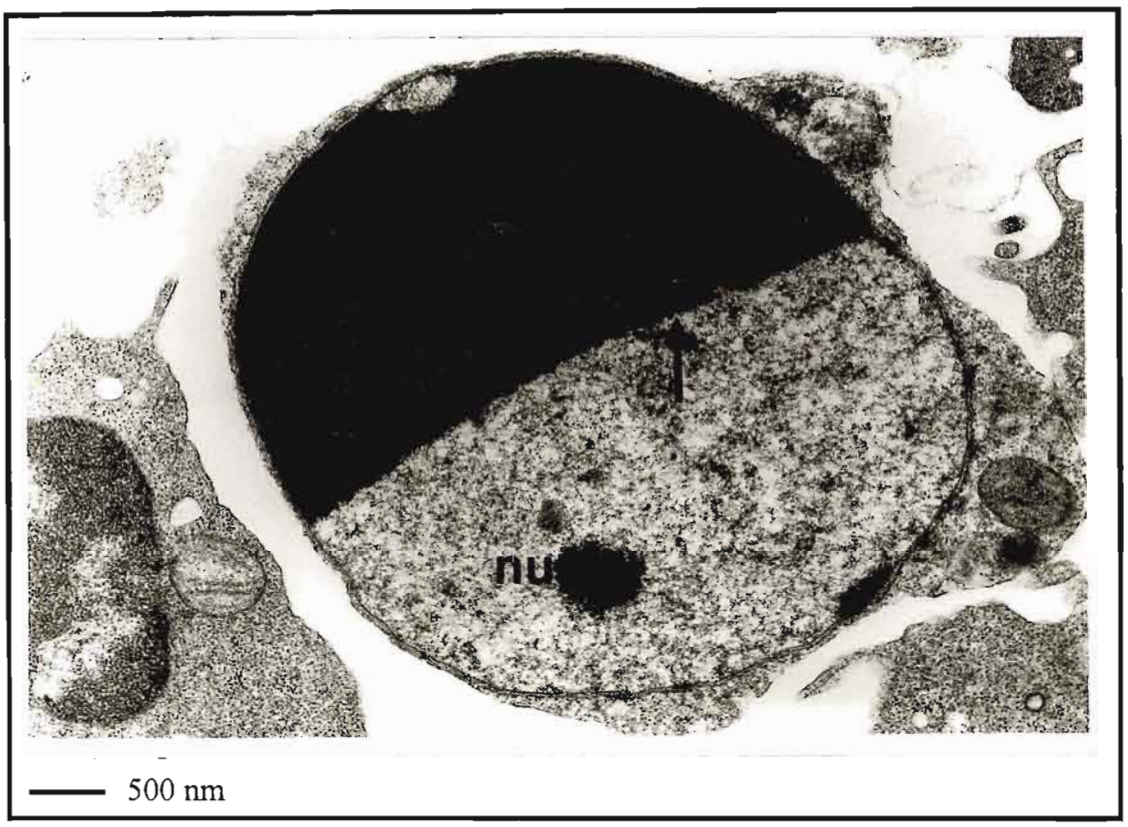

Figure 3.32 T-2 toxin treated lymphocyte (12 hours). A different stage of chromatin condensation (arrowed). A disintegrating nucleolus (nu) is also visible. There is a lack of cellular processes around the lymphocyte.

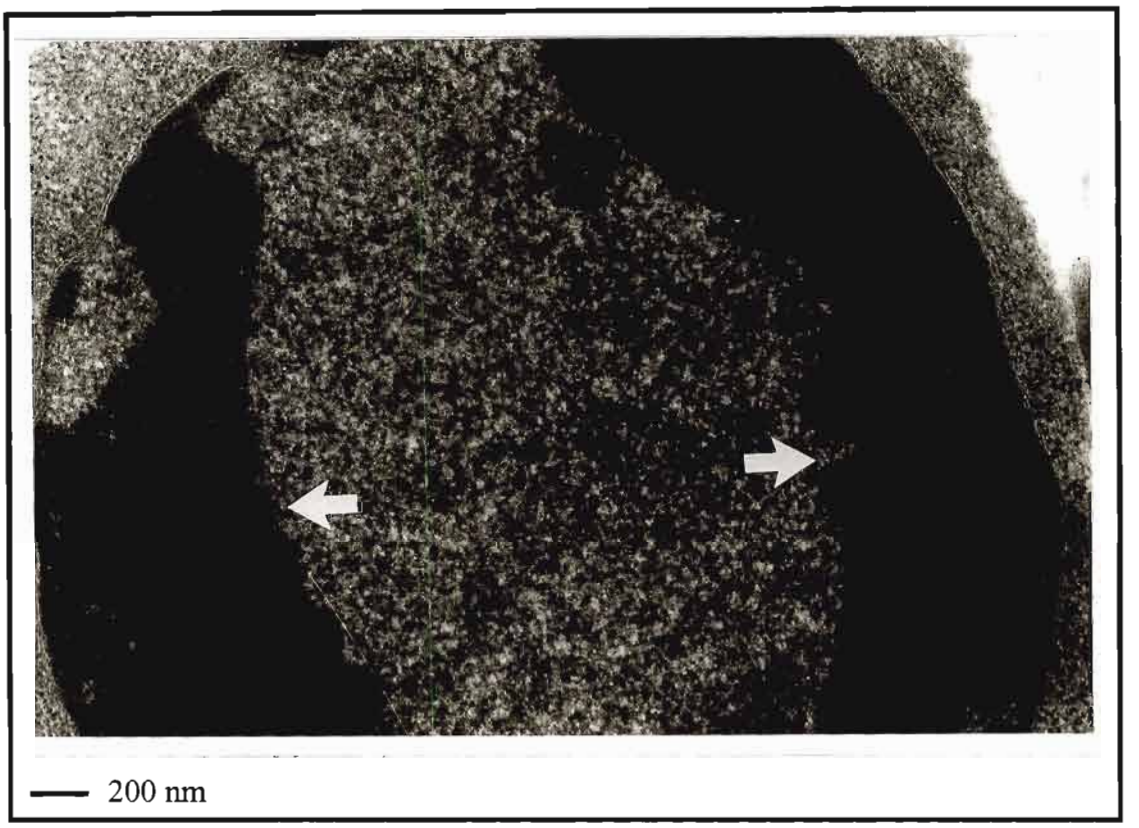

Figure 3.33 T-2 toxin treated lymphocyte (6 hours). Yet another state of condensation of the chromatin (arrowed). The crescents appear to still be forming.

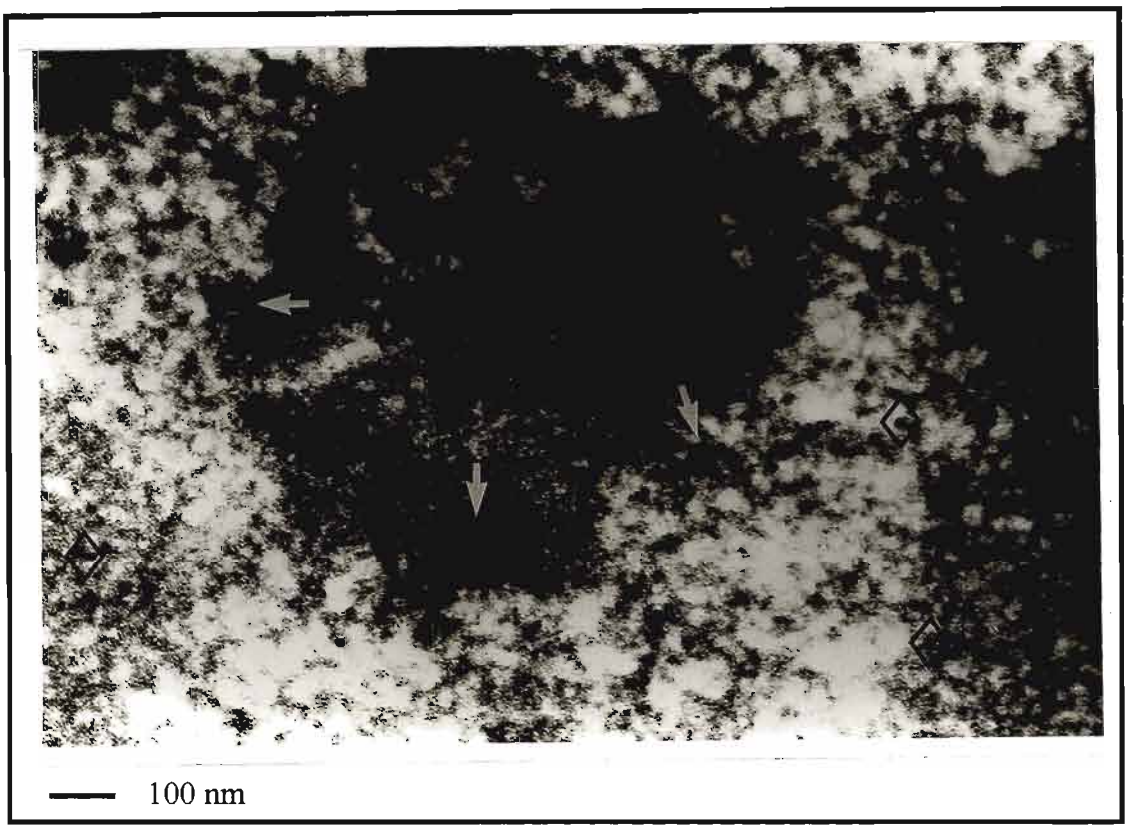

Figure 3.34 T-2 toxin treated lymphocyte (12 hours). Label within the nucleus (diamond) and nucleolus (arrowed) of a treated but apparently unaffected lymphocyte.

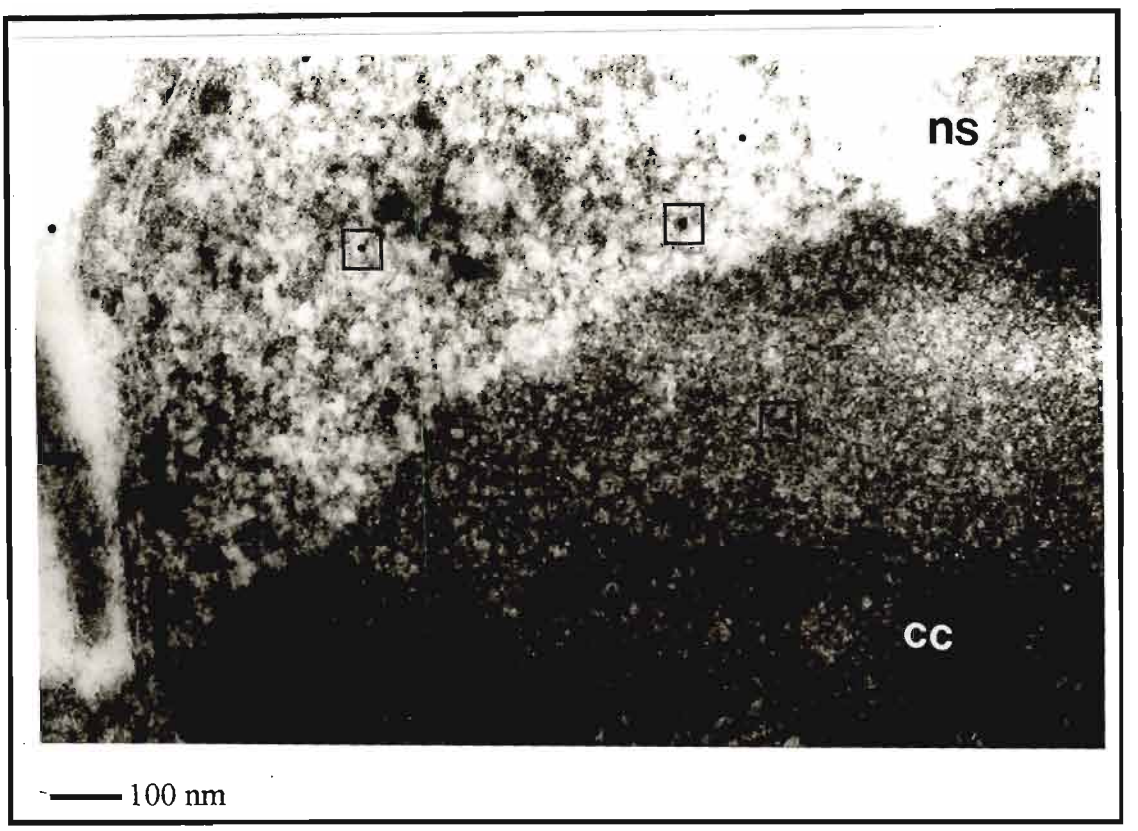

Figure 3.35 T-2 toxin treated lymphocyte (24 hours). Label was located within the darker condensed chromatin (cc) and nuclear sap (ns) of an apoptotic cell. Some of this label is indicated by (□). There was an abundance of label within the lymphocytes exposed to T-2 toxin for 24 hours.

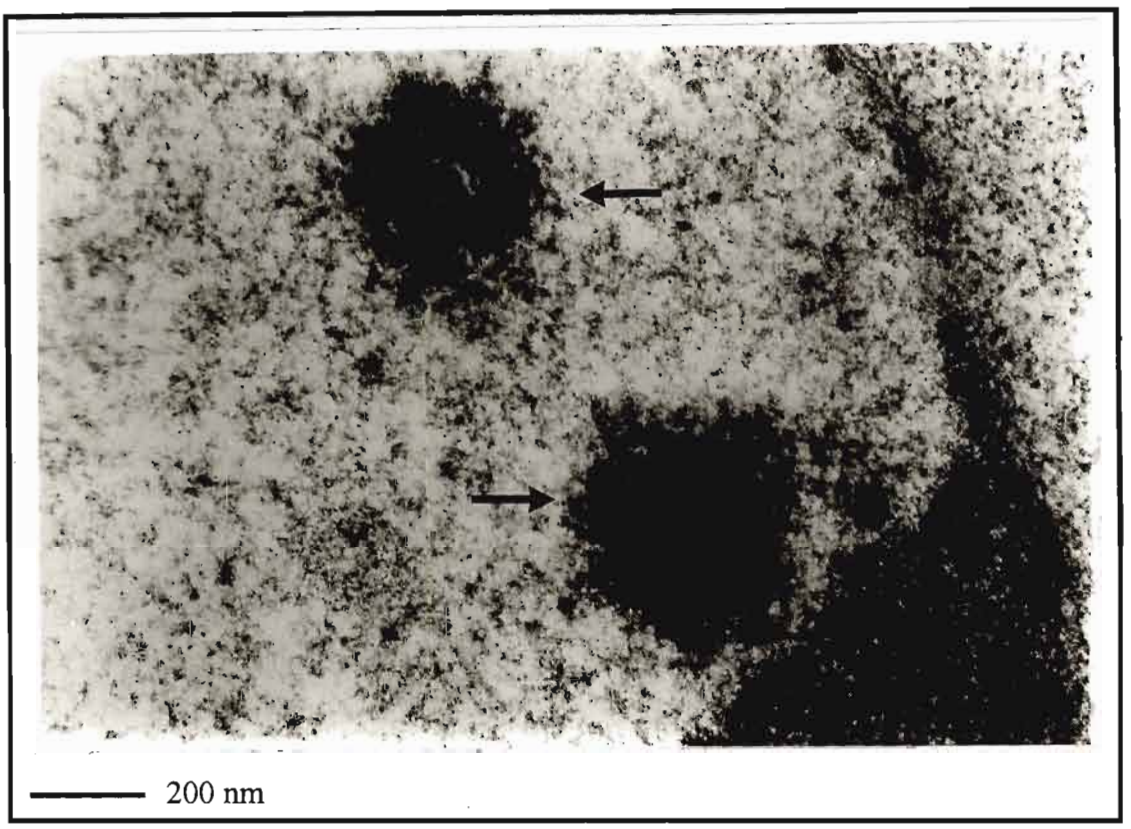

Figure 3.36 T-2 toxin treated lymphocyte (12 hours). Disintegration of the nucleolus (arrowed) was also associated with apoptotic lymphocytes.

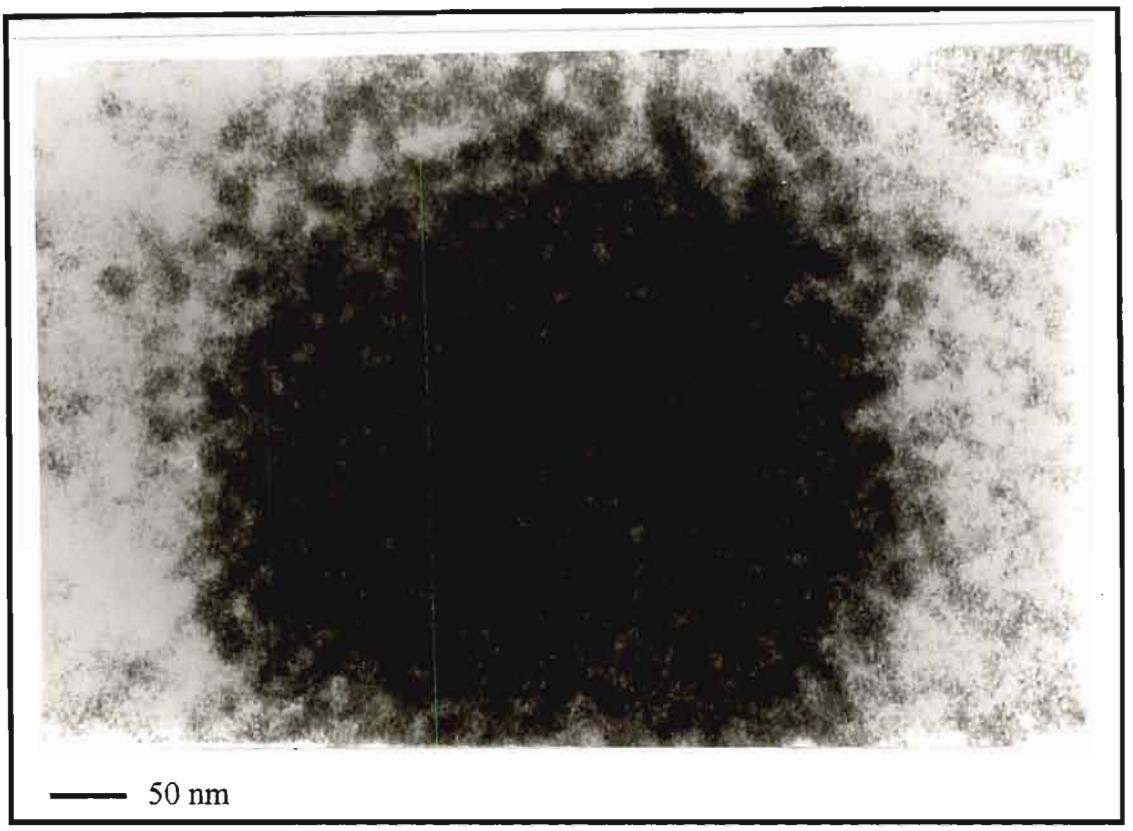

Figure 3.37 T-2 toxin treated lymphocyte (6 hours). Label (arrowed) within the disintegrating nucleolus of an apoptotic lymphocyte.

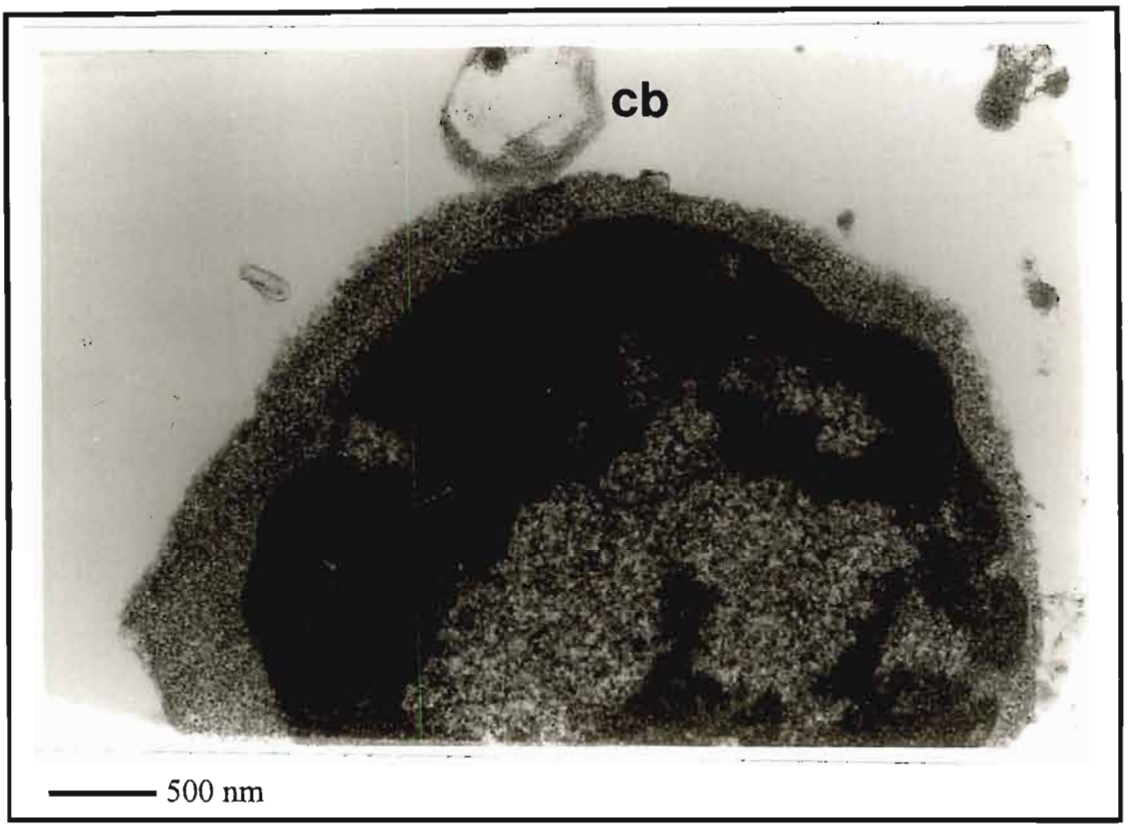

Figure 3.38 T-2 toxin treated lymphocyte (6 hours). Apoptotic cell with the formation of a cytoplasmic bleb (cb).

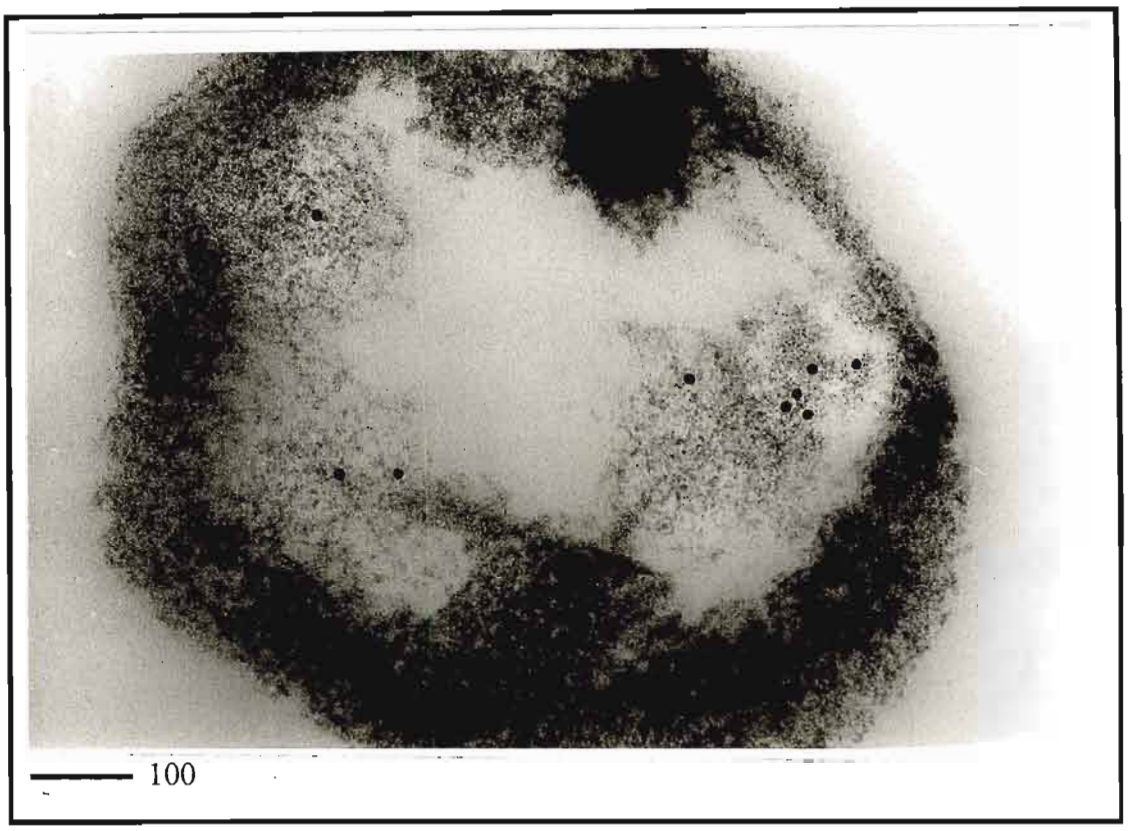

Figure 3.39 T-2 toxin treated lymphocyte (6 hours). An enlarged view of the bleb in figure 3.38. The black dots within the structure are the gold label. There are 12 of these within the bleb.

Damage to the T-2 treated lymphocytes (from minor damage to eventual death) seems to have greater occurrences at longer incubation times, i.e., at 6, 12 and 24 hours of treatment. The lymphocytes treated with T-2 toxin for 1 and 3 hours retain morphologies that are closer to those of the controls. There are a few occurrences of damaged and dead cells within the 1 and 3 hour treated samples, but these are too few to be of significance. The ICC results indicate that there was a smaller concentration of label within the samples treated for 1 and 3 hours than those for 6, 12 and 24 hours.

Nuclear indentation and the increase in the number of cellular processes appears to be the first ultrastructural signs of T-2 toxin damage. Hence it could be assumed that the first step in the lymphocyte interaction with the toxin is the increase in surface area. There seemed to be no apparent damage to cellular organelles, in particular the mitochondria, in lymphocytes treated for 1 and 3 hours. This could explain the high viabilities attained in the cytotoxicity assays, implying that although T-2 is inflicting changes in the lymphocytes, these changes are not sufficient to destroy the mitochondria, thereby not inhibiting cleavage of MTT.

The MTT assay (Fig. 3.3) also indicates that there is very little inhibition of MTT cleavage in the 6 hour samples. Although some dead cells were noted at this stage, as well as cells undergoing apoptosis, it seems that the majority of the mitochondria were still functional at this time, or perhaps not severely damaged, thus allowing the MTT salts to be cleaved. The TEM results also showed very little mitochondrial damage at 1 to 6 hours even in apoptotic lymphocytes. This could mean that the mitochondria were not primarily affected by T-2 toxin.

The samples incubated for 12 hours showed greater occurrences of apoptotic cells. This is in keeping with the cytotoxicity results which indicate that there is a large amount of dead cell and hence a decrease in mitochondrial function. Far more damaged mitochondria were viewed at this incubation time. The lymphocytes treated with T-2 toxin for 24 hours showed further increased signs of dead and dying cells, and the ICC experiments showed large amounts of label and therefore might indicate that the cells had reached saturation levels of T-2 toxin uptake. It is possible also that for these extended periods, the toxin had more time

in which to induce a full cytotoxic effect. Trichothecenes mycotoxins like T-2 toxin have demonstrated more discreet and in particular time-related cellular alterations (Reubel *et al.*, 1987).

It appeared that T-2 toxin was capable of inducing apoptosis and necrosis in normal human lymphocytes. It was qualitatively evident, however, that apoptosis was favoured. The criterion that were used to assess the appearance of possible necrotic cells, was the swelling of organelles, particularly the mitochondria. Care has to be taken however to discern between irreversible injury that leads to necrosis and reversible injury which manifests the same symptoms as necrotic cells (Walker *et al.*, 1988). It would therefore be safe to say that only the cells in severe state of vacuolation and autolysis were necrotic, whilst the cells with the swollen organelles may be representative of either reversible injury or irreversible injury (leading to necrosis).

The development of necrosis is associated with the irreversible and marked increase in the permeability of the mitochondrial and plasma membranes. The increase in membrane permeability is believed to involve the enzymatic degradation of membrane phospholipids. Following direct injury to the cell membrane by membrane active toxins, cations move across the membrane along concentration gradients. The accompanying fluid shifts causes cellular swelling, which may be reversible. In the cases of severe disturbances leading to necrosis, the increased concentrations of free cytosolic calcium results in activation of membrane-bound phospholipases, which degrade membrane phospholipids to fatty acids and lysophospholipids (Walker *et al.*, 1988).

In vitro findings on rat liver mitochondria by Pace (1983) suggests that mitochondria are a particular site of action of T-2 toxin. In a number of experimental systems, the early stage of apoptosis, i.e., the stage that precedes nuclear disintegration, is characterised by the breakdown of the inner mitochondrial transmembrane potential ($\Delta\Psi_m$). This $\Delta\Psi_m$ disruption may involve the mitochondrial permeability transition (Marchetti *et al.*, 1996). The mitochondrial permeability transition is self-sufficient to trigger apoptosis. This implies that there is a decrease in $\Delta\Psi_m$ which precedes nuclear signs of apoptosis. $\Delta\Psi_m$ disruption is

associated with (among others) an immediate shutdown of mitochondrial biogenesis and uncoupling of oxidative phosphorylation. Furthermore, mitochondria whose $\Delta\Psi_m$ is disrupted *in vitro* release an apoptogenic protein capable of inducing nuclear apoptosis (Marchetti *et al.*, 1996). It seems that T-2 toxin only affects mitochondria subsequent to 6 hours and 12 hours and over, because there was no noticeable decrease in the cytotoxicity assays of MTT cleavage prior to these exposures.

One of the most interesting and perhaps important points to note is that although T-2 toxin is an inhibitor of protein synthesis (Walker *et al.*, 1988), it does not appear to suppress apoptosis, which is dependent on protein synthesis. It was noted that T-2 toxin did not induce a reduction in the number of cytoplasmic polyribosomes. It could be argued that this retention of the polyribosomes is somehow related to the circumvention of the suppression of apoptosis in the T-2 treated lymphocytes. Polyribosomes are aggregates of ribosomes that are found in the cytoplasm and are responsible for protein synthesis (Wheater *et al.*, 1979 and Ghadially, 1982). The ribosomes are believed to be attached to specific sites along the length of a stand of messenger RNA which encodes the amino acid sequence for a specific protein being synthesised. Disaggregation of the polyribosomes is a recognisable sign of the arrest of protein synthesis (Ghadially, 1982).

It is likely that T-2 toxin initially reacts with receptors in the signal transduction pathway. The receptors with which the toxin may interact are either kinases or phosphatases that affect the phosphorylation and dephosphorylation of specific proteins. The apoptosis cell cycle regulators Bcl-2 and p53 are both phosphoproteins and could both be regulated through the signal transduction pathways (Payne *et al.*, 1995) which transpires in the plasmalemma and cytoplasm of the cell (Mathews and van Holde, 1990). Perhaps the epoxide group of the T-2 toxin molecule allows the molecule to mimic hormones that normally bind to the receptors.

Possible intracellular signalling mechanisms involved in the initiation of apoptosis includes the influx of calcium (Ca^{2+}) and an increase in intracellular free Ca^{2+} which is related to DNA fragmentation (Lucas *et al.*, 1994). Following this, p53 genes are activated (Kruyt *et*

al., 1996). The p53 protein is phosphorylated and the levels rise as a result of post-translational stabilisation (Hooper, 1994). The p53 protein activates Waf 1 and CIP1, which are inhibitors of cyclin-dependent protein kinases, and these throw the cell into cell cycle arrest (Fig. 1.12) (Nasmyth and Hunt, 1993). Thus although T-2 toxin is reported to be an inhibitor of protein synthesis, one of its first targets seems to be the cell cycle and signal transduction mechanisms, thereby increasing Ca^{2+} release. The subsequent DNA fragmentation could result in increased levels of protein regulators which initiate and regulate apoptosis.

The continued presence of polyribosomes, indicates either of two possibilities: 1) the rate of inhibition of protein synthesis in lymphocytes by T-2 toxin is slower than that in other cell lines or, 2) the inhibition of protein synthesis is not the primary target of T-2 toxin action in normal human lymphocytes. It seems that the initial steps of T-2 toxin action are: a) binding to the receptors on the surface of the cell membrane and possibly the nucleus and causing increases in the surface area; b) disruption of the mitochondrial permeability and hence the permeability transition during prolonged exposure times; c) the release of intracellular Ca^{2+} by inositol triphosphate in the signal transduction pathway (Mathews and van Holde, 1990); d) the disruption of permeability barrier of the ER plasmalemma resulting in the influx of Ca^{2+} (Schanne *et al.*, 1979) and; e) the destruction of the nucleus which manifests as chromatin condensation and DNA fragmentation.

The appearance of surface blebs are a predominant sign of apoptosis. The surface convolutions and the formation of blebs or apoptotic bodies are associated with the redistribution of cytoplasmic microfilaments (Walker *et al.*, 1988). They may also be associated with the presence of large amounts of cytosolic calcium ions (Ca^{2+}). Increases in cytosolic Ca^{2+} have been suggested to cause such alterations in the cell surface morphology by hepatotoxins, due to the interaction of Ca^{2+} with structural components of the cytoskeleton via Ca^{2+}-binding proteins or Ca^{2+}-dependent enzymes (Eriksson *et al.*, 1989).

It is also possible that other factors influence the initiation of apoptosis, for example, lysosomes remain intact during the early stages of apoptosis, and is likely that the release of lysosomal enzymes plays a part in the initiation of this type of cell death (Walker *et al.*, 1988).

The association of the anti-T-2 toxin conjugated label was evident in all the fractions in question, i.e., within the cellular processes and at the periphery of the cell (particularly at 1 and 3 hours), in association with the mitochondria, the ER, the cytoplasm (presumably with the polyribosomes) and the nucleus. The amount of label within the treated cells is directly proportional to the length of incubation of the samples, i.e., there was an increase in the amount of label observed with an increase in incubation time. It was of importance to note that the majority of the label at 1 and 3 hours was located at the periphery at the cytoplasm, within the cellular processes, and within the nucleus of the cells. This seems to confirm the assumption that the plasmalemma of the cell and the nucleus are the primary sites of action, and it is possible that from here the disruption of the signal transduction pathway occurred. Very little label was associated with the mitochondria at this stage. This may indicate that the primary initiation of apoptosis is associated with the cell cycle. The association of label along the periphery of the cell and outside the cell at 1 and 3 hours also concurs with the uptake and dissociation suggestions by Gyongyossy-Issa and Khachatourians (1984).

The TEM and ICC examinations were helpful in tracking the time response of lymphocytes to T-2 toxin exposure and in particular the evolution of cell death. Fluorescent cytometry using an *in situ* death detection kit is an alternative procedure because it is a precise and quantitative method of measuring apoptosis and necrosis, however it does not relate cellular apoptosis to histological localisation or provide morphological input apart from DNA damage (Boehringer Mannheim catalogue, 1996).

3.3 DNA Fragmentation Analyses

Fragmentation of the DNA of treated lymphocytes were observed in certain instances. The fragmentation patterns closely resembled those shown by Ueno *et al.* (1995) on the effects of T-2 toxin on promyelocytic leukaemia cells.

The initial observations were made on the DNA of lymphocytes treated with 1,56 μg/ml of T-2 toxin for 12 hours as well as the associated controls (Fig. 3.40). It was evident that T-2 toxin induced the fragmentation of the DNA of the treated cells, as they appeared to be a "smear" of fragments along the length of the gel. This image resembled those observed by Ueno *et al.*, 1995. The control DNA did not show evidence of fragmentation, and much of the DNA appeared to still be within the well indicating that the DNA was not cleaved and could therefore not run the length of the gel. This is in keeping with results shown by Ueno *et al.*, 1995.

Figure 3.41 represents the 0,8 % agarose gel, in which the fragmentation profiles of the different incubation times are compared. There seems to be no resulting fragmentation in the 1 and the 3 hour T-2 toxin treated samples, nor is there fragmentation in the controls. However at 6, 12 and 24 hours there is a marked increase in the fragmentation of the T-2 toxin treated lymphocytic DNA. At this stage it is just about discernible that there is a difference in the fragmentation profiles at 6, 12 and 24 hours.

Figure 3.42 shows that there is indeed a difference in the degree of fragmentation at 6, 12 and 24 hours. This is indicated by the "amount" of fragmentation which was judged by the amount of DNA in the lanes. Figure 3.42 shows that there is clearly more fragmentation at 24 hours than at 6 and 12 hours of incubation. The control lanes do not show evidence of DNA fragmentation.

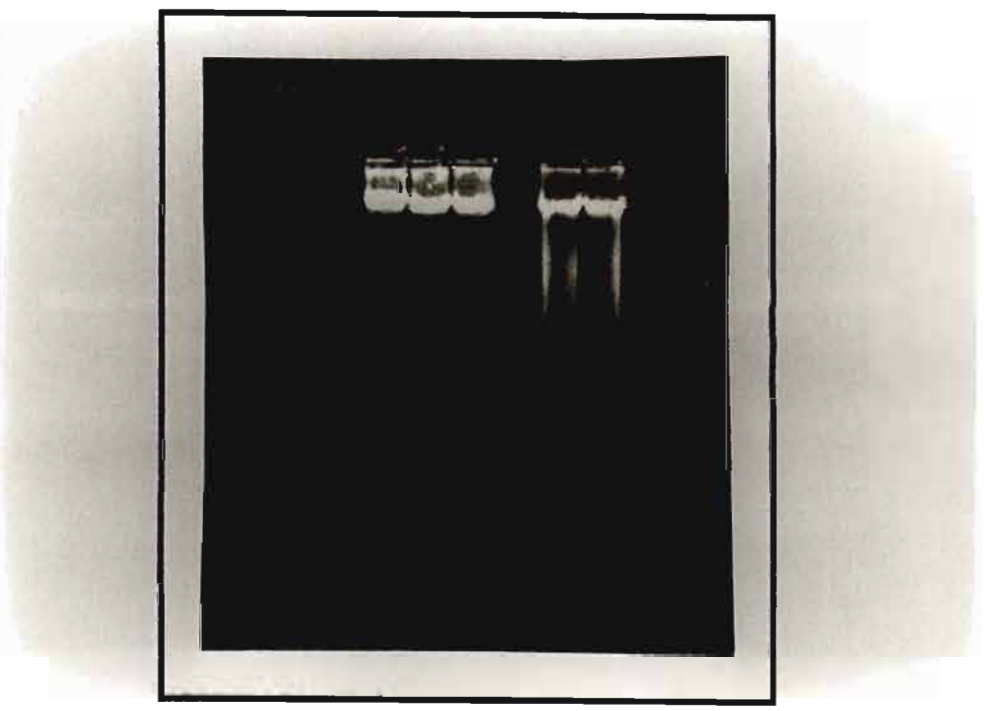

Figure 3.40 The first three wells were loaded with the control DNA, and no fragmentation was noted. The last two wells (right) were loaded with the DNA from cells treated with 1,56 μg/ml of T-2 toxin for 12 hours. Fragmentation is shown in the treated samples.

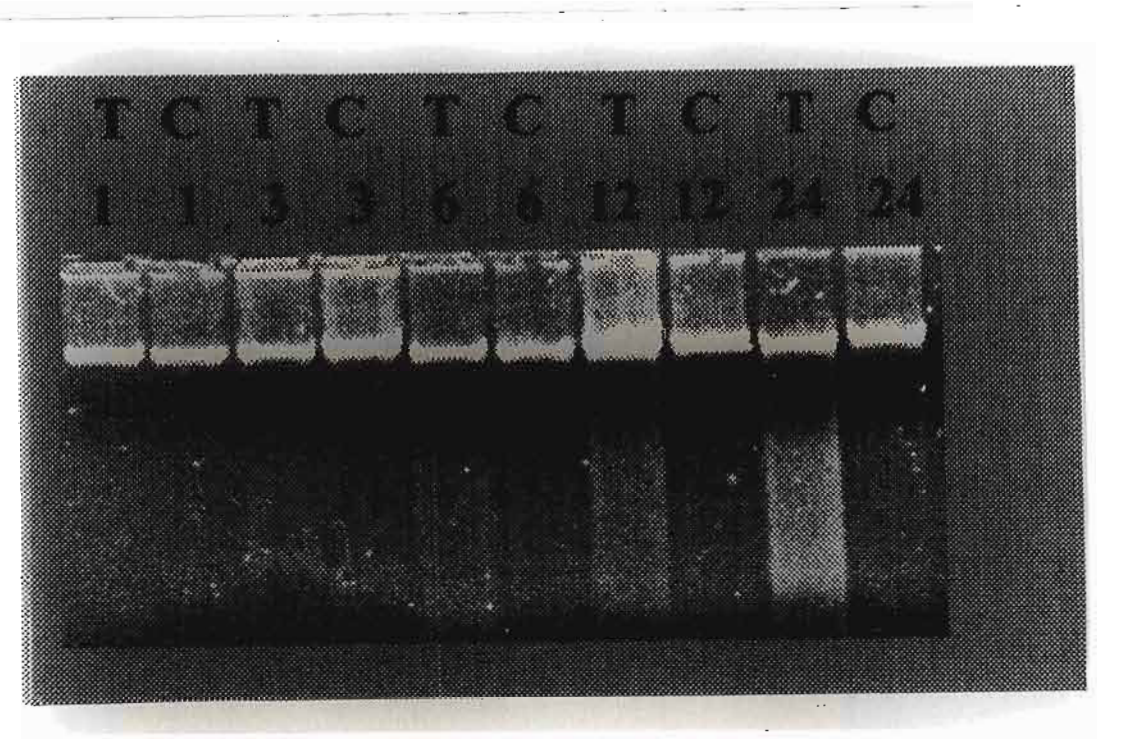

Figure 3.41 A 0,8 % agarose gel showing the differences in DNA fragmentation profiles of control (C) lymphocytes and lymphocytes treated with T-2 toxin (T) for 1, 3, 6, 12 and 24 hours. There seems to be an increase in fragmentation with an increase in incubation time. The controls as well as the 1 and 3 hour treated samples, do not show fragmentation.

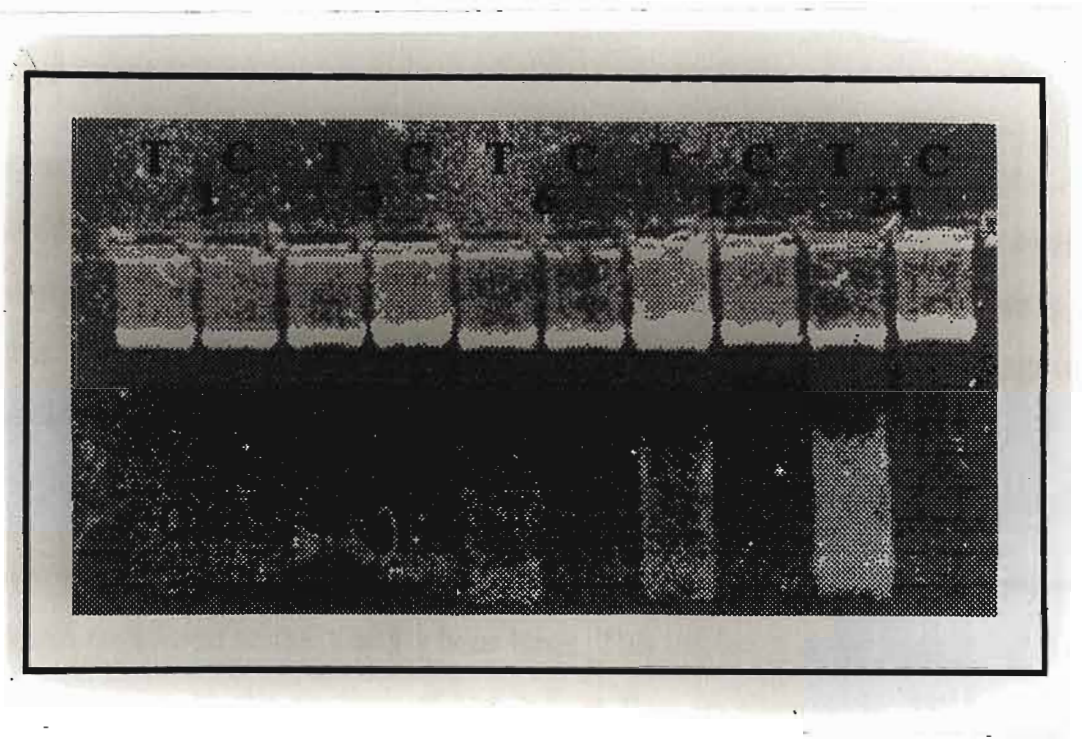

Figure 3.42 A 0,8 % agarose gel showing the DNA fragmentation profile of control lymphocytes (C) and T-2 treated lymphocytes (T) at incubation times of 1, 3, 6, 12 and 24 hours. There is an increase in the fragmentation patterns with an increase in incubation time. However, fragmentation is only observed at exposure times of 6 hours and above.

The 1 and the 3 hour T-2 treated lymphocytic DNA showed no evidence of fragmentation. The experiments were replicated at least four times in order to confirm these findings. They were consistent each time. The control DNA did not exhibit fragmentation to any significant degree, as the fragmentation profile was assessed in terms of visible fragmentation in the lanes. Furthermore, in situations in which there was no fragmentation, the DNA samples appeared not to have run in the gel, but rather remained in the wells. This appears to be due to a lack of fragmentation. This is clearly evident in the control samples shown in Ueno *et al.*, 1995.

The lack of fragmentation in the 1 and 3 hour sample is consistent with the almost negligible levels of toxicity in the cytotoxicity assays (Figs 3.1 and 3.2). It is also consistent with the paucity of dead or apoptotic lymphocytes in the electron microscopy studies at these exposure times. This means that at short incubation times at a concentration of 1,56 μg/ml, T-2 toxin has very little effect on normal resting human lymphocytes.

The lymphocytes treated with T-2 toxin for 6 hours show a remarkable fragmentation profile that when compared to the 1 and 3 hour lanes. This implies that between 3 and 6 hours there is indeed damage to the nucleus of the cell as thus to the cellular DNA by way of fragmentation. The cytotoxicity assays however show no significant cytotoxicity at the 6 hour incubation stage (Fig. 3.3). However the electron microscopy observations show that there is an increase in the amount of apoptotic cell death characteristics at 6 hours than when compared to the 1 and the 3 hour studies. The ICC shows that there is an increase in the amount of label within the nucleus and the cytoplasm within this time period as well, when compared to the 1 and the 3 hour studies. This implies that DNA fragmentation is an early sign of T-2 induced cellular injury (Ueno *et al.*, 1995) as opposed to mitochondrial damage.

The lymphocytes treated with T-2 toxin for 24 hours show a greater amount of fragmentation than the 12 hour incubation time (Figs. 3.41 and 3.42). The DNA incubated with T-2 toxin for 6 hours, indicates that although fragmentation of the DNA was occurring, the smears were less dense than those of the 12 and 24 hour incubation lanes. This implies that the extent of DNA fragmentation increased with an increase in incubation time. Ueno *et al.*, 1995 showed that there was an increase in the extent of fragmentation with an increase in incubation time and an increase in concentration of T-2 toxin administered to the cells.

Duke *et al.* (1983) used radiolabelling to specifically assay fragmented DNA. This was a sophisticated and accurate method. However the goal of this study was not to establish fragment length rather the effect of incubation time.. The method by Ueno *et al.* (1995) proved satisfactory and rapid. An alternate method is single cell gel electrophoresis (SCGE). The principle is similar to the DNA fragmentation assay, except that in the SGCE assay, whole isolated lymphocytes are used. Cells with an increase in DNA damage display increased migration of DNA from the nucleus to the anode and this is measured by the intensity of ethidium bromide fluorescence at fixed positions within the microscope (Singh *et al.*, 1988). The method employed by Ueno *et al.* (1995) afforded an overall view of DNA damage at a particular incubation time rather than of an isolated cell. This was more useful to determine the effects of incubation time in this case.

"Programmed cell death is a physiological process commonly defined by alterations in nuclear morphology (apoptosis) and/or the characteristic stepwise degradation of chromosomal DNA occurring before cytolysis" (Zamzami *et al.*, 1995). One of the apparent changes induced by apoptosis is the double strand cleavage of nuclear DNA (Walker *et al.*, 1988). The DNA is cleaved at internucleosomal linker regions , resulting in a "ladder" of 180-200 base pairs and multiples thereof on agarose gel electrophoresis (Payne *et al.*, 1995). The cleavage of the DNA generates two classes of chromatin fragments, i.e., 70 % of DNA exists as long histone H1-rich oligonucleosomes bound to the nucleus, and 30 % comprises of short oligonucleosomes and mononucleosomes, which are depleted in histone H1, and enriched in protein HMG1 and 2 and detached from the nucleus (Arends *et al.*, 1990).

The cause of the fragmentation of the DNA has been attributed to the activity of a Ca^{2+}/Mg^{2+} endonuclease, DNAse I or DNAse II (Payne *et al.*, 1995). An endonuclease is a protein that catalyses hydrolytic cleavages within the nucleic acid chain (Mathews and van Holde, 1990).

The nucleus is assumed to be one of the prime targets of programmed cell death (PCD) due to the following observations by Zamzami *et al.*, 1995:

- Direct DNA damage may cause PCD,
- During apoptosis, nuclear disintegration precedes cytolysis
- In numerous experimental models, the modulation of PCD is correlated with nuclear apoptosis,
- Agents that inhibit DNA-degrading enzymes, retard cytolysis of cells undergoing PCD, and
- A number of PCD-regulatory oncogene products function in the nucleus.

However it is also duly noted by recent studies that nuclear alterations are not obligatory events in PCD (Zamzami *et al.*, 1995). Payne *et al.*, 1995, maintain that apoptotic nuclear morphology is not always associated with fragmentation of the DNA. In this case however it is evident that the appearance of nuclear damage in the electron microscopy observations is directly related to the cellular DNA damage (fragmentation) shown in the electrophoresis studies.

The increase in the amount of fragmentation with an increase in treatment incubation time, is in direct relation to the increase in the occurrences of nuclear damage with an increase in incubation time as observed in the electron microscopy studies. It is of importance to consider that the proportional relationship between DNA fragmentation and incubation time, may be a significant indication of irreversible injury to the lymphocytes. This could mean that the DNA does not repair itself after 6 hours of toxic insult. There is no semblance of DNA damage in the 1 and 3 hour studies and this is in keeping with the cytotoxicity assays as well as the TEM studies.

An earlier event than cell death is the extensive fragmentation of the nuclear DNA into oligonucleosomal subunits (Cohen and Duke, 1984). It is possible that early damage to the nucleus and not early extensive damage to cellular mitochondria in apoptosis, prevent the mitochondria-dependent MTT assay from indicating cell death. In essence the cells might not in fact be dead but approaching death. The fragmentation requires RNA and protein. Both DNA fragmentation an cell death were prevented if protein or RNA synthesis were inhibited. It was evident from the electron microscopy results that although many lymphocytes did portray apoptotic profiles of chromatin condensation, there was still a presence of polyribosomes within the cytoplasm, indicating that protein synthesis might still be occurring. The endonuclease that cleaves the DNA at the chromosomal linker regions is found in the nuclei of thymocytes and depends on calcium and magnesium ions for its activation. It seems that the protein that is responsible for the death of thymocytes is dependent on the cytoplasm-to-nucleus calcium transport system (Cohen and Duke, 1984).

It appears that T-2 toxin induces an endonuclease and that this induction is susceptible to the inhibition to RNA and protein synthesis. The presence of Ca^{2+}/Mg^{2+} ions was shown to induce both chromatin condensation and DNA fragmentation in isolated mouse liver nuclei (Sun *et al.*, 1994). However when the endonuclease inhibitor $ZnCl_2$ was added to the cells, the DNA fragmentation induced by Ca^{2+}/ Mg^{2+} could be totally inhibited, but chromatin condensation still occurred. The results suggest that DNA fragmentation and chromatin condensation may be triggered through separate pathways during apoptosis (Sun *et al.*, 1994). DNA fragmentation and chromatin condensation both occur in lymphocytes treated with T-2 toxin. The occurrences are consistent in both the TEM studies and the DNA fragmentation analyses.

Suffice to say that the DNA fragmentation as well as the chromatin condensation induced by T-2 toxin on normal human lymphocytes was most probably due to the influx of intracellular Ca^{2+}/Mg^{2+} into the cytosol, and perhaps into the nucleus. This would induce the action of the endonuclease. It seems that in each type of cellular injury leading to apoptosis, the disruption of the plasma membrane occurs by widely differing mechanisms (Schanne *et*

al., 1979). This is followed by a common functional consequence possibly involving the influx of extracellular calcium across the damaged plasmalemma and down a steep concentration gradient (Schanne *et al.*, 1979). The intracellular Ca^{2+}/Mg^{2+} would flood into the cells due to damage to the ER or the release by inositol triphosphate. An alternative source could be extracellular Ca^{2+}/Mg^{2+}. Apoptosis results in damage to the plasmalemma. This might lead to an influx of Ca^{2+}/Mg^{2+} from the RPMI-1640-rich medium in which the lymphocytes are bathed (Appendix 2). The lack of fragmentation in the controls indicate that Ca^{2+} and Mg^{2+} in the medium did not appear to enter the lymphocytes in sufficient amounts to induce apoptosis. *In vivo*, the extracellular source of Ca^{2+} would be the fluid in which the cells and tissue bathe. However, the length of the incubation time is also an important factor, because the fragmentation increases with an increase in exposure time.

It is clear that regardless of the events leading to the manifestation of DNA fragmentation and chromatin condensation, both symptoms are present in lymphocytes treated with T-2 toxin. In both cases this predominantly occurs at the 6, 12 and 24 hour incubation times.

CHAPTER 4

4.0 CONCLUSION

Like the poor and taxes, mycotoxins are with us to stay. Prevention is the best "cure", but under certain climatic conditions, prevention is impossible (Bennett, 1989).

Pre- or postharvest contamination of various food crops by mycotoxigenic fungi is a common problem, and approximately 25% of the world's food supply is contaminated annually (Coulombe, 1993). It is therefore necessary to understand as much as possible about the mechanisms of the toxic actions of mycotoxins in the hopes of better preparing mankind for these inevitable battles.

T-2 toxin is cytotoxic to normal human lymphocytes. It inhibits mitochondrial activity and this is achieved in a time-dose dependent manner. The toxin has an acute effect on the lymphocytes (24 hours). However, these cytotoxic changes are only detectable at incubation times greater than 6 hours. The MTT assay is effective in determining the cytotoxicity of T-2 toxin, due to the ease, rapidity and sensitivity with which the test can be employed. Earlier beliefs due to the results of the cytotoxicity assay, was that T-2 toxin did not induce changes at 1 to 6 hours. However damage was shown at a nuclear level in the electron microscopy studies as well as the DNA fragmentation assays. This implies that although the MTT assay is a preferred assay for testing the mitochondrial viability of cells treated with T-2 toxin, it is possibly not the best for short incubation times.

The MTT assay may be misleading in terms of cellular damage, i.e., if there is no reduction of MTT cleavage, the cells are assumed to be healthy. Fluorescent cytometry with the use of a cell death detection assay, although an expensive method, is far more precise in determining cell death. However it does not provide insight into the morphological characteristics of cell death, which is of interest in this study.

T-2 toxin (1,56 μg/ml) induces apoptosis in normal human lymphocytes. This is verified by the morphological alterations consistent with those of documented apoptotic cells. The persistent presence of polyribosomes also confirms that the lymphocytes are undergoing apoptosis and possibly that there is negligible inhibition of protein synthesis if any at all. The initiation of apoptosis by T-2 toxin does not follow strict and predictable pathways. Instead the pathways and possibilities vary.

However as a result of the ultrastructural morphology, one can determine with some degree of clarity the mechanism of cell death. It seems that the major effect involves damage to the nucleus and DNA of the lymphocytes. This might involve the influx of either intracellular or extracellular Ca^{2+}/Mg^{2+} ions or both into the lymphocytes. The main type of cell death that is induced by T-2 toxin appears to be apoptosis.

The ICC results indicate that the amount of label associated with the cell is in direct proportion to the duration of incubation with T-2 toxin. Label was found within the cellular organelles, but more especially with the cytoplasm and nucleus of the treated lymphocytes. Label was associated with all areas of cellular damage, and particularly within the nuclei of apoptotic cells.

T-2 toxin (1,56 μg/ml) damages the DNA of lymphocytes irreversibly and forces the cells into apoptosis. The duration of the full cytotoxic effect is 24 hours although signs of damage are visible at 6 to 24 hours in the electron microscopy and DNA fragmentation studies. The DNA fragmentation provides undeniable evidence that T-2 toxin induces apoptosis in normal human lymphocytes. It seems also that the final common pathway towards apoptosis induced by T-2 toxin on normal human lymphocytes, involves the action of a Ca^{2+}/ Mg^{2+} activated endonuclease. This pathway manifests as the condensation of chromatin as seen in the electron microscopy studies, as well as the fragmentation of DNA. The results seem to indicate that nuclear patterns at short exposure times are independent of mitochondrial damage.

The fragmentation of the DNA appears to be time dependent, i.e., there is an increase in fragmentation with an increase in incubation time. This is of importance because it signifies that the damage to the lymphocytes is not reversible. These assumptions are aided by the cytotoxicity assays, which indicate significant increases in cell death at 12 and 24 hours.

T-2 toxin above all has proven to be strongly genotoxic, either by direct or indirect modes of action. The primary stages of cell death appear to involve damage to the nucleus and cleavage to the genomic DNA. The results obtained from the exposure times studied in this dissertation, indicates why T-2 toxin is such a potent mycotoxin. Its effects even at small doses such as 1,56 μg/ml, seem harsh and uncompromising and leave little room for doubt that its effects are acute and lethal.

APPENDICES

Appendix 1: The maintenance of a sterile work environment

All work requiring sterile handling was carried out in a cell and tissue culture laboratory that contained a laminar flow (Labotec).

Prior to each session hands were washed with hibitane (5%). This serves to sterilise and moisten the hands and thereby reduce the risk of dry skin blowing onto the cultures as well as reduce loosely adherent micro-organisms which are the greatest risk to the cultures (Freshney, 1983). Gloves were thereafter used and swabbed frequently with 70% ethanol. The laminar flow was swabbed down with an alcohol solution of 70% ethanol and 30% methanol.

Appendix 2:

Table a: (Biowhittaker Catalogue, 1993 and Biowhittaker Catalogue, 1996) A list of constituents of the media.

Components	**RPMI-1640 (mg/l)**	**HBSS (mg/l)**
$Ca(NO_3)_2.4H_2O$	100,00	
KCl	400,00	400,00
$MgSO_4.7H_20$	100,00	200,00
NaCl	5 000,00	8 000,00
$NaHCO_3$	2 000,00	350,00
$Na_2HPO_4.7H_2O$	1 512,00	90,00
$CaCl_2.2H_2O$		186,00
KH_2PO_4		60,00
Other components		
Glucose	2 000,00	1 000,00
Glutathione (reduced)	1,00	
HEPES	5 957,40	
Phenol Red	5,00	20,00
Amino Acids		
L-Arginine	200,00	
L-Asparagine.$2H_2O$	50,00	
L-Aspartic acid	20,00	
L-Cystine	50,00	
L-Glutamic Acid	20,00	
L-Glutamine	300,00	
Glycine	10,00	
L-Histidine	15,00	
Hydroxy L.Proline	20,00	
L-Isoleucine	50,00	
L-Leucine	50,00	

Appendix 3

The complete culture medium (CCM)

The CCM comprised of 82,66% RPMI-1640, 10,22% foetal calf serum and 7,12% Penicillin/streptomycin (5 000 u Pen/ 5 000 μg streptomycin/ml). The amount of Penicillin/streptomycin (pen/strep) added to the medium was determined by 4 ml of pen/strep for every 50 ml of RPMI-1640.

Appendix 4: The Haemocytometer Counts

Trypan Blue (0,4 g) was dissolved in 100 ml of distilled water (Sigma Chemical Company, 1996). The solution was filtered prior to use with a 0,45 μ filter (Millipore).

The cells suspension (50 μl) was mixed with trypan blue (50 μl). A few microlitres of this solution was placed on a Neubauer haemocytometer slide. This was covered with a Neubauer coverslip. The slide was viewed under an inverted microscope and the viable cells (fluorescent) were counted. The grid on the slide contained 5 large squares (Fig. a), each with smaller separations. One or all of the 5 larger squares were counted.

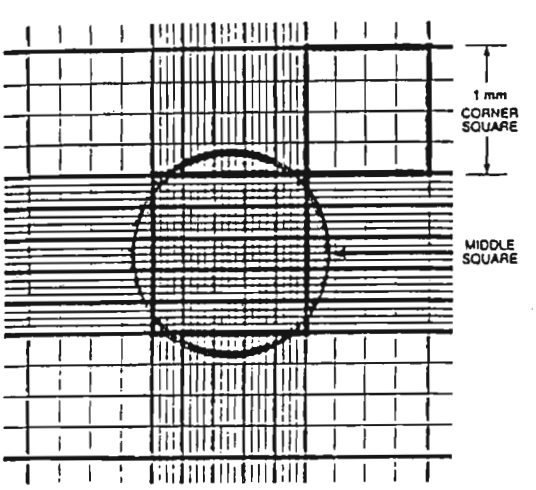

Figure a: (Sigma Chemical Company, 1996) The haemocytometer grid.

The number of cells was determined by the following equation :

cell/ml = n/v x dilution factor x 10^6

n = number of cells counted

v = area (number of big squares counted) x depth (0,1)

Dilution factor = 2 (equal volume of cell suspension and trypan blue)

Appendix 5: Preparation and Handling of T-2 toxin

T-2 toxin was stored in a lyophilised state at -20°C. Since the amounts of toxin required for experimentation were so small, the toxin was extracted in a weight per volume ratio with acetone. For example, if 250 μg of T-2 toxin was required, and there was a known volume of 1 mg in containment, then 1 ml of acetone was added to the stock amount and upon solubility, 250 μl would be extracted and placed in a clean vile. These solutions were dried with N_2 gas in a fume cupboard at room temperature.

T-2 toxin is a lethal mycotoxin and care was taken at all times in its handling. Gloves and laboratory coats were worn whilst extracting and working with the toxin. In situations were the toxin was handled outside a shielded area, masks and goggles were worn. Had spillages or skin contact occurred, activated charcoal and anti-T-2 toxin antibody were on hand. Waste was disposed of in medical waste bins and tanks.

Appendix 6: Students t-Test (Hoel and Jesson, 1982)

$$t = \frac{X_1 - X_2}{\sqrt{\frac{S_1^2}{n_1 - 1} + \frac{S_2^2}{n_2 - 1}}}$$

X_n = mean absorbance (group n) This was calculated from the LD_{50} value (50% viability)

S_n = standard deviation of group n

n_t = sample number

if $t > 2.26$ then $p < 0,05$.

Appendix 7: Electron Microscopy (reagents)

1% Glutaraldehyde: This was prepared by diluting 0,4 ml of 25% glutaraldehyde (SAARCHEM) in 9,6 ml of HBSS. The glutaraldehyde was prepared just prior to usage. Gloves were worn whilst working with glutaraldehyde. The 0,5% glutaraldehyde was prepared by mixing 0,2 ml in 9,8 ml of HBSS.

Phosphate buffered saline (PBS) pH 7,3-7,4: PBS tablets were dissolved in distilled water (1 tablet per 100 ml) by autoclaving the solution in a glass bottle. The bottle was lightly sealed and covered in foil. It was placed in the autoclave at 121°C for 20 minutes. The PBS was cooled to room temperature before use.

Osmium tetroxide (OsO_4): OsO_4 is a dangerous liquid that is stored in two containers at 4°C. It was only used in the fume cupboard and gloves and laboratory coats were worn at all times. Care was taken to avoid contact and inhalation.

Araldite resin: Gloves and laboratory coats were used whilst working with resin. The resin was made up from an araldite kit (TAAB, England). The resin was stored at -20°C in properly sealed vials. Great care was taken to ensure that the resin reached room temperature prior to

opening the vials, as condensation results in brittle specimens after embedding and these are difficult to section.

Uranyl acetate (Capital Enterprises): Ethanol (50%) was saturated with uranyl acetate (424,15 g/mol). This was filtered and centrifuged before use.

Lead citrate (Capital enterprises): Lead nitrate (2,66 g; 331,2 g/mol) was dissolved in 60 ml of distilled water. Sodium citrate (3,52 g; 294,1 g/mol) was added to this solution which immediately precipitated. The solution was left to stand 30 minutes at room temperature. Finally 16 ml of sodium hydroxide (1 N) was added to the solution. The solution cleared upon addition of sodium hydroxide. The lead citrate was filtered and centrifuged before use. The sections were covered at all times, in order to prevent mixing of lead citrate with CO_2, which would result in lead carbonate contamination of the grids and sections.

Appendix 8: Reagents for Immunocytochemistry

Tris-HCl stock buffer: Stock of 0,5 M Tris buffer. This was used to dilute the NaCl solution). Tris base (60, 6 g; molecular weight: 121,14) was dissolved in 800 ml of distilled water. The pH was adjusted to 7,2 with concentrated HCl. The volume was brought up to 1 litre with distilled water.

Stock-Tris NaCl: NaCl (8,764 g) was dissolved in 1 litre of distilled water to give a concentration of 0,15 M. The stock Tris buffer was diluted (1:10) in 0,15 M NaCl. A litre of this solution was prepared.

BSA for jet washes:

0,2% BSA in 50 mM Tris pH 7,2

1% BSA in 50 mM Tris pH 8,2 (this was also used to dilute the gold label)

0,2% - 0.2 g BSA in 100 ml Tris

1% - 1 g BSA in 100 ml in 100 ml Tris

Appendix 9: 1 x SDS gel loading buffer (Sambrook *et al.*, 1989)

50 mM Tris-HCl (pH 6,8)

2% SDS

10% glycerol

This was mixed and made up to 9 ml in distilled water. The solution was aliquoted at 900 µl into each of 10 sterile eppendorfs. These were frozen and stored. When the gel loading buffer was ready for use, 100 µl of 2-mercaptoethanol was added to each 900 ml of buffer used. The addition of 2-mercaptoethanol was carried out in a fume cupboard.

Appendix 10: TBE buffer (pH 8) (Sambrook *et al.*, 1989)

A 10x TBE buffer was made and stored in an autoclaved glass bottle.

The 10x buffer contained :

Tris base - 54 g

Boric acid - 27,5 g

EDTA, disodium - 4,65 g

This was made up to 500 ml with distilled water. For each experiment 100 ml of 10x TBE buffer was diluted with 900 ml of distilled water to give a 1x TBE buffer which was used in the gel and the electrophoresis tank.

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