

**MODULATING EFFECTS OF
FUMONISIN B₁ AND OCHRATOXIN A ON
IMMUNE CELLS IN HUMAN CARCINOMA**

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AUTHORS DECLARATION

This study represents original work by the author. It has not been submitted in any other form to any other Tertiary Institution. Where use of the work of others was made, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Department of Clinical Technology and Biotechnology, Faculty of Health Sciences, Durban Institute of Technology, Durban, South Africa under the supervision of Professor B. Odhav (Department of Biotechnology, Durban Institute of Technology).

SIGNED: _____

✓ J.K. ADAM

I hereby certify that the above statement is correct.

SIGNED: _____

PROFESSOR B. ODHAV (PhD)

DEDICATION

I dedicate this work to:

My late father, Mr Mohammed Omar Khan and my mother Mrs Rabia Khatoon Khan
for their inspiration and guidance;

My husband, Dr Ahmed Sadeq Adam, and my sons, Rayhaan, Waseem, Irfaan and
Nadeem, for their continuous support and encouragement; and

To Almighty Allah who makes all things possible.

ABSTRACT

Fumonisin B₁ (FB₁) and ochratoxin A (OTA) represent examples of mycotoxins of greatest public health and agro-economic significance. They exert adverse effects on humans, animals and crops that result in illnesses and economic losses. Fumonisin B₁ are cancer-promoting metabolites of *Fusarium proliferatum* and *F. verticillioides*, (formerly *moniliforme*), and are implicated in oesophageal cancer. Ochratoxins are metabolites of both *Aspergillus* and *Penicillium* species. These compounds are known for their nephrotoxic effects in all animal species and may promote tumours in humans. In man OTA exhibits unusual toxicokinetics, with a half-life in blood of 840 h (35 days) after oral ingestion. Although much is known regarding the toxicology of these toxins, little is known of the effects of these toxins on the immune system.

The aim of this study was to determine and compare the immunomodulating effects of FB₁ and OTA in human carcinoma. Initial experiments involved isolating lymphocytes and neutrophils from healthy volunteers. The isolated cells were exposed to either FB₁ or OTA on a dose and time dependent level and LD₅₀ of the toxins was determined. Thereafter, challenge tests were performed, whereby lymphocytes and neutrophils isolated from volunteers, oesophageal cancer patients and breast cancer patients were exposed to the LD₅₀ dose of either FB₁ or OTA for the appropriate time. The effect of the toxins was demonstrated by viability studies, light microscopy and electron microscopy. Cytokine receptors (CK, TNF and CSF) were evaluated by immuno-cytochemical methods and the levels of circulating cytokines (IL -1, IL-6, IL-8, IL-10 and TNF- α) were determined using ELISA kits.

The results of this study showed that fumonisin B₁ and ochratoxin A cause a decrease in the number of lymphocytes and neutrophils in a dose dependent manner. Ultrastructural changes observed were consistent with apoptosis i.e. cytoplasmic and chromatin condensation, chromatin margination, increased vacuolation and cell shrinkage. They also brought about a decrease in CSF, TNF, and CK receptors, and in circulating cytokine levels of IL-1, IL-6, IL-8, IL-10 and TNF- α . These changes were more pronounced in oesophageal and breast cancer patients. The results of this study clearly indicates that both FB₁ and OTA are immuno-suppressive agents, and in patients who already have cancer, they exacerbate the problem by impairing immune surveillance and thus increase cell proliferation of malignant cells. Future work with cytokine-linked immunotherapy may help patients with cancers.

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TABLE OF CONTENTS

AUTHORS DECLARATION	ii
DEDICATION	iii
ABSTRACT	iv
PUBLICATIONS ARISING FROM THIS DISSERTATION	vi
ACKNOWLEDGEMENTS	viii
TABLE OF CONTENTS	x
LIST OF FIGURES	xiv
LIST OF TABLES	xx
LIST OF ABBREVIATIONS	xxii
CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW	1
1.1 Study background	1
1.2 The immune response	9
1.2.1 General overview	9
1.2.2 Immune response modifiers	20
1.2.2.1 Interleukin-1	26
1.2.2.2 Tumour necrosis factor	27
1.2.2.3 Interleukin-6	27
1.2.2.4 Interleukin-8	28
1.2.2.5 Interleukin-2	28
1.2.2.6 Interleukin-12	29
1.2.2.7 Granulocyte-macrophage colony stimulating factor	29

1.2.2.8	Interferon- α	31
1.3	Action of mycotoxins on the immune system	32
1.3.1	Mechanisms involved in immunotoxicity	32
1.3.2	Immunotoxic effects of selected mycotoxins	33
1.4	Immune modulation by fumonisins and ochratoxins	37
1.4.1	Fumonisins	37
1.4.1.1	Occurrence	37
1.4.1.2	Characteristics	39
1.4.1.3	Toxicological effects	40
1.4.1.4	Mechanism of action	43
1.4.2	Ochratoxins	47
1.4.2.1	Occurrence	47
1.4.2.2	Characteristics	49
1.4.2.3	Toxicological effects	50
1.4.2.4	Mechanism of action	54
	CHAPTER TWO: MATERIALS AND METHODOLOGY	58
2.1	Sampling	58
2.2	Purification of lymphocyte and neutrophil	59
2.2.1	Specimen collection	59
2.2.2	Purification procedure	60
2.3	Enumeration of lymphocytes and neutrophils	61
2.4	Dose and time response FB ₁ and OTA on lymphocytes and neutrophils	63
2.4.1	Trypan blue assay	65
2.4.2	MTT assay	66

2.5	Effect of FB ₁ and OTA on oesophageal and breast cancer patients' neutrophils and lymphocytes	67
2.5.1	Effect of toxins on viability of neutrophils and lymphocytes	69
2.5.2	Morphological effects of FB ₁ and OTA	70
2.5.2.1	Light microscopy	70
2.5.2.2	Transmission electron microscopy	71
2.5.3	Effects of toxins on cytokine receptors	72
2.5.3.1	Light microscopy	76
2.5.3.2	Electron microscopy	77
2.5.4	Effect of toxins on cytokine levels	79
2.6	Statistical analysis	85
CHAPTER THREE: RESULTS		86
3.1	Effects of FB ₁ and OTA on lymphocytes and neutrophils from healthy volunteers	86
3.1.1	Effect on viability	86
3.1.2	Effect on morphology	92
3.1.2.1	Gross morphological changes	92
3.1.2.2	Ultrastructural changes	95
3.2	Effect of FB ₁ and OTA on lymphocytes and neutrophils from patients with oesophageal and breast cancer	102
3.2.1	Viability	102
3.2.2	Toxin effect on morphology	106
3.2.2.1	Oesophageal cancer patients	106

3.2.2.2 Breast cancer patients	114
3.2.3 Receptor localization	124
3.2.3.1 CK receptor localization by light microscopy	124
3.2.3.2 CK, TNF and CSF receptor localization by electron microscopy	127
3.2.4 Cytokine levels (IL-1, IL-6, IL-8, IL-10 and TNF- α)	135
3.2.4.1 Concentrations of IL-1, IL-6, IL-8, IL-10 and TNF- α in plasma samples	135
3.2.4.2 Concentrations of IL-1, IL-6, IL-8, IL-10 and TNF- α in the supernatant	137
CHAPTER FOUR: DISCUSSION	141
4.1 Effect of FB ₁ and OTA on cell viability of lymphocytes and neutrophils	143
4.2 Effect of FB ₁ and OTA on morphology of lymphocytes and neutrophils	145
4.3 Effect of FB ₁ and OTA on cytokine receptors on lymphocytes and neutrophils	148
4.4 Effect of FB ₁ and OTA on cytokines	150
CHAPTER FIVE: CONCLUSION AND FUTURE WORK	152
CHAPTER SIX: REFERENCES	156
CHAPTER SEVEN: APPENDICES	221

LIST OF FIGURES

Figure 1.1	Schematic representation of blood cell formation	10
Figure 1.2	Antigen recognition and antibody formation	13
Figure 1.3	The B-cell and T-cell response to non-self antigens	14
Figure 1.4	Dendritic cell interactions	19
Figure 1.5	Subdivisions of cytokines based on their biological actions	24
Figure 1.6	Structures of sphinganine, sphingosine and FB ₁	44
Figure 1.7	Schematic representation of the mechanism of action of FB ₁	45
Figure 2.1	Schematic representation showing separation of mononuclear cells and granulocytes with Histopaque 1077 and 1119 using differential centrifugation	61
Figure 2.2	Haemocytometer chamber	63
Figure 2.3	Algorithm depicting tests conducted on lymphocytes and neutrophils of cancer patients and volunteers to evaluate the effect of FB ₁ and OTA	69
Figure 2.4	A schematic presentation of the principle of the immunocytochemical immunolabelling protocol	73
Figure 2.5	A schematic representation of the ABC method	74
Figure 2.6	Schematic representation of chemokine's competitive enzyme immunoassay	80
Figure 3.1	Time-response of lymphocytes to different concentrations of FB ₁ (Trypan blue dye)	88
Figure 3.2	Time-response of lymphocytes to different concentrations of FB ₁ (MTT assay)	88
Figure 3.3	Time-response of neutrophils to different concentrations of FB ₁	89

	(Trypan blue dye)	
Figure 3.4	Time-response of neutrophils to different concentrations of FB ₁ (MTT assay)	89
Figure 3.5	Time-response of lymphocytes to different concentrations of OTA (Trypan blue dye)	90
Figure 3.6	Time-response of lymphocytes to different concentrations of OTA (MTT assay)	90
Figure 3.7	Time-response of neutrophils to different concentrations of OTA (Trypan blue dye)	91
Figure 3.8	Time-response of neutrophils to different concentrations of OTA (MTT assay)	91
Figure 3.9	Morphology of neutrophils	93
Figure 3.10	Morphology of lymphocytes	94
Figure 3.11	Electron micrographs of neutrophils unexposed to toxins	96
Figure 3.12	Electron micrographs showing neutrophils exposed to 25 µg/ml FB ₁ at 2 h	97
Figure 3.13	Electron micrographs showing neutrophils exposed to 50 µg/ml OTA for 23 h	98
Figure 3.14	Electron micrographs of control lymphocytes	100
Figure 3.15	Electron micrographs of lymphocytes exposed to 25 µg/ml FB ₁ at 2 h.	100
Figure 3.16	Electron micrographs of lymphocytes exposed to 50 µg/ml OTA at 23 h	101
Figure 3.17	Percentage viability of neutrophils exposed to 25 µg/ml FB ₁ for 2 h or	105

	50 µg/ml OTA for 23 h	
Figure 3.18	Percentage viability of lymphocytes exposed to 25 µg/ml FB ₁ for 2 h or 50 µg/ml OTA for 23 h	105
Figure 3.19	Electron micrographs of control lymphocytes	107
Figure 3.20	Electron micrographs of lymphocytes exposed to 25 µg/ml FB ₁ at 2 h.	108
Figure 3.21	Electron micrographs of lymphocytes exposed to 50 µg/ml OTA at 23 h	109
Figure 3.22	Electron micrographs of control neutrophils	111
Figure 3.23	Electron micrographs of neutrophils exposed to 25 µg/ml FB ₁ at 2 h	112
Figure 3.24	Electron micrographs of neutrophils exposed to 50 µg/ml OTA at 23 h	113
Figure 3.25	Electron micrographs of control lymphocytes	115
Figure 3.26	Electron micrographs of lymphocytes exposed to 25 µg/ml FB ₁ for 2 h	116
Figure 3.27	Electron micrographs of lymphocytes exposed to 50 µg/ml OTA	117
Figure 3.28	Electron micrographs of control neutrophils	119
Figure 3.29	Electron micrographs of neutrophils exposed to 25 µg/ml FB ₁ for 2 h	120
Figure 3.30	Electron micrographs of neutrophils exposed to 50 µg/ml OTA	121
Figure 3.31	Electron micrographs of lymphocytes at time zero and 2 h after exposure to 25 µg ml FB ₁ and 23 h after exposure to 50 µg/ml OTA	122
Figure 3.32	Electron micrographs of neutrophils at time zero and 2 h after exposure to 25 µg ml FB ₁ and 23 h after exposure to 50 µg/ml OTA	123
Figure 3.33	Light microscopic (LM) images of labelled (arrows) CK receptors on surfaces of lymphocytes from healthy individuals, oesophageal cancer	125

	and breast cancer patients exposed to 25 µg/ml FB ₁ for 2 h incubation or 50 µg/ml OTA for 23 h incubation.	
Figure 3.34	Light microscopic (LM) images of labelled (arrows) CK receptors on surfaces of neutrophils from healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 µg/ml FB ₁ for 2 h incubation or 50 µg/ml OTA for 23 h incubation.	126
Figure 3.35	Electron microscopic (EM) images of labelled (dots) CK receptors on surfaces of lymphocytes from healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 µg/ml FB ₁ for 2 h incubation or 50 µg/ml OTA for 23 h incubation.	129
Figure 3.36	E M images of labelled (arrows) CK receptors on surfaces of neutrophils of healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 µg/ml FB ₁ for 2 h incubation or 50 µg/ml OTA for 23 h incubation.	130
Figure 3.37	E M images of labelled (dots) CSF receptors on surfaces of lymphocytes from healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 µg/ml FB ₁ for 2 h incubation or 50 µg/ml OTA for 23 h incubation.	131
Figure 3.38	EM images of labelled (dots) CSF receptors on surfaces of neutrophils from healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 µg/ml FB ₁ for 2 h incubation or 50 µg/ml OTA for 23 h incubation.	132
Figure 3.39	EM images of labelled (dots) TNF receptors on surfaces of	133

	lymphocytes from healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 µg/ml FB ₁ for 2 h incubation or 50 µg/ml OTA for 23 h incubation.	
Figure 3.40	EM images of labelled (dots) TNF receptors on surfaces of neutrophils from healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 µg/ml FB ₁ for 2 h incubation or 50 µg/ml OTA for 23 h incubation.	134
Figure 3.41	Standard curve for IL-1	234
Figure 3.42	Standard curve for IL-6	234
Figure 3.43	Standard curve for TNF-α	235
Figure 3.44	Standard curve for IL-8	235
Figure 3.45	Standard curve for IL-10	236
Figure 3.46	Mean IL-1 concentration (ng/ml) in supernatant of lymphocytes and neutrophils at time zero and at 2 h exposure to 25 µg/ml FB ₁ exposure or 23 h exposure to 50 µg/ml OTA	138
Figure 3.47	Mean IL-6 concentration (ng/ml) in supernatant of lymphocytes and neutrophils at time zero and at 2 h exposure to 25 µg/ml FB ₁ exposure or 23 h exposure to 50 µg/ml OTA	138
Figure 3.48	Mean IL-8 concentration (ng/ml) in supernatant of lymphocytes and neutrophils at time zero and at 2 h exposure to 25 µg/ml FB ₁ exposure or 23 h exposure to 50 µg/ml OTA	139
Figure 3.49	Mean IL-10 concentration (ng/ml) in supernatant of lymphocytes and neutrophils at time zero and at 2 h exposure to 25 µg/ml FB ₁ exposure	139

	or 23 h exposure to 50 µg/ml OTA	
Figure 3.50	Mean TNF-α concentration (ng/ml) in supernatant of lymphocytes and neutrophils at time zero and at 2 h exposure to 25 µg/ml FB ₁ exposure or 23 h exposure to 50 µg/ml OTA	140
Figure 3.51	Summary of results	151

LIST OF TABLES

Table 1.1	Cells of the immune response	11
Table 1.2	Cytokines and chemokines	15
Table 1.3	Cellular receptors of the immune system	17
Table 1.4	Cellular expression and actions of cytokines	22
Table 1.5	Cytokine family and their criteria	25
Table 1.6	Selected examples of aflatoxin B ₁ immunotoxicity in livestock species and mice after oral treatments	34
Table 1.7	Influence of various tricothecenes on immune responses in various animal species	35
Table 1.8	Immunotoxic effects of miscellaneous mycotoxins	36
Table 2.1	Dose and time of exposure of FB ₁ and OTA	65
Table 2.2	Method for the processing of samples for TEM	71
Table 2.3	Characteristics of receptor antibodies used in ICC	75
Table 2.4	Protocol for immunolabelling of slides for light microscopy	77
Table 2.5	Protocol for the processing/preparation of tissues for electron microscopy	78
Table 2.6	Immunolabelling procedure for electron microscopy: colloid gold method	79
Table 2.7	Serial dilution of cytokine standards (IL-1, IL-6, IL-8, IL-10 and TNF- α)	83
Table 3.1	Lethal dose for lymphocytes and neutrophils exposed to FB ₁	87
Table 3.2	Lethal dose for lymphocytes and neutrophils exposed to OTA	87
Table 3.3	Lymphocytes and neutrophils of healthy individuals exposed to	228

	25 µg/ml FB ₁	
Table 3.4	Lymphocytes and neutrophils of healthy individuals exposed to 50 µg/ml OTA	229
Table 3.5	Lymphocytes and neutrophils of oesophageal cancer patients exposed to 25 µg/ml FB ₁	230
Table 3.6	Lymphocytes and neutrophils of oesophageal cancer patients exposed to 50 µg/ml OTA	231
Table 3.7	Lymphocytes and neutrophils of breast cancer patients exposed to 25 µg/ml FB ₁	232
Table 3.8	Lymphocytes and neutrophils of breast cancer patients exposed to 50 µg/ml OTA	233
Table 3.9	Immunolabelling results of TNF, CSF and chemokine receptor localization on neutrophils, and lymphocytes	128
Table 3.10	Mean concentrations of IL-1, IL-6, IL-8, IL-10 and TNF-α in plasma samples of volunteers, oesophageal cancer patients and other cancer patients	136

LIST OF ABBREVIATIONS

ABC	-	avidin - biotin complex
AFB ₁	-	aflatoxin B ₁
APC	-	antigen presenting cells
BLK	-	function blocking assays
CCM	-	complete culture medium
CD	-	cluster of differentiation
CK	-	chemokine
CNS	-	central nervous system
Con A	-	concanavalan A
CSF	-	colony stimulating factor
CTL	-	cytolytic T lymphocyte
CYP	-	cytochrome P
DAB	-	diaminobenzidine
DMSO	-	dimethyl sulphoxide
DNA	-	deoxyribonucleic acid
DTH	-	delayed type hypersensitivity
EDTA	-	ethylene diamine tetracetic acid
EIA	-	enzyme immunoassay
ER	-	endoplasmic reticulum
EtOH	-	ethanol
F	-	<i>Fusarium</i>
FB ₁	-	fumonisin B ₁
FB ₂	-	fumonisin B ₂

GM-CSF	-	granulocyte macrophage-colony stimulating factor
HBSS	-	Hanks balanced salt solution
HLA	-	human leucocyte antigen
IARC	-	International Agency for Research on Cancer
IB	-	immunoblotting
IBH	-	inclusion body hepatitis
ICC	-	immunocytochemistry
IL	-	interleukin
IL-1RA	-	interleukin-1 receptor antagonist
INF	-	interferons
IP	-	intraperitoneal
LD	-	lethal dose
LM	-	light microscopy
LPS	-	lipopolysaccharide
mbGM-CSF	-	membrane bound granulocyte macrophage-colony stimulating factor
MHC	-	major histocompatibility complex
mRNA	-	messenger RNA
MTT	-	methylthiazol tetrazolium
Neut	-	neutralizing
NK	-	natural killer
OD	-	optical density
O ₅ O ₄	-	osmium tetroxide
OTA	-	ochratoxin A
PAP	-	peroxidase-anti-peroxidase
PBS	-	phosphate buffered saline

PCT	-	proximal convoluted tubules
PGE ₂	-	prostaglandin E ₂
PHA	-	phytohaemagglutinin A
PKC	-	protein kinase C
PMN	-	polymorphonuclear leucocytes
ppm	-	parts per million
PPs	-	pPhosphates
RBC	-	red blood cells
RES	-	reticular endothelial system
RNA	-	ribonucleic acid
RT	-	room temperature
SBTI	-	soybean trypsin inhibitor
SEM	-	standard error of the mean
SRBC	-	sheep red blood cells
TEM	-	transmission electron microscope
TGF	-	transforming growth factor
Th	-	T helper cell
TNF	-	tumour necrosis factor
WBC	-	white blood cells

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 STUDY BACKGROUND

Mycotoxins are a group of structurally diverse fungal secondary metabolites that elicit a wide spectrum of toxicologic effects (Pestka *et al.*, 2004). Of particular interest is the capacity of some mycotoxins to alter normal immune function when present in foods at levels below observable overt toxicity (Pestka and Bondy, 1990), and the fact that the immunotoxicity data for fungal toxins varies considerably for different toxins (Cooray, 1984).

The most well studied toxins are the aflatoxins (Boonchuvit and Hamilton, 1975; Hoerr and D'Andrea, 1983; Michael, *et al.*, 1973; Miller, *et al.*, 1978; Pier, *et al.*, 1970; 1972; 1980; Reddy *et al.*, 1987). These are immunomodulating agents that act primarily on cell-mediated immunity and phagocytic cell function (Giambrone, *et al.*, 1978; Paul *et al.*, 1977). Studies indicate that effects on haematopoietic cells may contribute to aflatoxin (AFB₁)-induced immunomodulation *in vivo*. Interleukin-1 (IL-1) production by peritoneal macrophages from rats given a single intraperitoneal (ip) injection of 1 mg/kg AFB₁ increased 1 d after dosing (Cukrova *et al.*, 1992). In contrast, fumonisin toxicity has been characterized relatively recently in comparison to aflatoxin and ochratoxin, and fumonisin-induced immunotoxicity is an area of active research. The fumonisin toxins are produced primarily by *Fusarium verticillioides* and *F. proliferatum* (Bacon and Nelson, 1994). Of particular note is the apparent species specificity of fumonisin toxicity. Purified fumonisin B₁ (FB₁) causes leukoencephalomalacia in horses (Marasas *et al.*, 1988) and pulmonary oedema in pigs (Harrison *et al.*, 1990). In mice the primary target is the liver, whereas in rats the kidneys are more sensitive (Gelderblom *et al.*, 1991; Voss *et al.*, 1993; 1995). In spite of these species

differences, the available data on immunotoxicity indicate that fumonisin-induced changes in immune function are not species specific, and appear to involve aspects of humoral, cellular, and innate immunity (Creppy *et al.*, 2004).

In vitro studies have focused on the effects of FB₁ on macrophage function and on lymphocyte proliferation (Tryphonas *et al.*, 1997). Fumonisin B₁ extracted from *F. verticillioides*-infected corn kernels reduced the viability and phagocytic activity of chicken peritoneal macrophages (Chatterjee and Mukherjee, 1994). Microscopic examination revealed increased nuclear disintegration in treated macrophages (Chatterjee *et al.*, 1995). This confirms earlier studies by Dombrink-Kurtzman *et al.*, 1994. Fumonisin B₁ inhibits murine spleen lymphocyte and turkey peripheral lymphocyte proliferation (Dombrink-Kurtzman *et al.*, 1994; Martinova and Merrill, 1995). The most striking morphological change in turkey lymphocytes was cytoplasmic vacuolization in treated cells (Dombrink-Kurtzman *et al.*, 1994). Similarly, bone-marrow lymphocyte cytoplasmic vacuolization has been observed consistently in rats and mice treated with FB₁ *in vivo* (Bondy *et al.*, 1996; 1997; 1998).

Some of the immunomodulatory effects of fumonisins may be the result of changes in the expression of cell surface markers important in immune cell communication. In mice given a single injection of 5 or 20 pg FB₁, CD3 receptor expression and sphingomyelin levels were decreased in thymocytes but not in splenocytes (Martinova *et al.*, 1995). Fumonisin-induced immunomodulation may also be due to changes in cytokine secretion. Selective induction of tumor necrosis factor (TNF)- α mRNA and secretion of TNF- α protein were observed in peritoneal macrophages from mice injected subcutaneously with FB₁ (0.25 to 6.75 mg/kg/d for 5d). Interleukin-1 α and interferon (INF) γ expression and secretion were not affected (Dugyala *et al.*, 1998).

In mammalian cells, FB₁ inhibits N-acyltransferase (ceramide synthase), which is the enzyme catalyzing the amide linkage of a fatty acid to sphinganine to form the complex sphingolipid dihydroceramide. This results in accumulation of substrate (free sphinganine) and depletion of product (dihydroceramide), as well as increased products associated with free sphinganine metabolism (Riley *et al.*, 1994; Wang *et al.*, 1991). Sphingolipid metabolites are involved in multiple signal transduction pathways and in the generation of intracellular second messengers (Spiegel & Merrill, 1996). Fumonisin is structurally similar to the protein kinase C (PKC) inhibitor sphingosine, and has been shown to inhibit PKC activity in CV-1 (African green monkey kidney) cells (Huang *et al.*, 1995) and to cause redistribution of PKC from cytosol to membrane in rat cerebral cortex slices (Yeung *et al.*, 1996). In CV-1 cells fumonisin also inhibits the transcription of cyclin dependent kinase inhibitors and induces cell-cycle arrest (Ciacci-Zanella *et al.*, 1998). *In vivo*, FB₁ has been shown to inhibit transcription of cyclin D1 and to increase transcription of the cyclin kinase inhibitor p27 in the livers of treated rats. Since immune responses are critically dependent on intracellular signalling, the relationship between fumonisin-induced disruption of sphingolipid metabolism and immunomodulation requires further exploration. Fumonisin B₁ has been used as a tool to show that ceramide production is a part of the signal pathway involved in T-cell-receptor-induced IL-2 production and apoptosis (Tonnetti *et al.*, 1999).

Fumonisin B₁ is a worldwide corn contaminant and has been epidemiologically linked to the high incidence of human oesophageal cancer in South Africa and China (Wang *et al.*, 2000). It is also hepatocarcinogenic in rats (Gelderblom *et al.*, 1991). Inhibition of ceramide synthase and disruption of membrane phospholipids have been shown to be mechanisms of its toxicity (Fukuda *et al.*, 1996; Wang *et al.*, 1991). The mechanism by which FB₁ acts as a carcinogen was reported by Ramljak and colleagues (2000). They demonstrated an overexpression of

cyclin D1 protein in both pre-neoplastic and neoplastic specimens obtained from a long-term feeding study of FB₁ in rats. In addition elevated Cdk4 activity was also shown. Pellagalli *et al.* (1999) demonstrated that FB₁ alters integrin adhesive activity, affecting all cellular integrin-dependent functions. Evidence for induction of apoptosis by FB₁ was first obtained when C6 glioma cells were incubated with fumonisin B₁ causing DNA fragmentation profiles showing laddering in gel electrophoresis and apoptotic bodies revealed with acridine orange and ethidium bromide. Further confirmation experiments and comet assays have been performed under similar conditions (Mobio *et al.*, 2000). Seegers *et al.* (2000) have shown that FB₁ influenced the effects of arachidonic acid, prostaglandins E2 and A2 on cell cycle progression, apoptosis induction, tyrosine and CDC2-kinase activity in oesophageal cells.

Ochratoxin A (OTA) has been characterized as a carcinogenic toxin, which can also cause kidney damage when consumed by humans (Nill, 2001; Wafa *et al.*, 1998). When ingested as a food contaminant, OTA is very persistent in humans with a blood half-life of thirty five days after a single oral dosage due to unfavourable elimination toxicokinetics. This renders the toxin among the most frequent mycotoxin contaminant in human blood (Petzinger and Ziegler, 2000; Schlatter *et al.*, 1996). Ochratoxin A is neither stored nor deposited in the body, but heterogenous distribution may impose damage to the kidneys (Petzinger and Ziegler, 2000). Human exposure occurs mainly through consumption of contaminated grain and pork products. Overall, the contamination of foods with OTA is still a widespread problem (Ehrlich *et al.*, 2002). The compound causes nephrotoxic effects in animals; furthermore it is hepatotoxic, immunosuppressive and teratogenic (Peraica *et al.*, 1999). Reviews of mycotoxin- induced immunotoxicity indicated that OTA acts on more than one aspect of the immune system, which parallels its multiple effects at the cellular level; it inhibits humoral, cellular and innate immunity, including cellular depletion of lymphoid

organs in broiler chicks, depressed delayed hypersensitivity responses in turkeys, depressed serum immunoglobulin concentrations in broiler chicks (Atroschi *et al.*, 2000; Bondy & Pestka, 2000) and depressed blood monocyte phagocytic activity in turkey poults (Chang and Hamilton, 1980).

To the best of my knowledge, no reports on experimental fumonisin and ochratoxins exposure on humans is available, although evidence exists linking these toxins to alterations in immune functions in animals (Bondy and Peska, 2000; Creppy *et al.*, 1983). In these models these toxins are immunomodulatory and mostly immunosuppressive (Berek *et al.*, 2001; Creppy *et al.*, 2004). This research is of vital importance as it is becoming evident from other studies that mycotoxins play a role in diseases of unknown aetiology and hence the human immune responses to these toxins need investigation. Hence, in this research we seek to investigate and understand the effect of the mycotoxins, fumonisin B₁ and ochratoxin A on the cells of the immune system, i.e., lymphocytes and neutrophils.

HYPOTHESIS

It is hypothesized that mycotoxins (FB₁ and OTA) impair the immune system, by affecting the mechanisms involved in cell cycle or by affecting cell communications by altering the expression of IL-1, TNF- α , IL -6, IL-8 and IL-10 in lymphocytes and neutrophils.

AIM

The aim of this study is to investigate *in vitro*, the effects of FB₁ and OTA on the functioning of the cells of the immune system, i.e., neutrophils and lymphocytes and secondly, to correlate the level of immune response to cells present in blood of patients with cancer (oesophageal and other).

RESEARCH OBJECTIVES

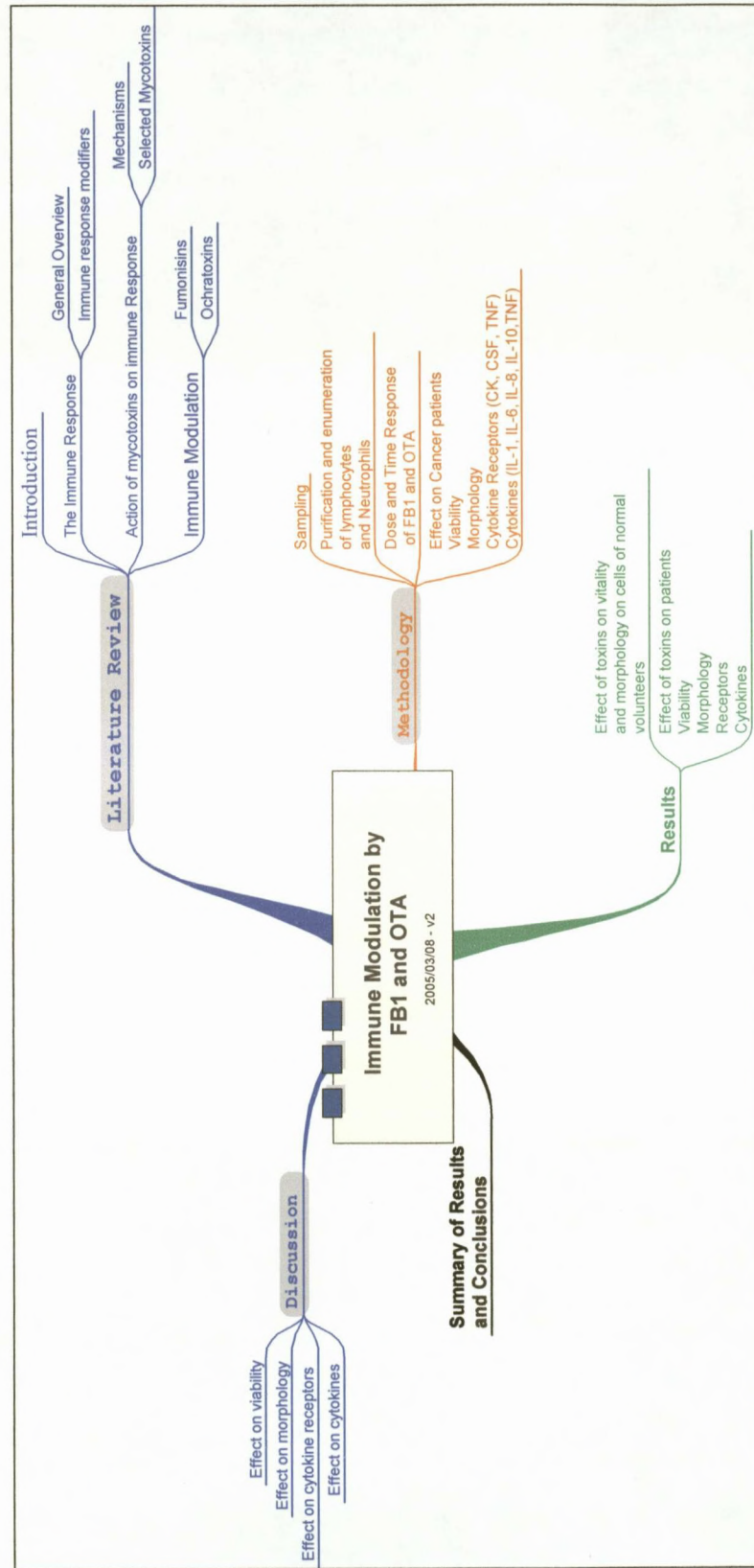
- To assess the effect of toxins on the viability and morphology of lymphocytes and neutrophils from healthy volunteers
- To investigate the effect of fumonisins and ochratoxins on the expression of cytokine receptors, *i.e.*, chemokine (CK), colony stimulating factor (CSF) and tumour necrosis factor (TNF), on the immune cells harvested from healthy individuals and from two patient groups.
- To determine the effect of the toxins in cancer patients, by evaluating differences in viability and morphology in healthy volunteers and patients with oesophageal cancer and other cancers.
- To compare levels of circulating cytokines (IL-1, TNF-alpha, IL-6, IL-8 and IL-10) in healthy individuals and two patient groups.

- To measure the effects of fumonisins and ochratoxins on the release of IL-1, TNF-alpha, IL-6, IL-8 and IL-10 from lymphocytes and neutrophils isolated from healthy individuals and from patients with oesophageal cancer and those with non-oesophageal cancer.

LIMITATIONS OF THE STUDY

- Immune function is usually determined by the use of lymphocyte proliferation assays and macrophage inhibitory assays. However, as results of these are evident in animal models, this study focuses on morphological and cytokine changes in cancer patients.
- All patients recruited in this study having other cancer, *i.e.*, non-oesophageal cancers were female patients with breast cancers.
- All experiments were not conducted on the same patients due to the fact that the patients do not return to the same hospital.

STRUCTURE OF DISSERTATION



1.2 THE IMMUNE RESPONSE

1.2.1 General overview

We live in a microbial world and our bodies are constantly being exposed to bacteria and their toxins, fungi and their toxins, parasites and viruses (Viljoen *et al.*, 1993). It is the function of the immune system to scan the body in order to identify any substance: natural or synthetic, living or inert- that it considers foreign and potentially harmful to the body. It distinguishes self from non-self and initiates the appropriate response. The immune response is the body's mechanism of controlling the spread of infection. Immune responses are mediated by specific cells with defined functions. The characteristics of the most important cells of the immune system and their appearances are presented in Figure 1.1 and Table 1.1.

The blood is comprised of four major components: plasma/serum, platelets, leucocytes and erythrocytes. The leucocytes, specifically, are the cells making up the immune system. The main types of leucocytes are: lymphocytes, granulocytes (comprised of neutrophils, basophils and eosinophils) and macrophages.

The lymphocytes and the neutrophils were the two cell types isolated for this study. These two cells are the main cell types that fight against infection. Neutrophils are phagocytic cells that engulf microbes present in inflamed tissue before the infection spreads (Harvey *et al.*, 1996). The lymphocytes are highly complex cells, comprised of B cells (cells maturing in the bone marrow) and T cells (cells maturing in the thymus). Lymphocytes are the cells responsible for producing antibodies against a particular antigen, thus aiding to protect the body from disease (Capra *et al.*, 1999).

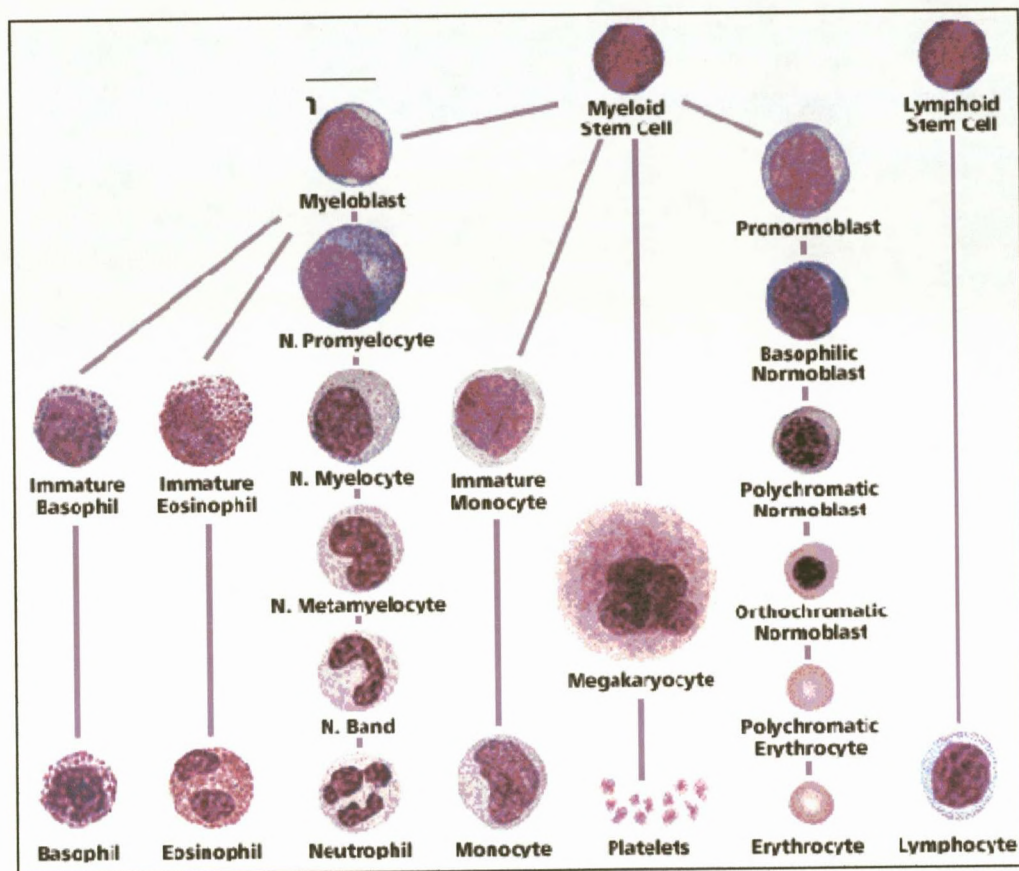


Fig. 1.1 Schematic representation of blood cell formation

Table 1.1 Cells of the immune response

Cells	Characteristics	Markers	Functions
Natural Cytolytic cells Natural killer cells	Large granular lymphocytes	Fc receptors for antibody; CD16, CD56, CD57	Kill antibody-decorated cells and virus-infected or tumour cell (no MHC restriction)
Phagocytic Cells Neutrophil (polymorphonuclear leukocytes) Eosinophils Macrophages	Granulocytes with short life span, multilobed nucleus and granules, segmented band forms (more immature) Bilobed nucleus, heavily granulated cytoplasm See below	- - -	Phagocytose and kill bacteria
Antigen-Presenting Cells (APCs) Monocytes* Macrophages* Langerhans' cells* Dendritic cells* Microglial cells* Kupffer cells* B cells	Found in lymphocytes, blood, lungs, and other organs Possible residence in tissue, spleen, lymph nodes, and other organs; activated by interferon- γ and TNF Present in the skin Lymph nodes, tissue CNS and brain Presence in liver See below	Class II MHC Expressing Cells Horseshoe-shaped nucleus, lysosomes, granules Large, granular cells; Fc and C3 receptors - - - -	Present Antigen to CD4 T cells Are precursors to macrophage-lineage, cytokine release Initiate inflammatory and acute phase response; activated cells are antibacterial and have antiviral and antitumour activities Transport antigen to lymph nodes Are efficient antigen presenters Produce cytokines Filter particles from blood (e.g. viruses)
Antigen-Responsive Cells T cells (all) CD4 T cells CD8 T killer cells CD8 T cells (suppressor cells)	Mature in thymus; large nucleus, small cytoplasm Helper/DTH cells; activation by APC's through class II MHC antigen presentation TH1 subtype TH2 subtype Recognition of antigen presented by class I MHC antigens Recognition of antigen presented by class I MHC antigens	CD2, CD3, T-cell receptor CD2, CD3, T-cell receptor, CD4 IL-2, Ifn- γ , LT production IL-4, IL-5, IL-6, IL-10 production CD2, CD3, T-cell receptor, CD8 CD2, CD3, T-cell receptor, CD8	Produce IL-2, other cytokines; stimulate T- and B-cell growth; promote B-cell differentiation (class-switching), antibody production Promote initial defenses (local), DTH, T killer cells Promote later humoral responses Kill viral tumour, non-self (transplant) cells; secrete TH1 lymphokines Suppress T- and B-cell response
Antibody-Producing Cells B cells Plasma cells	Mature in Peyer's patches, bone marrow, bursal equivalent; large nucleus, small cytoplasm; activation by antigens and T-cell factors Small nucleus, large cytoplasm	Surface antibody, class II MHC antigens -	Produce antibody and present antigen Are terminally differentiated, antibody factories
Other Cells Basophils/mast cells	Granulocytic	Fc receptor for IgE	Release histamine, provide allergic response, are antiparasite

* Monocyte, macrophage lineage.

APCs, antigen presenting cells; CNS, central nervous system, DTH, delayed-type hypersensitivity, Ig, immunoglobulin; IL, interleukin; LT, lymphotoxin; MHC, major histocompatibility complex; TNF, tumour necrosis factor

Immune cells communicate by direct cell-to-cell interactions (touch), and by the sensing of soluble molecules that include molecules such as cytokines, chemokines, interferons, steroids and prostaglandins (Murray *et al.*, 2002). The cells of the immune system function in an integrated manner through two pathways: innate (natural) immunity, and acquired immunity (Blaney and Howard, 2000).

Innate immunity involves a large number of different cell populations including: epithelial cells, monocytes, macrophages, dendritic cells, polymorphonuclear leucocytes (PMN), natural killer (NK) cells, and various lymphocyte subpopulations, which bridge the divide between innate and acquired immunity. These cells generally arise from precursor cell populations in the bone marrow. Humoral systems are also important and include many cytokines (Table 1.1), certain enzymes (e.g. lysozyme), metal-binding proteins, integral membrane ion transporters, complex carbohydrates, and complement pathways.

Acquired immunity, or adaptive immunity, comprises a unique cellular process whereby genetic 'mutation' occurring in two specialised cell populations, B- and T-lymphocytes (Figure 1.2), produces numerous molecular 'shapes' that are expressed as antibodies and T-cell receptors. If these molecules bind to structurally related proteins called antigens, and provided costimulatory signals are present, proliferation of antigen-specific lymphocytes occurs and a specific immune response is generated (Adam *et al.*, 2003).

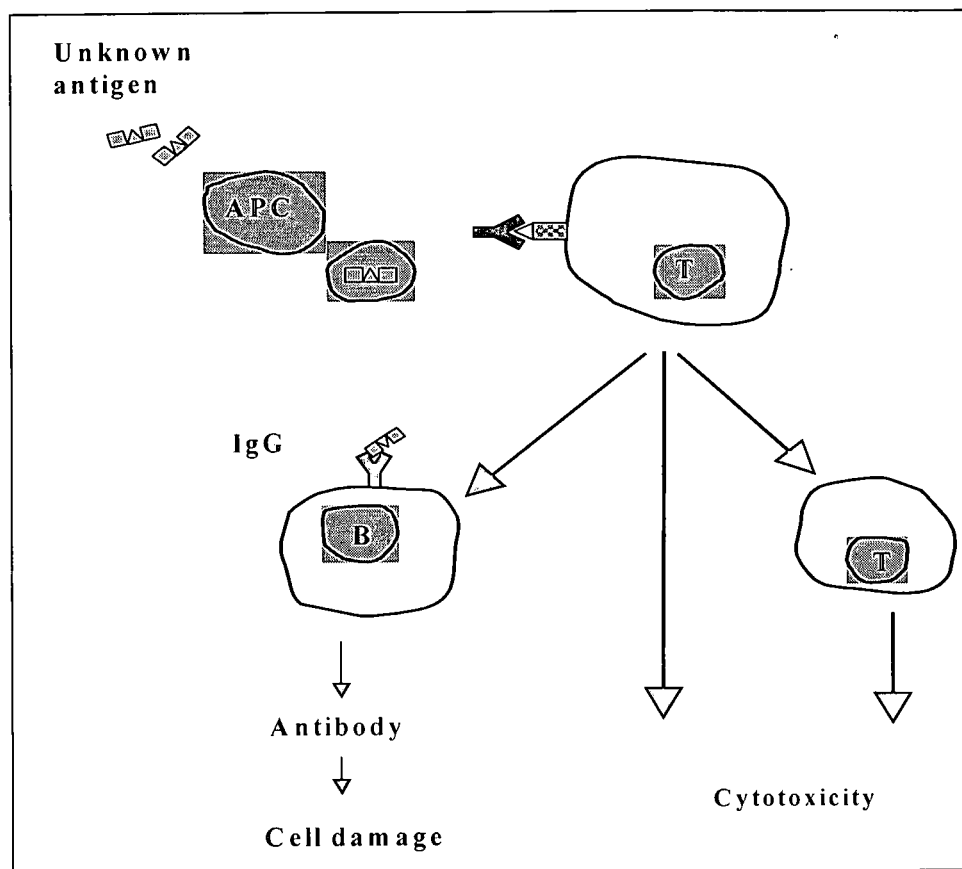


Fig. 1.2 Antigen recognition and antibody formation

The specificity of immune response resides in selective clonal proliferation of lymphocytes. Figure 1.3 is a schematic representation of the events that occur in the immune response at the cellular level.

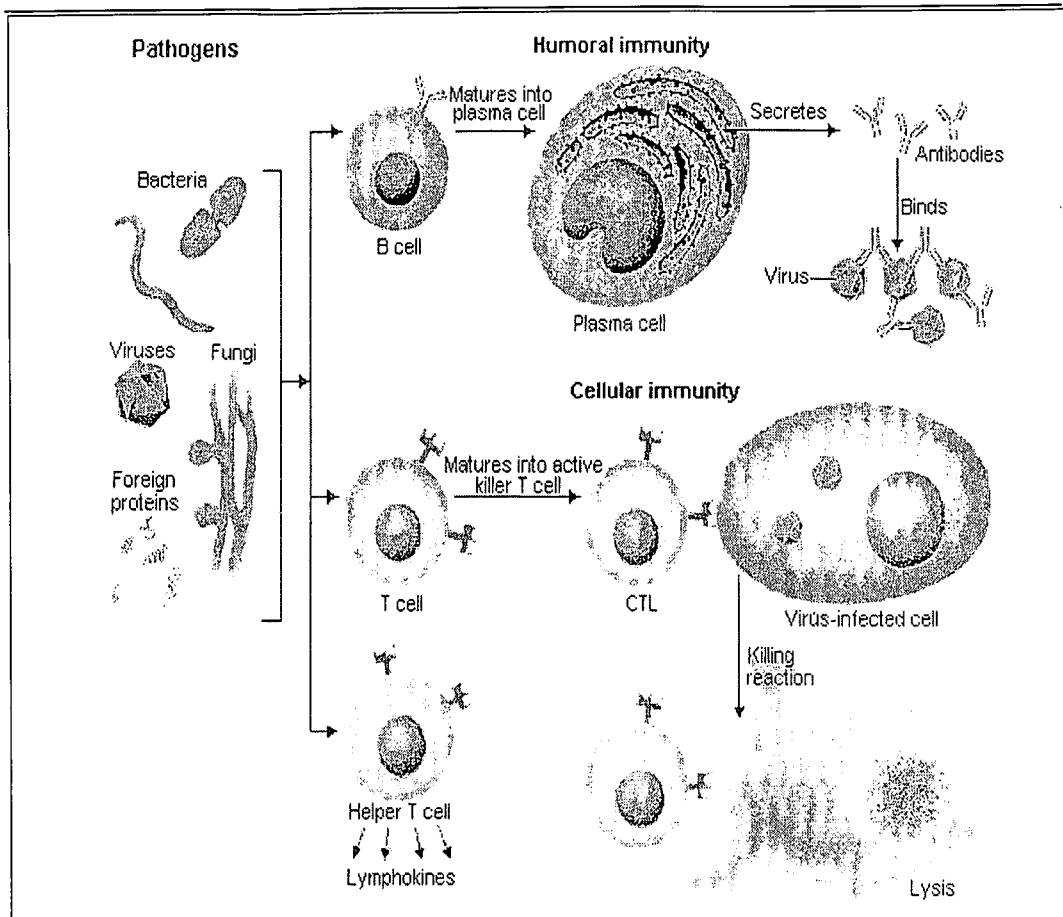


Fig. 1.3 The B-cell and T-cell response to non-self antigens

Two further families of molecules play major roles in acquired immunity: major histocompatibility complex (MHC) gene products, and cytokines (Borish and Steinke, 2003). Cytokine formation, release, and target interactions form an important arm of the cellular response in growth, repair, and cell proliferation. The source, major target cells and function of the major cytokines are listed in Table 1.2.

Table 1.2 Cytokines and chemokines

Factor	Source	Major target	Function
Interferon- α , interferon- β	Leucocytes, fibroblasts	Virally infected cells, tumour cells; NK cells	Induction of antiviral state; activation of NK cells, enhancement of cell-mediated immunity
Interferon- γ	CD4 TH1 cells, NK cells	Macrophages, * T cells	Activation of macrophage, promotion of inflammation, promotion of TH1 and inhibition of TH2 responses
IL-1 α , IL-1 β	Macrophage, fibroblasts, epithelial cell, endothelial cells	T cells, B cells, PMN, tissue, central nervous system	Many actions: promotion of inflammatory and acute phase responses, fever, activation of T cells
TNF- α (cachectin)	Similar to IL-1	-	Similar to IL-1, antitumour and wasting (cachexia-weight loss) functions
TNF- β	T cells	PMN, tumours	Lymphotoxin: tumour killing, activation of PMN
Colony-stimulating factors (e.g., GM-CSF)	T cells, stromal cells	Stem cells	Growth and differentiation of specific cell types
IL-2	CD4 T cells (TH0, TH1)	T Cells, B cells, NK cells	T- and B-cell growth
IL-3	CD 4 T cells (keratinocytes)	Stem cells	Differentiation
IL-4	CD4 (TH0, TH2), T cells	B and T cells	B-cell growth and differentiation; Ig production; TH2 responses
IL-5	CD4 TH2 cells	B cells, eosinophils	B-cell growth and differentiation, IgA and IgE production, eosinophil production, allergic responses
IL-6	Macrophage, T and B cells, fibroblasts, epithelial cells, endothelial cells	T cells and B cells, hepatocytes	Stimulation of acute-phase and inflammatory responses, fever, Ig secretion, B-cell growth and development
IL-7	Bone marrow, stroma	Presursor cells and stem cells	Growth of pre-B cell, thymocyte, T cell, and cytotoxic lymphocyte
IL-10	CD4 TH2 cells	B cells, CD4 TH1 cells	B-cell growth, inhibition of TH1 response
IL-12	Macrophage	NK cells, CD4 TH1 cells	Activation
TGF- β	CD4 TH3 cells	B cells	IgA production; immunosuppression of B, T and NK cells and macrophages; promotion of oral tolerance, wound healing
A-chemokines: C-X-C chemokines – two cysteines separated by one amino acid (IL-8; IP-10; GRO- α , GRO- β , GRO- γ	Many cells	Neutrophil, T cells, macrophages	Chemotaxis, activation
β -chemokines: C-C chemokines – two adjacent cysteines (MCP-1; MIP- α ; MIP- β ; RANTES.	Many cells	T cells, macrophages, basophils	Chemotaxis, activation

* Applies to one or more cells of the monocyte-macrophage lineage.

GM-CSF, granulocyte macrophage-colony stimulating factor; GRO, growth related oncogene; Ig, immunoglobulin; IL, interleukin; IP, interferon- α protein; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; NK, natural killer; PMN, polymorphonuclear leucocytes; RANTES, regulated on activation, normal T expressed and secreted; TNF, tumour necrosis factor

The notable cytokines modulating the proliferation of tumour cells are probably transforming growth factor- β (TGF- β) family, epithelial growth factor, and colony stimulating factors (Waller and Ernstoff, 2003).

The cytokines provide an essential link for cell-to-cell communication and component of the acquired as well as the innate immune response (Clark and Ledbetter, 1994). The cell surface protein CD40 with its receptor provides a costimulatory signal for the interaction between T-cells and antigen presenting cells (APC) (Chen *et al.*, 2002).

Cell populations play a major role in both innate and acquired immunity; however, the two key cell populations that essentially define acquired immunity are the B- and T-lymphocytes (Gowans *et al.*, 1962). Acquired immunity, therefore, involves a wide range of antigen receptors expressed on the surface of T- and B-lymphocytes that detect non-self molecules.

Molecules that act as recognition 'receptors' in innate immunity comprise humoral proteins (c-reactive protein, serum amyloid, mannose binding protein, and CD14) and cellular receptors (scavenger and mannose receptors, dendritic cell targets, CD14, CD35, CD21, and CD11b [Table 1.3]).

Table 1.3 Cellular receptors of the immune system

CD number	Function
CD1a/1b/1c	Peptide, lipid antigen
CD2	presentatation
CD3	T-cell adhesion to APC
CD4	T-cell activation
CD5	Th activation
CD8	T-cell activation
CD11a/CD18	T-cell subpopulation marker
CD11b/CD18	Leucocyte adhesion protein
CD11c/CD18	Leucocyte adhesion protein
CD14	Leucocyte adhesion protein
CD16	LPS binding protein
CD19	Phagocytosis and ADCC
CD23	B-cell activation/proliferation
CD28	B-cell activation/IgE regulation
CD54	Costimulatory molecule
CD62E	Adhesion molecule
CD71	Vascular adhesion molecule
CD80	Receptor for transferring
CD86	Costimulatory molecule
CD88	Costimulatory molecule
CD152	C5a receptor
	Costimulatory molecule

T-cells instruct affected host cells to either shut down protein synthesis or self-destruct. B-cells respond to antigens by secreting their own antigen receptors as antibodies. Antibodies also call on the innate immune system for help. Stimulation of antigen through the B-cell receptor followed by T-cell activation drives proliferation and differentiation of antigen-

specific naive B-lymphocytes into memory B-cells and plasma cells. Memory B-cells mediate secondary immune responses and plasma cells sustain antibody production for several months (Blalock and Smith, 1985).

The different subpopulations of T-cells (Mason, 1987) are recognized largely by their expression of surface proteins (CD markers). All T-cells express CD3, a hetero-oligomeric protein that is part of the T-cell receptor complex, and can be further subdivided into those cells that express CD4 and those that express CD8. The CD4 lymphocyte population can be further functionally subdivided into two subpopulations termed Th₁ and Th₂ lymphocytes (Mason, 1988). Th₁ and Th₂ cells are initially discriminated not on the basis of cell surface markers but on the basis of the patterns of cytokines they produce. In very recent years, it has proved possible to identify these distinct CD4 subpopulations on the basis of their expression of chemokine receptors.

Dendritic cells constitute a family of APC defined by their morphology and their capacity to initiate primary immune response (Rafiq *et al.*, 2002). Langerhans cells are paradigmatic dendritic cells, described in 1868 by Paul Langerhans, in Berlin. Langerhans cells are present with epithelial cells in the epidermis, bronchi, and mucosae (Figure 1.4). After antigenic challenge, the dendritic cells migrate into the T-cell areas of proximal lymph nodes where they act as professional APC. Langerhans cells originate in the bone marrow and CD34+ haematopoietic progenitors are present in cord blood or circulating blood. They are actively involved in skin lesions of allergic contact dermatitis or atopic dermatitis in cancer immunosurveillance (Schmitt, 2001). Recently, several studies have shown that vaccine therapy using dendritic cells (DCs) genetically engineered to express a surrogate

tumour antigen can effectively induce antitumour immunity (Candido *et al.*, 2001; Nakamura *et al.*, 2002; Paul, *et al.*, 2002; Tirapu *et al.*, 2002).

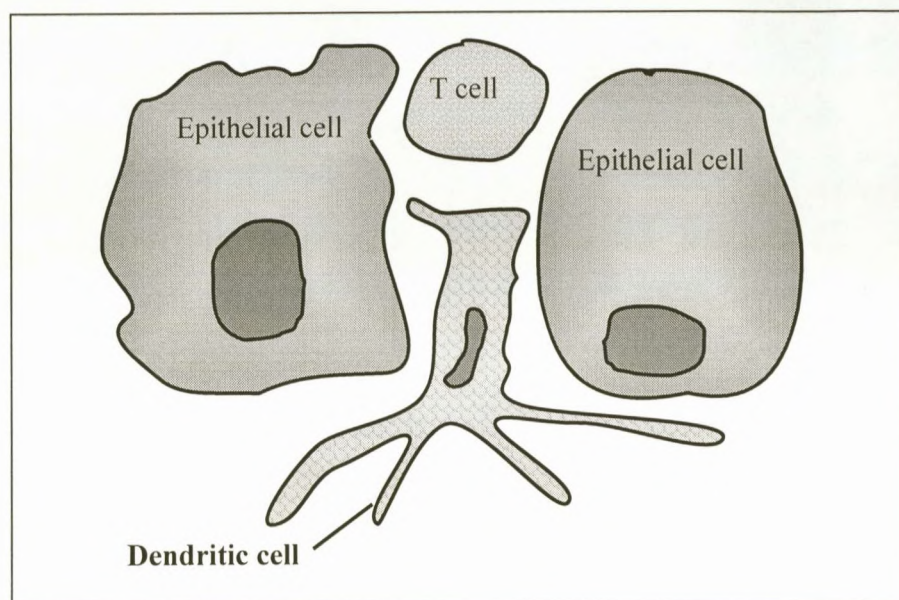


Fig. 1.4 Dendritic cell interactions

Human neutrophil transcribes and secretes peptides termed α -defensin-1, -2, and -3 in response to non-self proteins. Defensins have the ability to signal activation of cells involved in adaptive immunity, specifically CD8⁺ T. Alpha-defensin and β -chemokines (MIP-1 α , MIP-1 β , and RANTES) may be cooperatively involved in cell defense. Beta-defensins are small peptides of the innate immune system. Beta-defensins may act directly on immature dendritic cells, as an endogenous ligand for Toll-like receptor 4, to up-regulate dendritic cell maturation, thereby triggering adaptive immune responses. It is suggested that β -defensins may provide molecular immunosurveillance against tumour antigens.

The role of macrophages in tumour growth and development is complex and multifaceted (Gough *et al.*, 2001). Whilst there is limited evidence that tumour-associated macrophages can be directly tumouricidal and stimulate the antitumour activity of T-cells, there is now contrasting evidence that tumour cells are able to block or evade the activity of tumour associated macrophages at the tumour site (Bingle *et al.*, 2002).

1.2.2 Immune response modifiers

These are molecules, either extrinsic or intrinsic to the host, that affect the immune response. One group of extrinsic modifiers is referred to as *immune potentiators*. These include BCG, *Clostridium parvum* and endotoxin, which are all microbes or microbial products that have been shown to modify the immune response and, under certain conditions, to cause tumours to regress or grow more slowly than usual. The intrinsic group, known as *biological response modifiers*, includes IL-1, IL-2, IFN (α , β and γ), TNF, B-cell growth factors, and haematopoietic growth factors (such as colony stimulating factors). These agents exert their influence at different stages of the immune response (Gupta and Kanodia, 2002; Minter *et al.*, 2000).

Haematopoietic growth factors are often combined with chemotherapy and radiotherapy to restore bone marrow function. Thalidomide, which suppresses TNF- α production and has antiangiogenic properties, is currently under evaluation in several cancers. Currently, there are more than 20 IL and at least 5 other proteins have just been found which are likely to be termed IL. The cellular sources and functions of these IL are provided in Table 1.4. Cytokines, the messengers of the immune system, are either proteins or glycoproteins, secreted by immune cells. They have autocrine and paracrine functions, so

that they function locally or at a distance to enhance or suppress immunity. Currently, in cancer therapy, cytokines are used to enhance immunity (Paul *et al.*, 2002; Tirapu *et al.*, 2002). They also regulate the adaptive immune system, the T- and B-cell immune responses. In the immune system, cytokines function in cascades. Thus, clinical trials of individual cytokines are rarely useful, since cytokines tend not to work individually but probably cooperatively (Muller and Pawelec, 2003; Verheyen *et al.*, 2000). Some of the individual cytokines that have been tested and found ineffective for cancer treatment include IL-1 β , although it may be useful still because it helps to mediate the severe toxicity of IL-2. Interleukin-2 is the most widely studied IL and is used for immunostimulation in metastatic renal cell carcinoma and malignant melanoma (Lohr *et al.*, 2001). Although the use of TNF certainly sounded promising, but because it caused severe hypotension when used systemically, its value is limited. Interleukin-4 shows minimal anticancer activity and is toxic. Interleukin-6 has some activity against cancer cells but turns out to be a growth factor for myeloma cells. Granulocyte macrophage-colony stimulating factor (GM-CSF) used primarily in stem cell transplant to reconstitute the myeloid series has been studied for melanoma with controversial results.

Which cytokines are important for cancer?

Cytokines are a family of peptide molecules that are responsible for direct cell-to-cell communication. They mediate interactions between leucocyte populations and between leucocytes and tissue cells (Table 1.4). Cytokines of the IL family, derived from APC (namely, IL-1 and IL-6), also provide costimulatory signals that result in T-cell activation. T-cell derived lymphokines and the contact between T- and B-cells through specific pairs of receptors and co-receptors provide signals essential for B-cell stimulation (Clark and

Ledbetter, 1994; Davies and Gillies, 2003). T-cell proliferation is the result of IL-2 expression that is dependent on T-cell activation.

Table 1.4 Cellular expression and actions of cytokines

Cytokine	Source	Actions
IL-1	Macrophages, fibroblasts, synovial lining cells, T- and B-lymphocytes, endothelial cells	<ul style="list-style-type: none"> - Stimulates production of IL-6 and TNF-α - Augments T-cell proliferation and B-cell activation - Induces hepatic production of acute phase proteins - Activates neutrophils to synthesize and release PG - Increases binding of lymphocytes and monocytes to endothelial cells - Induces endothelial cell proliferation
TNF	Macrophages, monocytes	<ul style="list-style-type: none"> - Stimulates production of IL-1, -6, and -8 - Increases PGE₂ and collagenase production - Increases plasminogen activity - Increases release of FGF - Modulates PMN function such as the release of oxygen metabolites, phagocytosis, adhesion to endothelium, and ability to degrade cartilage
IL-6	Neutrophils, monocytes, fibroblasts, T- and B-cells, endothelial cells	<ul style="list-style-type: none"> - Stimulates release of hepatic acute phase proteins - Induces activated B-cells to differentiate into plasma cells
IL-8	Neutrophils, fibroblasts, hepatocytes, epithelial and endothelial cells	<ul style="list-style-type: none"> - Stimulates and attracts neutrophils
GM-CSF	Macrophages, fibroblasts, endothelial cells, and activated lymphocytes	<ul style="list-style-type: none"> - Stimulates secretion of IL-1, TNF-α, and PGE₂ - Activates chemotaxis, phagocytosis, antibody cytotoxicity, and oxidative metabolism in granulocytes - Induces HLA-DR expression in monocytes

Abbreviations: IL, interleukin; TNF, tumour necrosis factor; PG, prostaglandin; FGF, fibroblast growth factor; GM-CSF, granulocyte-macrophage colony stimulating factor.

The net result of cytokine production is the emergence of antigen specific, tissue infiltrating, and destructive T-cells (Chen *et al.*, 2003). Cytokines also activate macrophages and other inflammatory cells and the production of antibodies by stimulated T-cells. Cytokines can amplify the ongoing immune response by up-regulating the expression of human leucocyte antigen (HLA) and costimulatory molecules (such as 137) on parenchymal cells and APC. The costimulators direct T-cell differentiation, for example, into a CD4⁺ Th₁ cell, which secretes lymphokines, facilitating cytolytic T lymphocytes killing of cells (Somasundaram *et al.*, 2002), or differentiates into a CD4⁺ Th₂ cell, which stimulates antibody production by B-cells (Dallman, 1995). Cell killing may occur via specific T-cell products, such as granzyme B (a serine esterase protein) and perforin (a pore-forming lytic protein), which have been reported to correlate closely with acute rejection of grafts (Clement *et al.*, 1994). The type of organ grafted, HLA matching between donor and host and the degree of presensitisation, influence the acute rejection process. CD4⁺ T-helper cells are the primary, initiating, and organizing component of host immunoresponsiveness against grafts. CD8⁺ cells are recruited secondarily to the site to complete the acute rejection process (Mason and Morris, 1986; Mason, 1987). It is considered that these cellular and molecular responses observed during graft rejection will apply to transformed cells as they become carcinogenic.

In this study the expression of the following cytokines were studied: IL-1, IL-6, IL-8, IL-10 and TNF- α . Interleukin-1, IL-6, IL-8 and TNF- α are proinflammatory and IL-10 is anti-inflammatory (Figure 1.5). At present, cytokines are divided into six families (Henderson, *et al.*, 2000). The subdivisions are based upon a number of criteria including historical subdivisions and biological actions. These families are depicted in Table 1.5.

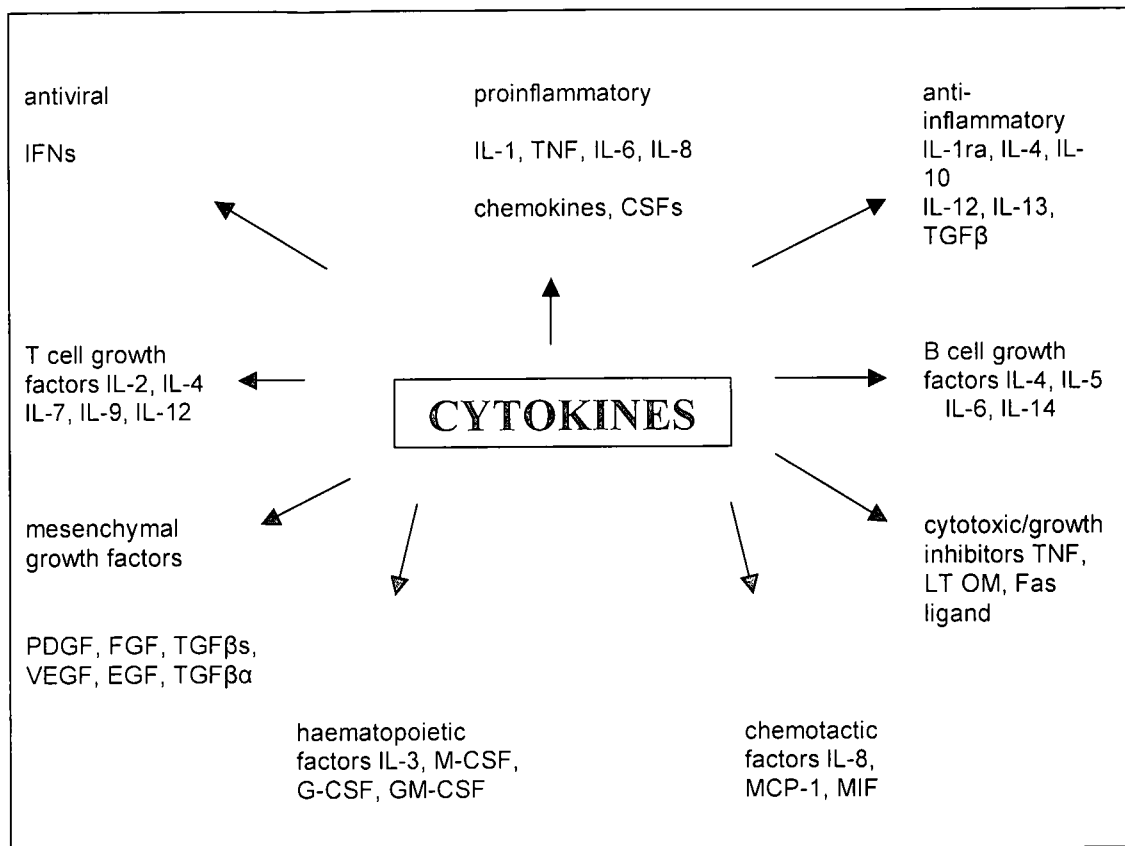


Fig. 1.5 Subdivision of cytokines based on their biological actions

Table 1.5 Cytokine family and their criteria

No.	Cytokines Families	Historical subdivisions and biological actions
1.	Interleukins (e.g. IL-1, IL-8)	Many interleukins are growth factors for lymphocytes but this family of proteins also contains a colony stimulating factor (IL-3) and a chemokine (IL-8). This only shows that the name given to a particular cytokine defines its major biological function.
2.	Cytotoxic cytokines (e.g. TNF- α , TNF- β , CD40)	TNF, now recognised as a key protein in infection, was identified in endotoxin- injected mice as an activity which could kill certain tumour cell lines. It was reported that injection of bacterial filtrates into cancer patients could cure them. Experiments have been repeated over the past decade with recombinant TNF and have shown some clinical benefit.
3.	Interferons (e.g. alpha, beta, lamda INF)	These have a major function in inhibiting the growth and spread of viruses and toxins. The alpha INF's have the most potent antiviral actions.
4.	Colony stimulating factors (CSF)	These are a small group of cytokines which are involved in the growth and differentiation. These cytokines also have actions on the mature cells.
5.	Growth factors	The term growth factor encompasses a very large number of proteins. These cytokines can act on fibroblasts as well as epithelial cells. However, some of the growth factors are able to inhibit the growth of certain cells.
6.	Chemokines	This is a large group of peptide chemotactic factors.

1.2.2.1 Interleukin-1

The IL-1 family consists of IL-1 α , IL-1 β and IL-1 receptor antagonist (IL-1RA), which are structurally related to each other and have similar affinity for IL-1 receptors on cells (Dinarello, 1994). Interleukin-1 α and IL-1 β are potent agonists that elicit various biological responses, whereas IL-1RA blocks the effect of the agonists by competing for binding sites on the cell surface receptors (Arend, 1993). Interleukin-1 α , IL-1 β and IL-1RA are encoded by separate genes; designated as *IL1A*, *IL1B*, and *IL1RN*, respectively. The three genes are clustered on the long arm of human chromosome 2 in a region (q13-q21) that spans more than 430 kilobases (Nicklin *et al.*, 1994). Three related cell surface proteins are involved in IL-1 binding and signalling; type 1 IL-1R, type 2 IL-1R and the IL-1R associated protein (Sims and Dower, 1994). The IL-1R genes are members of the large immunoglobulin supergene family, and are located in the same region of human chromosome 2 as their ligands.

Interleukin-1 is produced during antigen presentation and is secreted by macrophages, fibroblasts, endothelial cells, as well as T and B lymphocytes (Lorenzo, 1991). It has a wide range of biological actions, and acts via modulating gene expression in target cells. Both IL-1 α and IL-1 β possess co-mitogenic properties, recruit cells to the cancer site, and stimulate the production of pro-inflammatory mediators, including IL-6 and TNF (Woods *et al.*, 1998). Interleukin-1 has been shown to augment T-cell proliferation and B-cell activation in response to antigenic challenge (Dinarello *et al.*, 1986). The cytokine activates neutrophils to synthesize and release prostaglandins (Rossi *et al.*, 1985), enhance binding of lymphocytes and monocytes to endothelial cells and induce neovascularization, a process that may encompass tumour initiation of new blood vessels.

1.2.2.2 Tumour necrosis factor

Tumour necrosis factor is produced principally by macrophages and monocytes. The production of TNF is stimulated by several factors, including lipopolysaccharide (LPS), Interleukin-1, GM-CSF, and mediates its effects by interaction with two related membrane receptors: TNF-R1 and TNF-R2 (or type I and type II). Tumour necrosis factor α is thought to be the controlling element in the 'cytokines network', and is responsible for the production of other cytokines for example, IL-1, IL-6 and IL-8 (Brennan *et al.*, 1992). A deficiency of TNF- α in mice promoted cancer (Suganuma, 1999).

1.2.2.3 Interleukin-6

Interleukin-6 is the end product of a cytokine signaling cascade, monocytes, T lymphocytes, and fibroblasts and is secreted by specialized immune cells during inflammation. It has great influence on many functions, including differentiation, stimulation, activation of immune cells, or other cells of neuroendocrine origin. Thus, IL-6 serves as a key messenger in communication with the neuroendocrine system, and serves as a potent activator of the hypothalamic-pituitary-adrenal axis at all levels. Changes in the levels of expressions of this cytokine and its receptor have been observed during chronic inflammatory disease, and have been associated with tumorigenesis (Hayashi *et al.*, 2002; Willenberg *et al.*, 2002). The synthesis of this cytokine is induced by IL-1 and TNF α (Wong and Clark, 1988). Interleukin-6 stimulates activated B cells to differentiate into plasma cells which produce immunoglobulins (Arend and Dayer, 1990). Circulating levels of IL-6 are elevated in prostate carcinoma (Adler *et al.*, 1999) and lymphoma (Kurzrock *et al.*, 1993), and reduced in undifferentiated thyroid carcinoma (Basolo *et al.*, 1998).

1.2.2.4 Interleukin-8

Interleukin-8 is a member of the chemokine supergene family. The chemokines belong to two related polypeptide families, C-X-C and C-C chemokines, as defined by the location of the two cysteine residues at the amino terminus. In the C-X-C family, the cysteine residues are separated by a non-conserved amino acid and in the C family the cysteine residues are in juxtaposition (Baggiolini *et al.*, 1994). The C-X-C chemokines are clustered on human chromosome 4. It is a potent neutrophil attractant and stimulator, and is produced by neutrophils, fibroblasts, hepatocytes, epithelial and endothelial cells (Baggiolini *et al.*, 1989). Interleukin-1, TNF, and LPS stimulated neutrophils exhibit increased expression of IL-8 mRNA and IL-8 production (Chen *et al.*, 2003; Strieter *et al.*, 1992).

1.2.2.5 Interleukin-2

The lymphokine, IL-2 is essential in stimulating T and B cell populations to divide and expand their clones. Interleukin-2 is produced by antigen-stimulated T lymphocytes and must be present in sufficient quantities in order to mount an effective counterattack against cancer cells. Increased formation of IL-2 is an important way of expanding of Tc lymphocyte population. These killer cells can accomplish lysis of tumour cells through cell-to-cell contact. Two categories of this cell type are believed to exist, namely, (1) antigen-specific, MHC-restricted (well established) and (2) broad specificity, non-MHC-restricted Tc lymphocytes. Thus, antigen-specific killer cells would recognise cells with specific tumour markers, whereas those with broad specificity could lyse a variety of different targets on the tumour cell.

Current clinical trial data suggests that combined IL-2 and IFN- α administered subcutaneously in accordance with specified regimes achieves long-term survival benefits in subsets of patients with metastatic renal cell carcinoma (Atzpodien *et al.*, 2002). Combining immune modulators may be a way forward for metastatic carcinomas (Liu *et al.*, 2002; Wigginton *et al.*, 2002). The immunobiological agents: IL-2 and IFN- α , when combined with meroxyprogesterone, produce good response rates and low toxicity (Naglieri *et al.*, 2002). However, it should be remembered that clinical trials restricted to IL-2 or IFN- α alone have given contradictory results in the treatment of metastatic renal cell carcinoma (Ravaud *et al.*, 2002).

1.2.2.6 Interleukin-12

Interleukin-12 is a very exciting cytokine. It is a heterodimeric protein that promotes NK and T-cell activity and is a growth factor for B-cells. Interleukin-12 plays a central role in T-cell-mediated immune responses (Portieljie *et al.*, 2003). Endogenously formed IL-12 confers T-cells with a tumour migratory capacity and at the same time entices tumour cells to accept tumour-migrating T-cells (Uekusa *et al.*, 2002). It has demonstrated antitumour activity in mouse models. Alone, IL-12 shows minimal potential for therapeutic effect (Atkins *et al.*, 1997). Essentially, the efficacy of IL-12 is dependent on stimulating Th₁ cells to release IFN- γ (Segal *et al.*, 2002).

1.2.2.7 Granulocyte-macrophage colony stimulating factor

Granulocyte-macrophage colony stimulating factor is a growth factor that is synthesized by macrophages, fibroblasts, endothelial cells and activated lymphocytes (Groopman *et al.*,

1989). It was first characterised based on promotion of growth and differentiation of granulocytes and macrophages. Granulocyte-macrophage colony stimulating factor stimulates the secretion of IL-1 and enhances the secretion of TNF- α and prostaglandin E₂ (PGE₂) from macrophages (Fischer *et al.*, 1988; Heidenreich *et al.*, 1989). In addition, GM-CSF activates chemotaxis, phagocytosis, antibody dependent cytotoxicity and oxidative metabolism in granulocytes (Firestein, 1994), and induces HLA-DR expression on monocytes (Xu *et al.*, 1989).

Cancer vaccines composed of tumour cells engineered to secrete the cytokine, GM-CSF, are currently being clinically evaluated. Although immune recognition of tumours is known to occur, the failure of the host to either suppress or attenuate progression of the disease may reflect limited immunogenicity arising from the absence of critical determinants like the tumour augmenting family of cytokines (Dranoff, 2002). To enhance the immunogenicity of GM-CSF-secreting tumour cell vaccines, a novel approach expressing GM-CSF as a membrane-bound form (mbGM-CSF) on the tumour cell surface has been investigated. The intent is to enhance antigen presentation by increasing interactions between the tumour cell lines in the vaccine and GM-CSF receptor-positive APC, notably the patient's Langerhans cells (dendritic cells) residing within the intradermal injection site. Tumour cells have been engineered to express either membrane-bound or secreted GM-CSF (Galea and Cogne, 2002; Yei *et al.*, 2002). Granulocyte-macrophage colony stimulating factor has been approved also for use in stem cell and bone marrow transplant to reconstitute the myeloid series. Granulocyte-macrophage colony stimulating factor is also being evaluated as an adjuvant for vaccine therapy (Rini *et al.*, 2003).

1.2.2.8 Interferon- α

Interferon was isolated in 1970 from white cells and called IFN because it interfered with viral infection. Interferon- α is actually a family of molecules comprising at least two types. They are encoded by closely related genes on chromosome 9, encoding proteins that are variably glycosylated. These comprise of about 150 amino acids and bind to certain receptors on the surface of immune cells. They are known to have profound and diverse effects on gene expression. Interferon- α has many roles. It up-regulates MHC class I, tumour antigens, and adhesion molecules. It is also an antiangiogenic agent, which is very active in the immune system, promoting B- and T-cell activity. Interferon- α stimulates macrophages and even dendritic cells and up-regulates Fc receptors. The mode of action of IFN- γ appears to be through activation of the host immune system, which depends on the intrinsic immunogenicity of the target tumour cell (Gri *et al.*, 2002).

Interferon's activity in cancer has been well documented (Allan *et al.*, 1995; Dorval *et al.*, 1987; Foon *et al.*, 1986; Kirkwood *et al.*, 1996; 2000; 2001; Quesada *et al.*, 1986) and is indicated for the treatment of certain leukaemias (Foon *et al.*, 1986; Zinzani *et al.*, 1997) and Kaposi's sarcoma in order to inhibit tumour proliferation and angiogenesis. The efficacy of IFN- α has been well established for the treatment of advanced melanoma (Huang *et al.*, 1996; Martinez-Escribano *et al.*, 2002) and renal cell carcinoma patients (Bukowski *et al.*, 2002).

1.3 ACTION OF MYCOTOXINS ON THE IMMUNE SYSTEM

Mycotoxins act as immunomodulators, mostly as immunosuppressors (Richard, 1991; cited by Bondy and Pestka, 2000). The importance of this immunosuppressive activity is difficult to assess because immunosuppression is likely to be overshadowed by an infectious disease (Corrier, 1991). Experimental evidence suggests that this mycotoxin activity is of potential significance to both human health (Bauer, 2004; Kovacs, 2004) and economic losses in livestock production (Bennett and Klich, 2003), particularly where aflatoxins, some trichothecenes and OTA are involved (Fink-Grimmels, 1999; Sharma, 1984; 1991).

1.3.1 Mechanisms involved in immunotoxicity

Little information exists on how mycotoxins produce immunotoxicity. Some mycotoxins such as AFB₁ and fusarium T-2 toxin inhibit protein synthesis and cell proliferation (Ueno *et al.*, 1995). This inhibition may not be the primary mechanism involved in their immunotoxic effects: both have selective effects on various subpopulations of lymphocytes. Several mycotoxins are cytotoxic to lymphocytes *in vitro*, perhaps because of their effects on membranes (including those involving lymphocytic receptors) or interference with macromolecular synthesis and function. Cytochalasins are highly cytotoxic and act on cytokinesis (perhaps by binding to filamentous actin), but their immunotoxic potential has not been ascertained.

Mycotoxins can indirectly influence the immunologic functions (Blalock and Smith, 1985). Some of the compounds are neurotoxic or cause other organ pathology, and these compounds may activate the endocrine mechanisms (Sharma, 1984; 1985; 1991). The stress-

induced release of corticosteroids by *Fusarium* T-2 toxin inhibits immune functions (Taylor *et al.*, 1989).

Mycotoxins or their metabolites in mammals may be highly reactive and may destroy tissues. The immune system probably responds to altered proteins or other biological molecules formed by binding with reactive chemicals. However, no experimental evidence exists of this mechanism involving mycotoxins. Antibodies against mycotoxins conjugated with proteins have been produced and are utilized in analytical immunoassays.

The influence of exogenous chemicals on immune responses may be highly variable and may increase, decrease or fail to affect the response, depending on the testing protocol and dose (Sharma and Zeeman, 1980).

1.3.2 Immunotoxic effects of selected mycotoxins

Many episodes of mycotoxin poisoning in livestock species resulted in the death of animals from infectious organisms. Tables 1.5-1.7 show selected examples of mycotoxin effects on immune functions in various species.

The most widely studied immunotoxic agent is AFB₁, which was consistently immunosuppressive in various animal models (Concova *et al.*, 2003; Reddy *et al.*, 1983; Thaxton *et al.*, 1974). The immune responses mediated by T cells appear to be more sensitive to AFB₁; although both helper T cells and suppressor T cells can be affected, depending on the challenge dose of the mycotoxin (Hatori *et al.*, 1991). As indicated in Table 1.5 peripheral bovine lymphocytes are susceptible to AFB₁ (Reddy and Sharma, 1989). In most

experiments, a suppression of antibody formation occurred, generally against T-dependent antigens like sheep red blood cells.

Table 1.5 Selected examples of aflatoxin B₁ immunotoxicity in livestock species and mice after oral treatments

Species	Effects reported	Reference
Bovine	Suppressed mitogen-induced stimulation of peripheral lymphocytes	Paul <i>et al.</i> , 1977
Swine	Decreased lymphocyte response to mitogens, inhibited macrophage migration, and decreased DTH antibody titer to SRBC Resulted in swine clinical disease and pathogenic challenge	Hoerr and D'Andrea, 1983 Miller <i>et al.</i> , 1978
Chicken	Decreased antibody formation against SRBC Increased mortality with <i>Salmonella</i> Impaired phagocytic and bactericidal activity of heterophils Decreased antibody formation Reduced phagocytic activity in RES cells	Thaxton <i>et al.</i> , 1974 Boonchuvit and Hamilton, 1975 Chang and Hamilton, 1979 Giambrone <i>et al.</i> , 1978 Michael <i>et al.</i> , 1973
Turkey	Reduced acquired resistance to <i>Pasteurella Multocida</i> Resulted in thymic involution	Pier and Heddleston, 1970 Pier <i>et al.</i> , 1972
Mice	Decreased lymphocytic response mitogens, decreased antibody response to SRBC, and impaired DTH Decreased DNA, RNA, and protein synthesis in cultured lymphocytes	Reddy <i>et al.</i> , 1987 Reddy and Sharma, 1989

DTH = Delayed type sensitivity; RES = reticular endothelial system; SRBC = sheep red blood cells.

Interest has increased in immune suppression by T-2, a mycotoxin that also inhibit protein synthesis. In toxic doses, T-2 produced necrosis of lymphatic organs in most of the species evaluated is shown in Table 1.6. In lymphocyte cultures, T-2 inhibited blastogenesis (Taylor

et al., 1987); however, T-2 may also include indirect mechanisms involving the hypothalamic-pituitary-adrenal axis (Taylor *et al.*, 1989). Oral exposure of mice to this mycotoxin produced inflammation of the gut mucosa, leading to a systematic endotoxemia and triggering a stresslike response that increased concentrations of glucocorticoids. However, indirect effects on immune system explain only part of the immunotoxic effects of T-2 (Ueno *et al.*, 1995). Some studies have concentrated on the immunotoxicity of other tricothecenes, including those that are macrocyclic. Selected examples are listed in Table 1.6.

Table 1.6 Influence of various tricothecenes on immune responses in various animal species

Species	Tricothecene	Effects	Reference
Cattle	T-2	Reduced neutrophil function and reduced lymphocyte blastogenesis	Mann <i>et al.</i> , 1984
		Decreased response of lymphocyte to PHA	Buening <i>et al.</i> , 1982
		Resulted in necrosis in lymphoid organs and tissues	Beasley, 1984
Sheep	T-2	Resulted in lymphopenia and leucopenia	Friend <i>et al.</i> , 1983
Swine	T-2	Resulted in necrosis in B-cell regions of lymphoid tissues	Beasley, 1984
	T-2	Decreased lymphocytic proliferation with mitogens	Pang <i>et al.</i> , 1987a
	(inhalation)	Resulted in transient alteration of immune responses	Pang <i>et al.</i> , 1987b
	T-2 (topical)	Decreased leucocyte count and antigen-induced lymphocyte transformation	Rafai and Tuboly, 1982
	T-2	Resulted in massive lymphocyte necrosis	Weaver <i>et al.</i> , 1978a
	DAS	Resulted in mild and inconsistent leucopenia and necrosis of germinal centers in mesenteric lymph nodes and splenic white pulp	Weaver <i>et al.</i> , 1978b
Chicken	T-2	Increased mortality to pathogenic bacterial challenge	Boonchuvit <i>et al.</i> , 1975
		Resulted in lymphopenia and lymphatic necrosis	Hoerr <i>et al.</i> , 1982a,b
Turkey	T-2	Resulted in lymphatic necrosis	Richard <i>et al.</i> , 1978
Mice	T-2	Decreased antibody formation	Taylor <i>et al.</i> , 1985
		Interfered with <i>in vitro</i> mitogen-induced blastogenesis	Taylor <i>et al.</i> , 1987
	Macrocyclic tricothecene	Resulted in inconsistent effects, not well correlated with acute toxicity	Hughs <i>et al.</i> , 1989, 1990

PHA = Phytahaemagglutinin

Macrocyclic tricothecenes, even those that are very toxic, did not uniformly affect immune responses (Hughes *et al.*, 1988; 1989).

The immunotoxicity of most mycotoxins has not been evaluated. The studies concerning miscellaneous compounds are listed in Table 1.7.

Table 1.7 Immunotoxic effects of miscellaneous mycotoxins

Mycotoxin	Species	Effects	Reference
Ochratoxin A	Swine	Resulted in necrosis of gut-associated lymph nodes	Szczecz <i>et al.</i> , 1973a
	Dog	Resulted in necrotic lymph nodes in mesentery	Szczecz <i>et al.</i> , 1973b
	Chicken	Resulted in leucopenia and impaired phagocytosis by heterophils	Chang <i>et al.</i> , 1979
	Turkey	Resulted in leucocytopenia, heterocytopenia and thymic atrophy	Chang <i>et al.</i> , 1981
Patulin	Mice	Increased resistance to <i>Candida albicans</i> and decreased concentrations of circulating immunoglobulins	Escoula <i>et al.</i> , 1988a
	Rabbits	Decreased serum immunoglobulins and resulted in reduced blastogenesis of lymphocytes and reduced chemiluminescence of peritoneal leucocytes	Escoula <i>et al.</i> , 1988b
Cutrinin	Mice	Resulted in transient stimulation of lymphoproliferative response, increased antibody against SRBC	Reddy <i>et al.</i> , 1988
Rubratxin B	Mice	Resulted in leucopenia and decreased lymphoproliferation	Taylor <i>et al.</i> , 1983

SRBC = Sheep red blood cells

Ochratoxin A and patulin may have immunosuppressive effects. The lympholytic effect of oral doses of OTA was largely limited to the gut-associated lymphatic tissue (Szczecz *et al.*, 1973a; b) and often required doses that were systemically toxic. Patulin inhibited DNA

synthesis in peripheral lymphocytes: these were mitigated by cysteine, which suggested that sulphydral binding was involved in patulin-induced toxicity (Cooray *et al.*, 1982).

The response to citrinin was marked by immunostimulation (Reddy *et al.*, 1988), but its effects were reversible and appeared to be related to the nephrotoxic potential of this mycotoxin (Reddy and Sharma, 1984). Rubratoxin B may also suppress the immune system (Taylor *et al.*, 1983).

1.4 IMMUNE MODULATION BY FUMONISINS AND OCHRATOXINS

1.4.1 Fumonisin

1.4.1.1 Occurrence

Fumonisin represents a new group of mycotoxins first described and characterised in 1988 (Bezuidenhout *et al.*, 1988; Gelderblom *et al.*, 1988). Fumonisin is produced mainly by *F. verticillioides* (known previously as *F. moniliforme*) and *F. proliferatum* (Alberts *et al.*, 1993; Marasas *et al.*, 2001; Rheeder *et al.*, 2002). At the present time, fumonisins B₁, B₂, B₃, B₄, and A₂ are known (Scott, 1993). The most abundantly produced member of the family is FB₁. Fumonisin B₁ can constitute up to 70% of all fumonisins in the food and is the most frequent cause of fumonisin toxicosis in animals (Riley *et al.*, 1993). Fumonisin contaminates maize based foods and feeds throughout the world (Bullerman, 1996; Marasas, 1993; 1995; 1996). In the U.S. and Canada some *Fusaria* have been found in wheat, sorghum and corn (Kuiper-Goodman *et al.*, 1996; Pohland, 1996). Studies show clearly that *F. verticillioides* is widespread in the Midwestern and Southeastern U.S. corn seeds and plants. Studies of mycotoxin occurrence in Indiana corn found that fumonisins were the most frequently

detected mycotoxins in corn from 1991-1993. However, fumonisin contamination varied greatly from year to year, for example, in 1991, 96% of 328 samples evaluated had some *Fusarium* ear rot. Of the 113 most severe samples, 44% had fumonisin levels above 5 ppm (parts-per-million). In contrast to 1991, all lots tested were below 5 ppm in 1992 (Vincelli and Parker, 1994). In India, a single outbreak of acute foodborne disease caused by FB₁ had been reported. In the 27 villages involved, the individuals affected were from the poorest social strata who had consumed maize and sorghum harvested and left in the fields during unseasonable rains (Bhat *et al.*, 1997; WHO, 2000). These findings were of concern because of a report from China in which these metabolites were identified in 100% of 24 corn samples consumed as food collected from patients with oesophageal cancer. Fumonisin B₁ is carcinogenic in rats and mice. Ecological studies have linked consumption of fumonisin-contaminated maize with oesophageal cancer in human populations in *Transkei (South Africa)*, South Africa, China, Iran and northeast Italy (Chu and Li, 1994; Marasus *et al.*, 1981; Peraica *et al.*, 1999; Shephard *et al.*, 2000; Turner *et al.*, 1999; Yoshizawa *et al.*, 1994)

Finally, fumonisins can cause neural tube defects in experimental animals and may also have a role in human cases. It has been hypothesized that a cluster of anencephaly and spina bifida cases in southern Texas may have been related to fumonisins in corn products (Hendricks, 1999; Hendricks *et al.*, 1999; Missmer *et al.*, 2002). The International Agency for Research on Cancer has evaluated the risk of fumonisins to humans and classified them as group 2B (probably carcinogenic) (Rheeder *et al.*, 2002).

1.4.1.2 Characteristics

Fumonisin B₁ is a mycotoxin produced by the fungus, *Fusarium verticillioides*, which is associated with symptoms caused by contaminated corn and corn-based foods (Dutton and Kinsey, 1995; Dutton, 1996; Leslie *et al.*, 1990; Stockman, 2001). Fumonisin B₁ and Fumonisin B₂ (FB₂) were first reported in South Africa (Gelderblom *et al.*, 1992a,b) and are associated with oesophageal cancer, which is prevalent in Transkei (South Africa) and in China (Marasas *et al.*, 1981; Sydenham *et al.*, 1990), and with primary liver cancer (Ueno *et al.*, 1997). Fumonisin B₁ is also responsible for lung oedema in pigs and leucoencephalomalacia in equine species (Nijs *et al.*, 1999). In addition to their adverse effect on the brain, liver and lungs (Smith *et al.*, 1996), fumonisins affect kidneys, pancreas, testes, thymus, gastrointestinal tract and blood cells (Vincelli and Parker, 1994). An episode of human gastroenteritis in India was associated with the consumption of fumonisin-contaminated grain (Bhat *et al.*, 1997).

Fusarium is characterized by the production of three types of spores: macroconidia, microconidia, and chlamydospores. Macroconidia are typically canoe-shaped (tapered at the ends). Colony colours vary from pinkish to purple for *Fusarium verticillioides* (Myburg, 1998).

Unlike most known mycotoxins, fumonisins are highly polar compounds and are soluble in polar solvents for example, water, ethanol, and acetonitrile but insoluble in organic solvents. This makes them difficult to study. They are hydrolysed by strong acids and alkalis (Jackson *et al.*, 1996). Usually they are extracted in aqueous methanol or aqueous acetonitrile (Blackwell *et al.*, 1996). High-performance liquid chromatography with fluorescent detection

is the most widely used analytic method (Plattner *et al.*, 1996). The major toxic effects by FB₁ are based solely on its disruptive effects on sphingolipid biosynthesis resulting in cell damage (Wang *et al.*, 1991).

1.4.1.3 Toxicological effects

Of particular interest is the apparent species specificity of fumonisin toxicity. Purified FB₁ causes leucoencephalomalacia in horses (Marasas, 1993; 1995; Marasas *et al.*, 1988) and pulmonary oedema in pigs (Harrison *et al.*, 1990). In mice the primary target is the liver, whereas in rats the kidney is more sensitive (Gelderblom *et al.*, 1991; 1995; Voss *et al.*, 1993; 1995; Ribar and Mezaric, 1998). Voss *et al.* (1996) reported hepatotoxic changes in lambs, rabbits, and mink, as well as an increased rate of apoptosis in the liver and kidney. The National Toxicology Program (Howard *et al.*, 2001) found FB₁ to be a renal carcinogen in male F344/N rats and a hepatocarcinogen in female B6C3F.

Equine leucoencephalomalacia manifests as a neurological disorder of horses and is accompanied by inflammation and oedema of the CNS (Uhlinger, 1997). Primary signs of the acute disease include blindness, paresis or paralysis of the facial, oral, or glosopharngeal muscles, locomotor abnormalities (ataxia), hyperaesthesia, and stupor. Death usually occurs several hours after the appearance of the neurological signs (Wilson and Marapot, 1971). In regions, where corn is the primary constituent of the human diet, the occurrence of oesophageal carcinoma has been reported (Voss *et al.*, 1996).

When FB₁ was administered to horses, within eight days the horses exhibited signs of blind staggers. *F. verticillioides* is common even in food-grade corn and is often abundant in ground feeds and silage. Growing pigs fed a ration containing 78 to 82% corn heavily colonized by *F. verticillioides* grew as well as the sound pigs fed a ration of sound corn. It is therefore likely that fumonisin is not always present when the fungus is, or that pigs are not sensitive to FB₁ (Jacobsen *et al.*, 1993). In spite of these species differences, the available data on immunotoxicity indicate that fumonisin-induced changes in immune function are not species specific, and appear to involve aspects of humoral, cellular, and innate immunity.

Poultry are relatively resistant to the effects of fumonisins, and show few signs of toxicity after ingestion of feed contaminated with less than 75 mg FB₁/kg feed. Hepatic changes, including hepatocellular hyperplasia, are the most notable changes in ducklings or turkey poults ingesting feed contaminated with greater than 75 mg FB₁/kg (Bermudez *et al.*, 1995; Ledoux *et al.*, 1992). Fumonisin-associated immunotoxicity has been seen in birds ingesting feed contaminated with mycotoxins including fumonisins (Javed *et al.*, 1995). Turkey poults ingesting feed contaminated with *F. verticillioides* culture material containing fumonisins at levels of 100 or 200 mg/kg feed displayed lesions indicative of immunotoxicity, including diffuse thymic cortical thinning, mild bursal follicular atrophy, and mild splenic lymphocyte depletion (Weibking *et al.*, 1993). There is limited evidence that ingestion of culture material contaminated with lower fumonisin levels is immunotoxic to poultry (Quereshi *et al.*, 1995). A dose of 10 mg of FB₁ given as a pure substance, or 30 mg in natural form per kilogram of the food, is toxic for young poultry (Weibking *et al.*, 1993). Reduced white blood cell counts (WBC) were observed in chicks given a relatively high

dose of FB₁. Fumonisin B₁ toxicosis of broiler chickens intoxicated with 30 or 300 mg of the toxin isolated from a culture of *F. verticillioides* (or 10 mg of FB₁ in pure substance) per kilogram of food was characterized by a reduction in the prothombin time, an increase in plasma fibrinogen, and in the activity of antithrombin 3. Simultaneously, a decrease in serum albumin and increased serum globulins were observed (Espada *et al.*, 1997).

In pigs, ingestion of fumonisin-contaminated corn at levels of 33 mg/kg for 21 d resulted in suppressed lymphocyte blastogenesis and titers to pseudorabies virus at 14 d but not at 21 d (Osweiler *et al.*, 1993b). Lymphocyte blastogenesis was also suppressed in pigs given fumonisin-contaminated culture material at levels resulting in 100 mg FB₁/kg feed (Harvey *et al.*, 1996), and after 28 d PHA-induced lymphocyte blastogenesis was suppressed. Administration of a sublethal dose of the toxin resulted in the inhibition of pulmonary intravascular macrophages (Smith *et al.*, 1996).

In calves, ingestion of feed experimentally adulterated with fumonisin contaminated corn screenings containing up to 148 µg/g total fumonisins inhibited neutrophil migration but not phagocytosis or antibody dependent cytotoxicity (Osweiler *et al.*, 1993a).

In general, mice were not as sensitive to FB₁ as rats (Bondy *et al.*, 1996; 1997; 1998). Mice given 1 to 75 ppm purified FB₁ for 14 d showed minimal signs of immunotoxicity. A few females had mild thymic cortical lymphocytolysis in the 35 and 75 mg/kg dose groups, and there were significant numbers of vacuolated bone-marrow cells including lymphocytes, but there were no marked changes in the spleen

or lymph nodes and no changes in numbers of circulating blood cells or serum total immunoglobulins (Bondy *et al.*, 1997).

In vitro studies have focused on the effects of FB₁ on macrophage function or on lymphocyte proliferation. Fumonisin B₁ extracted from *F. verticillioides*-infected corn kernels reduced the viability and phagocytic activity of chicken peritoneal macrophages at levels of 6 to 18 µg/ml in macrophage cultures (Chatterjee and Mukherjee, 1994). Microscopic examination revealed increased nuclear disintegration in treated macrophages (Chatterjee *et al.*, 1995). This confirms earlier studies in which cytotoxicity, nuclear disintegration and cytoplasmic blebbing, and reduced phagocytosis were observed in chicken peritoneal macrophages exposed to FB₁ at concentrations of 0.5 to 10 µg in culture (Quereshi and Hagler, 1992). Fumonisin B₁ inhibits murine spleen lymphocyte and turkey peripheral lymphocyte proliferation at levels as low as 0.1 µg/ml (Dombrink-Kurtzman *et al.*, 1994; Martinova and Merrill, 1995). In turkey lymphocytes, FB₂ was a more potent inhibitor of proliferation than FB₁ *in vitro* (Dombrink-Kurtzman *et al.*, 1994). The most striking morphologic change in turkey lymphocytes was cytoplasmic vacuolization in treated cells (Dombrink-Kurtzman *et al.*, 1994). Similarly, bone-marrow lymphocyte cytoplasmic vacuolization has been observed consistently in rats and mice treated with FB₁ *in vivo* (Bondy *et al.*, 1996; 1997; 1998).

1.4.1.4 Mechanism of action

Some of the immunomodulatory effects of fumonisins may be the result of changes in the expression of cell surface markers important in immune cell communication (Engelhardt *et al.*, 1989). In mice given a single intraperitoneal (IP) injection of 5 or 20

μg FB_1 , CD3 receptor expression and sphingomyelin levels were decreased in thymocytes but not in splenocytes (Martinova *et al.*, 1995). Fumonisin-induced immunomodulation may also be due to changes in cytokine secretion. Selective induction of TNF- α mRNA and secretion of TNF- α protein were observed in peritoneal macrophages from mice injected subcutaneously with FB_1 (0.25 to 6.75 mg/kg/d for 5 d). Interleukin-1 α and INF- γ expression and secretion were unaffected (Dugyala *et al.*, 1998).

Fumonisin B₁ also causes intracellular membrane degeneration and plasma membrane changes, which suggests that the cell membrane may be an early target of these toxins (Scott, 1993; Ferrante *et al.*, 2002). The chemical structure of the backbone of the fumonisin compounds is similar to that of the cellular sphingolipids (Figure 1.6).

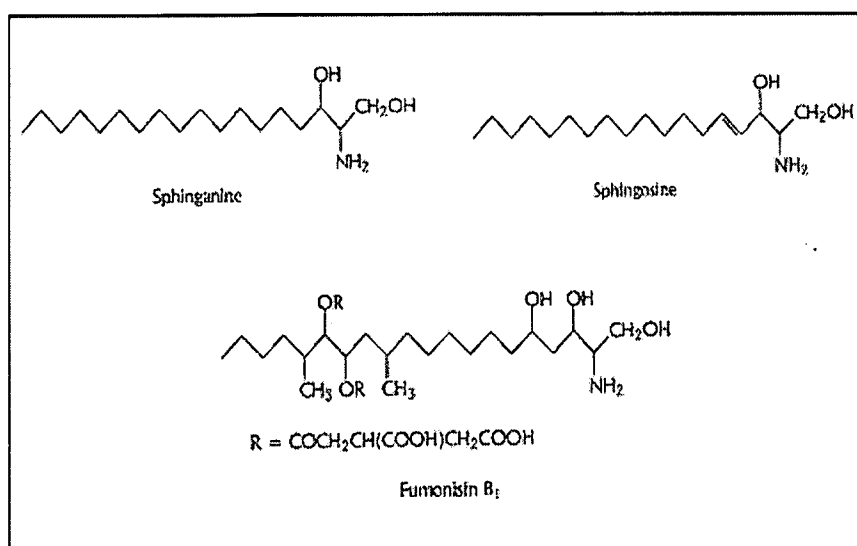


Fig. 1.6 Structures of sphinganine, sphingosine and FB_1

In mammalian cells, FB₁ inhibits *N*-acyltransferase (ceramide synthase), which is the enzyme catalyzing the amide linkage of a fatty acid to sphinganine to form the complex sphingolipid dihydroceramide (Figure 1.7). This results in accumulation of substrate (free sphinganine) and depletion of product (dihydroceramide), as well as increased products associated with free sphinganine metabolism (Riley *et al.*, 1994; 1996; Wang *et al.*, 1991). Accumulation of sphinganine, a compound that is toxic to many cells, may account for the evidence of varied effects such as toxicity and induction of apoptosis in the liver, kidney of rats, mice and rabbits (Gelderblom *et al.*, 1996).

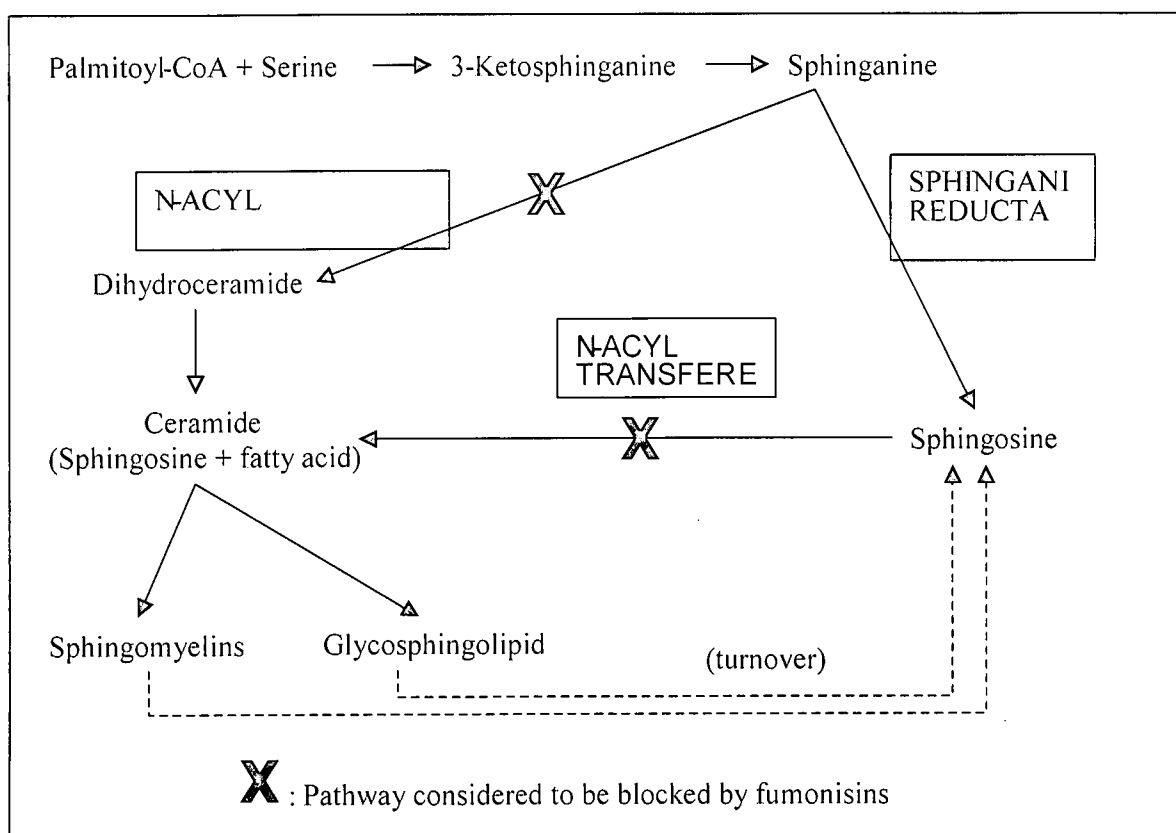


Fig. 1.7 Schematic representation of the mechanism of action of FB₁

Sphingolipid metabolites are involved in multiple signal transduction pathways and in the generation of intracellular second messengers (Spiegel and Merrill, 1996). Since

fumonisin is structurally similar to the PKC inhibitor sphingosine, it has been shown to inhibit PKC activity in CV-1 cells *i.e.* African green monkey kidney cells (Huang *et al.*, 1995), and to cause redistribution of PKC from cytosol to membrane in rat cerebral cortex slices (Yeung *et al.*, 1996). In CV-1 cells fumonisin also inhibits the transcription of cyclin dependent kinase inhibitors and induces cell-cycle arrest (Ciacci-Zanella *et al.*, 1998).

Since immune responses are critically dependent on intracellular signaling, the relationship between fumonisin-induced disruption of sphingolipid metabolism and immunomodulation requires further exploration. Fumonisin B₁ has been used as a tool to show that ceramide production is a part of the signal pathway involved in T-cell-receptor-induced IL-2 production and apoptosis (Tonnetti *et al.*, 1999). The further use of FB₁ to study the role of sphingolipids in immune cell signalling will undoubtedly provide valuable data on the immunomodulatory effects of this toxin. Cells treated with FB₁ *in vitro* undergo a mixture of necrotic and apoptotic cell death (Tolleson *et al.*, 1996). In mice, apoptosis was found in liver and kidney after a short-term treatment with FB₁ (Sharma *et al.*, 1997) and the overall severity of liver and kidney lesions was closely correlated with the disruption of sphingolipid metabolism (Tsunoda *et al.*, 1998).

There is considerable evidence that TNF- α signalling pathways also play an important role in fumonisin-induced toxicity *in vitro* and *in vivo*. For example, Ciacci-Zanella and Jones (1999) reported that fumonisin cytotoxicity was effectively prevented by inhibition of caspases, mediators of TNF- α cellular signaling. Murine macrophages cells treated *in vitro* produced TNF- α , and TNF- α was also induced in mouse liver

after short-term repeated treatment with FB₁ (Sharma *et al.*, 2002). Furthermore, the acute haematological effects of FB₁ in mice were reversed by anti-TNF- α antibodies (Dugyala *et al.*, 1998).

Fukuda *et al.* (1996) investigated the effects of FB₁ on the activity of protein serine/threonine phosphatases (PPs), (PP1, PP2A, PP2B, PP2C and PP5) in rats. Inhibition of dephosphorylation was observed for all five PPs with IC₅₀ of 80 μ M-300 μ M. Among the PPs examined, PP5 was the most sensitive with an IC₅₀ of 80 μ M. Inhibition of PP5 could thus play a role in the toxicity and carcinogenic action of FB₁.

1.4.2 Ochratoxins

1.4.2.1 Occurrence

Ochratoxins are fungal metabolites of *Aspergillus* (Abarca *et al.*, 1994; Bayman *et al.*, 2002) and *Penicillium* (Chu, 1974; Pitt, 1987) strains. The most frequently found mycotoxin in this group is OTA, which is produced by *Aspergillus ochraceus* and *Penicillium verrucosum*. Ochratoxin A was discovered as a metabolite of *Aspergillus ochraceus* in 1965 during a large screen of fungal metabolites that was designed specifically to identify new mycotoxins (Van der Merwe *et al.*, 1965). Shortly thereafter, it was isolated from commercial corn sample in the United States (Shotwell *et al.*, 1969) and recognized as a potent nephrotoxin (Krogh, 1992). As with other mycotoxins, the substrate on which the moulds grow as well as moisture level, temperature, and presence of competitive microflora interact to influence the level of toxin produced. It is commonly found in cereals, oleaginous seeds, coffee, cocoa, beer, wine and as

a result of carryover from contaminated animal feed (Ehrlich *et al.*, 2002; Rosa *et al.*, 2004; Serra Bonvehi, 2004).

South African chemists were the first to characterize OTA. The toxin has been identified in several plant products in South Africa, North America, Asia and Europe. Agricultural commodities in which OTA has been found include corn, wheat, rye, barley, oats, sorghum, ground nuts and coffee beans. Ochratoxin A and other mycotoxins have also been detected in grain dust (Betina, 1989).

Ochratoxin A is a nephrotoxin to all animal species studied to date, and is most likely toxic to humans, who have the longest half-life for its elimination of any of the species examined (Creppy, 1999; Steyn, 1971). In addition to being a nephrotoxin, animal studies indicate that OTA is a liver toxin, an immune suppressant, a potent teratogen, and a carcinogen (Beardall and Miller, 1994). Ochratoxin A disturbs cellular physiology in multiple ways, but it seems that the primary effects are associated with the enzymes involved in phenylalanine metabolism, mostly by inhibiting the enzyme involved in the synthesis of the phenylalanine-tRNA complex (Bunge *et al.*, 1979; Marquardt and Frohlich, 1992). In addition, it inhibits mitochondrial ATP production (Meisner and Meisner, 1981) and stimulates lipid peroxidation (Rahimtula *et al.*, 1988).

Ochratoxin has been detected in blood, other animal tissues and in milk, including human milk (Marquardt and Frohlich, 1992). It is frequently found in pork intended for human consumption (Fink-Gremmels *et al.*, 1995). Ochratoxin is believed to be responsible for a porcine nephropathy that has been studied intensively in the Scandinavian countries. The disease is endemic in Denmark, where rates of porcine nephropathy and ochratoxin

contamination in pig feed are highly correlated (Krogh, 1987). In addition, ochratoxin is associated with disease and death in poultry (Burns and Dwivedi, 1986; Chang *et al.*, 1979; Hamilton *et al.*, 1982). There has been speculation that ochratoxins are involved in a human disease called endemic Balkan nephropathy (Hult *et al.*, 1982; Krogh, 1987). This condition is a progressive chronic nephritis that occurs in populations who live in areas bordering the Danube River in parts of Romania, Bulgaria, and the former Yugoslavia (Palli *et al.*, 1999; Petkova-Bocharova and Castegnaro, 1991). In one Bulgarian study, ochratoxin contamination of food and the presence of ochratoxin in human serum were more common in families with endemic Balkan nephropathy and urinary tract tumours than in unaffected families (Castegnaro *et al.*, 1987). In addition to ochratoxin poisoning, this curious disease has been attributed to genetic factors, heavy metals, and possible occult infectious agents. The current consensus is that endemic Balkan nephropathy is of unknown etiology, but many mycotoxin reviews list it, without caveat, as an ochratoxicosis.

It has also been hypothesized that the gene for phenylketonuria might occur in relatively high frequency because of a heterozygous advantage against ochratoxin poisoning and that ochratoxin might be a risk factor for testicular cancer (Petzinger and Weidenbach, 2002).

1.4.2.2 Characteristics

Ochratoxin is a colourless, crystalline compound. It is a widespread mycotoxin produced mainly by the mould fungi, *Aspergillus ochraceus* and *Penicillium verrucosum* during the storage of cereals, cereal products and other plant derived products such as herbs and spices (Betina, 1989; Krogh *et al.*, 1973).

1.4.2.3 Toxicological effects

The toxicity of OTA in rats, guinea pigs, beagle dogs, swine and to rainbow trout has been described (Boorman *et al.*, 1984; Dirheimer and Creppy, 1991; Luster *et al.*, 1987). Studies in rats have shown that the half-life of the toxin is 55 hours and that free metabolites are the excreted compounds (Betina, 1989).

The ability of OTA exposure to increase susceptibility to infectious agents has been confirmed by the observation that chicks infected with inclusion body hepatitis (IBH) virus and concurrently exposed to OTA (0.5 ppm for up to 35 d) had more pronounced haematological, biochemical, and histopathological changes than chicks exposed to the virus or to OTA alone (Sandhu *et al.*, 1995; 1998).

When pigs ingested 2.5 mg OTA/kg feed for 35 d, cell-mediated and phagocytic cell responses were suppressed (Harvey *et al.*, 1992). Cutaneous basophil hypersensitivity to phytohaemagglutinin A (PHA) was decreased after 24 h. Delayed hypersensitivity to tuberculin was suppressed in OTA-treated gilts at 48 h but not at 24 or 72 h. In peripheral lymphocyte cultures, PHA-stimulated proliferation and ConA-stimulated IL-2 production were both suppressed in OTA-treated pigs. Changes in anti-chicken red blood cell (RBC) antibody titers or antibody isotypes, which would be indicative of humoral effects, were not observed in OTA-treated pigs (Harvey *et al.*, 1992).

Administration of OTA to mice has been shown to be immunosuppressive (Boorman *et al.*, 1984), although the literature indicates that there are inconsistencies in responses based on route of administration, as well as discrepancies between dose and response in different studies. In mice given OTA intraperitoneally (IP), for up to 17 d (1 to 6 mg OTA/kg body weight/d), changes included decreased phagocytosis in circulating phagocytic cells, decreased splenic antibody producing cells, and serum antibody titers to sheep red blood cell (SRBC) and *Pasteurella multocida*. Parameters that were unchanged included numbers of circulating lymphocyte subpopulations, measured by flow cytometry, and cell-mediated immunity, measured by changes in foot-pad swelling responses to SRBC or *Pasteurella* antigen. Significant changes occurred at either 3 or 6 mg OTA/kg body weight (Muller *et al.*, 1995). Although the dosing period was shorter, the toxin levels causing statistically significant changes were within range of those used by Prior and Sisodia (1982), who observed suppressed antibody responses to *Brucella abortus* antigen at doses of 5 mg/kg body weight after 50 d of IP OTA administration. In contrast, single IP injections of 0.005 µg OTA/kg body weight (Haubeck *et al.*, 1981) or 1 µg OTA/kg body weight (Creppy *et al.*, 1983) inhibited antibody responses to SRBC. These doses are comparatively lower than the levels of OTA required to elicit immunotoxic responses in mice and in other animal models.

Until recently the literature indicated that oral exposure to OTA was not effective in generating immunotoxicity in mice. Antibody responses to *Brucella abortus* antigen were actually increased in mice exposed to OTA in feed (50 d, 4 ppm; Prior and Sisodia, 1982), whereas IP administration of OTA to mice suppressed antibody responses to SRBC (single dose, 1 µg/kg body weight; Creppy *et al.*, 1983) and *Brucella abortus* antigen (50 d, 5 mg/kg body weight; Prior and Sisodia, 1982). However, recent research indicates that immune

suppression does occur in mice exposed to OTA orally. Antibody responses to SRBCs were suppressed in mice receiving 250 or 2600 µg OTA/kg diet for 28 d; after 90 d of dietary OTA exposure mice had lower proportions but not lower total numbers of thymic CD4+ and CDB+ cells (250 and 2600 µg OTA/kg diet) and decreased spleen and thymus lymphocyte proliferative responses to ConA (6, 250, and 2600 µg OTA/kg diet) (Thuvander *et al.*, 1995).

Ochratoxin A administered to pregnant female rodents either IP or by gavage at doses ranging from 1 to 10 mg/kg body weight causes fetal mortality and a range of developmental abnormalities in pups, including skeletal malformations and brain, central nervous system, craniofacial, and ocular abnormalities (Kuiper-Goodman and Scott, 1989). Prenatal exposure to lower doses of OTA has been shown to affect the immune system of developing mice and rats (Thuvander *et al.*, 1996a; 1996b; 1996c).

In rodents, exposure to OTA during lactation results in transient immunostimulation (Thuvander *et al.*, 1996b; 1996c), demonstrating that OTA is not wholly immunosuppressive. The observation of immunosuppression in rodents exposed prenatally to OTA and transient immunostimulation in rodents exposed during lactation highlights the complex nature of interactions between OTA and the developing immune system. This suggests a need for further studies of immune responses in adult animals exposed perinatally to OTA.

The embryonic potential of OTA was studied after administering single mounting doses of the mycotoxin to chicken embryos on days two, three and four. The beginning of the embryotoxicity dose range was found to be between 0.01 to 0.05 mg. The maximum response occurred after administration on day three. In addition to significant growth retardation of

fetuses, cleft beak, reduction deformities of the limbs, and abdominal wall and ventricular septal defects were encountered on day eight of incubation (Vesela *et al.*, 1983).

In vitro, OTA has been shown to abolish human B- and T-lymphocyte proliferation and to inhibit IL-2R expression and IL-2 production in T lymphocytes (Lea *et al.*, 1989). Furthermore, OTA was shown to inhibit the late stages of T-cell activation such as IL-2-induced proliferation, but not the early stages, for example, increased cytoplasmic Ca^{2+} levels or activation of protein kinase C (Stormer and Lea, 1995). These events could be connected to the ability of OTA to inhibit both DNA and protein synthesis under certain conditions. Ochratoxin A has also been shown to induce DNA degradation associated with apoptosis in PHA-stimulated human blood lymphocytes (Seegers *et al.*, 1994a), indicating the potential involvement of programmed cell death in OTA-induced immunotoxicity.

Ochratoxin A induced renal carcinomas in rats and mice (Lock and Hard, 2004), and in the latter species additionally hepatocellular carcinomas. How common is human exposure to ochratoxin? Studies from Canada, Sweden, West Germany, Tuscany and Yugoslavia detected ochratoxin in human blood and serum (Kuiper-Goodman and Scott, 1989; Palli *et al.*, 1999). Analyses of urine from children in Sierra Leone detected both ochratoxin and aflatoxin throughout the year (Jonsyn-Ellis, 2000).

Several detailed risk assessments have been conducted for OTA (Kuiper-Goodman and Scott, 1989). Given the known human exposure and the abundance of toxicological data from animal studies, the European Union Scientific Committee has recommended that OTA levels be reduced to below 5 ng/kg of body weight per day (Sweeney *et al.*, 2000). In addition,

several European countries have proposed individual regulations, with maximum tolerated concentrations varying greatly from country to country (Jelinek *et al.*, 1989; Van Egmond, 1991).

Based on the results of carcinogenicity studies and on evidence of effects in humans, the IARC classified OTA as a possible human carcinogen (category 2B) (Beardall and Miller, 1994; Ehrlich *et al.*, 2002; Petkova-Bocharova and Castegnaro, 1991).

1.4.2.4 Mechanism of action

The mechanisms of tumour induction in rodent kidney by OTA have been addressed in many studies, including investigations of the role of biotransformation and bioactivation and the formation of OTA-derived nucleic acid derivatives in target and non-target organs for toxicity. The results diverge, as do those of the studies on mutagenicity. Although no definite mechanism for the carcinogenicity of OTA to rodent kidney has been described, non-genotoxic events make a major contribution to the induction and progression of OTA-derived renal tumours (Bendele *et al.*, 1985).

Several studies have addressed the biotransformation of OTA and its role in its toxicity. Biotransformation has been postulated to be involved in the DNA binding and renal tumorigenicity of OTA, and a variety of cytochrome P450s (CYPs), peroxidases, and glutathione S-transferases have been suggested to catalyse the transformation of ochratoxin A to reactive intermediates (Fink-Gremmels *et al.*, 1995; Grosse *et al.*, 1997; El Adlouni *et al.*, 2000). However, none of these studies assessed the capacity of the respective enzymes to transform OTA to metabolites or suggested the structure(s) of a reactive metabolite

(Castegnaro *et al.*, 1998; 2003). Most studies assessed potentially relevant end-points in the toxicity of OTA and their modulation by changes in xenobiotic-metabolizing enzyme activities. Because of these limitations, no conclusions can be drawn about the mechanisms of OTA induced tumour formation in rat kidney.

The possible biotransformation reactions of OTA have been postulated on the basis of rigorous analytical chemistry. Formation of an OTA-derived reactive quinone was suggested (Gillman *et al.*, 1999), but this metabolite was formed only by a chemical system that mimics the CYP system.

The OTA-derived reactive quinone was not detected by the use of isolated enzymes and microsomes with high activity for specific CYPs, and only 4R- and 4S-hydroxy-OTA were formed at very low yields (Gautier *et al.*, 2001; Mally *et al.*, 2004; Zepnik *et al.*, 2001; 2003). Subcellular fractions rich in prostaglandin synthase activity or purified CYP enzymes also did not catalyse the formation of reactive OTA metabolites (Gautier *et al.*, 2001).

The known mechanisms of formation of OTA metabolites (insertion of an oxygen into a carbon-hydrogen bond) do not suggest formation of reactive and toxic intermediates. The lack of involvement of CYP-mediated oxidation in the toxicity of OTA is supported by the observation that increasing the rates of biotransformation of the toxin by induction of CYP decreases its renal toxicity (Omar *et al.*, 1996), and the observation of typical toxic effects of OTA in cell systems with very low or no CYP activity (Dopp *et al.*, 1999; Hoehler *et al.*, 1996; Seegers *et al.*, 1994b). The formation of OTA-derived radicals capable of interacting with macromolecules is also not indicated. In contrast, the electron spin resonance spectra suggest the formation of hydroxy radicals (Hoehler *et al.*, 1996; 1997).

Formation of DNA adducts has also been postulated as an important event in the tumourigenicity of OTA (Lock and Hard, 2004). The formation of spots interpreted as OTA-derived DNA adducts was observed in target tissues in rodents by the very sensitive ^{32}P -postlabelling assay. The nature of the DNA damage and/or mutations caused by OTA is unknown (Grosse *et al.*, 1995; 1997). The end-points in many of the studies on the mechanisms of tumourigenicity of OTA was the possible formation of DNA adducts (spots by ^{32}P -postlabelling). However, a role of DNA binding of OTA is not supported by the results of studies of biotransformation cited above or of experiments to investigate the binding of radiolabelled OTA to nucleic acids (Gautier *et al.*, 2001). Studies of DNA binding with [^3H]OTA revealed no binding of 'metabolically activated' OTA to calf thymus DNA *in vitro* or to DNA from rat liver or kidney *in vivo*. The sensitivity of these experiments was similar to that of the postlabelling studies. Lack of DNA binding of OTA or its metabolites was observed *in vivo* after administration of a single dose of [^3H]OTA (Rasonyi *et al.*, 1999).

In summary, these data cast doubt on the hypothesis that OTA causes renal tumours by covalent binding of reactive intermediates to DNA. The hypothesis that DNA damage induced by OTA is due to oxidative stress represents an alternative explanation for the discrepant data and is more consistent with the observations. Several experimental observations support this hypothesis. An unusually large number of DNA adducts (up to 30 individual adducts) was formed from OTA in low yields in various experimental systems (Castegnaro *et al.*, 1998; De Flora *et al.*, 1996).

Patterns of modifications similar to those observed with ochratoxin A by postlabelling were observed in kidney DNA of rodents exposed to iron(III) nitrilotriacetate (Randerath *et al.*,

1995), a renal carcinogen that acts through oxidative stress, or in DNA exposed to hydrogen peroxide (Randerath *et al.*, 1996). Some of these results are consistent with a major role of oxidative stress in the toxicity of OTA. For example, antioxidants prevent the induction of DNA damage by OTA in mice (Grosse *et al.*, 1997). Induction of renal toxicity, oxidative stress due to mitochondrial dysfunction, and persistent cell proliferation represent an alternative mechanism for the renal carcinogenicity of OTA. The toxin is known to induce oxidative stress (Aleo *et al.*, 1991) and the formation of hydrogen peroxides (Omar *et al.*, 1996).

In addition, mechanisms linked to long-term renal toxicity and oxidative stress are known to play an important role in tumour induction in rat kidney (Hard, 1998). Several non-genotoxic chemicals that do not undergo bioactivation reactions induce renal tumours in rodents. For example, DNA damage and cellular toxicity mediated by oxidative stress seem to be involved in the renal carcinogenicity of iron(III) nitrilotriacetate and potassium bromate in rodents. These compounds are potent renal carcinogens and induce renal tumours in rodents in high yields after short exposure. Sex differences in tumour incidences are also seen with these compounds. For example, as seen with OTA, male rats are more susceptible to renal tumour induction by potassium bromate (Umemura *et al.*, 1998).

CHAPTER TWO: MATERIALS AND METHODOLOGY

The aim of this study was to determine and compare the immunomodulating effects of FB₁ and OTA in human carcinoma. In order to achieve this lymphocytes and neutrophils were first isolated from volunteers. Thirty healthy individuals participated in this aspect of the project. The isolated cells were exposed to either FB₁ or OTA on a dose and time dependent level. The viability of the cells was demonstrated by the Trypan blue dye exclusion test method and the methylthiazol tetrazolium (MTT) assay. The toxic effects of the mycotoxins at which 50% cell death (LD₅₀) occurred, was determined. Thereafter challenge tests were performed, whereby lymphocytes and neutrophils isolated from volunteers (30), oesophageal cancer patients (30) and other cancer patients (30), were exposed to the LD₅₀ dose of either FB₁ or OTA for the appropriate time. The effect of the toxins was demonstrated by viability studies, morphological studies, changes in cytokine receptors and cytokine analysis.

2.1 SAMPLING

Patients with cancer were recruited from two South African State Hospitals in KwaZulu-Natal, and volunteers (healthy individuals) were recruited from the Durban Institute of Technology (DIT), ML Sultan campus. Any seriously ill patients, or volunteers with common colds, influenza or any other complications which could affect the immune system were excluded from the study. Oesophageal cancer patients were recruited from the medical ward of the King George V Hospital after obtaining permission from the superintendent of the hospital. Breast cancer patients as well as oesophageal cancer patients were recruited from the surgical ward of the King Edward VIII Hospital under the consultant care of Professor A. A. Hafejee. Volunteers were students and employees of the DIT. A total of 90 individuals participated in this study and the population comprised both males and females. An informed-consent form (Appendix A) was completed by all participants. All the individuals were black,

between the ages of 25 - 75 years. Cancers had been confirmed by histological examination of biopsy specimens. Ethical approval for this study was obtained from the Durban Institute of Technology Ethics Committee.

The sample size was distributed as follows:

Group I -	oesophageal cancer patients	30
Group II -	non-oesophageal cancer patients	30
Group III -	volunteers (healthy individuals)	60

2.2 PURIFICATION OF LYMPHOCYTES AND NEUTROPHILS

2.2.1 Specimen collection

Blood samples were collected early in the morning. Venous whole blood (12 ml) was obtained from the three groups of individuals with a 15 ml sterile syringe and transferred immediately into 3 x 5 ml Vacu-test tube (Radem Medical, Sandton, SA) containing 0.5 ml of 3.8% sodium citrate and 100 µl of cocktail medium [0.12 mM ethylene diamine tetra-acetic acid (EDTA), 6 mM phenanthraline, 8 µg/ml soybean trypsin inhibitor (SBTI), 0.44 µg/ml captopil and 1.08 µg/ml phosphoramidon] in order to preserve basal layers of cytokines. One tube of blood was immediately centrifuged at 1750 x g for 10 min and the plasma was collected in eppendorf tubes containing 100 µl of cocktail medium. This was stored at -70°C for cytokine analyses. The remaining two tubes of blood were used to isolate neutrophils and lymphocytes.

2.2.2 Purification

The lymphocytes and neutrophils were isolated from whole blood using differential centrifugation technique as outlined by Boyum (1968):

- Blood (3 ml) was overlaid onto Histopaque 1119 and Histopaque 1077 (Sigma, St. Louis) in the ratio 1:1:1 in 15 ml centrifuge tubes. This is represented graphically in Figure 2.1.
- These samples were then centrifuged at 700 x g for 30 min at RT.
- After centrifugation, the lymphocytes present in the buffy coat - mononuclear layer and neutrophils present in the buffy coat – granulocyte layer were collected separately with sterile Pasteur pipettes into two separate centrifuge tubes (Figure 2.1).
- These neutrophils and lymphocytes were then washed by the addition of an equal volume of oxygenated (95% O₂ and 5% CO₂ mixture - Afrox) 0.01 M phosphate buffered saline (PBS) pH 7.4 by centrifugation at 225 x g for 15 min. The supernatant containing excess histopaque was discarded. This washing process was repeated twice.
- The cells (lymphocytes/neutrophils) were then resuspended in 1 ml oxygenated PBS (pH 7.4) and then aspirated gently with a Pasteur pipette for even distribution of the cells without harming or destroying them.
- Cell numbers were determined using the method of Freshney (1984) using a haemocytometer with trypan blue.

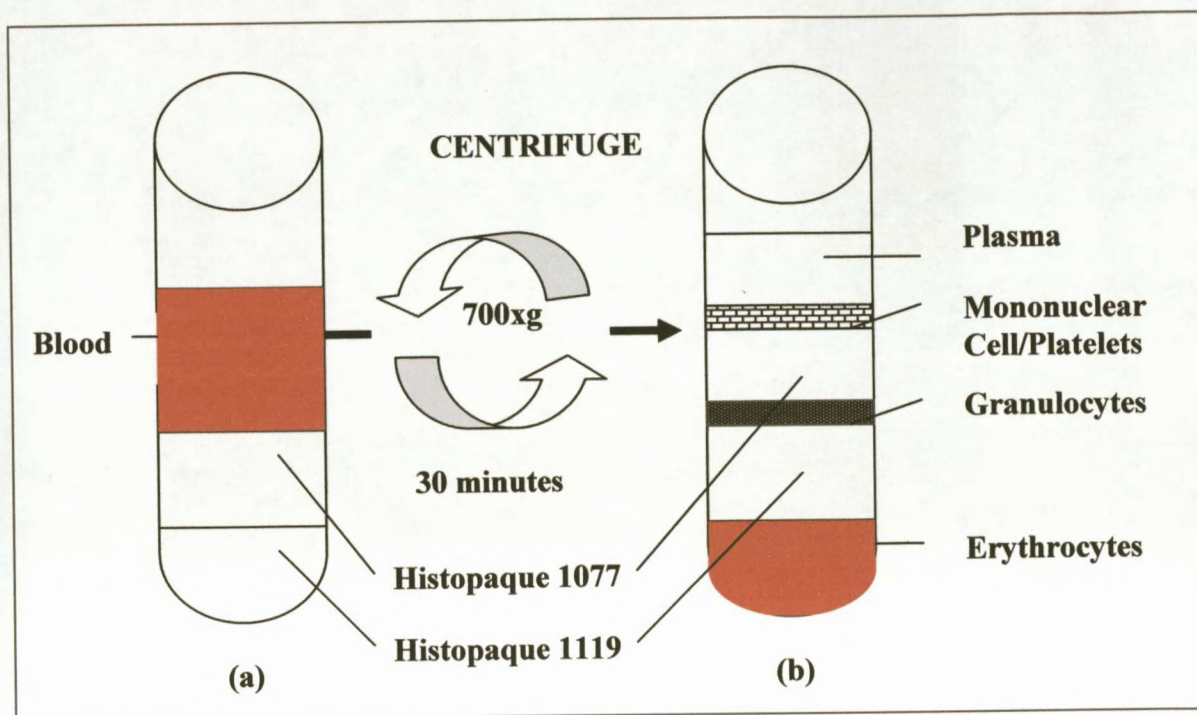


Fig. 2.1 Schematic representation showing separation of mononuclear cells and granulocytes with Histopaque 1077 and 1119 using differential centrifugation

2.3 ENUMERATION OF LYMPHOCYTES AND NEUTROPHILS

Principle of test

Trypan blue (Sigma, St. Louis) is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This test determines the proportion of living cells or organisms in a sample. The method is based on the principle that living (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Staining also facilitates the visualisation of cell morphology.

Protocol

- A 0.2% trypan blue solution was made up in distilled water using the trypan blue dye crystals.

- Fifty microlitres of each cell suspension (lymphocytes or neutrophils) were mixed with 50 μ l of the 0.2% trypan blue solution in eppendorf tubes and incubated for 1 min at RT.
- With the Neubauer cover-slip in place, a microcapillary tube was used to transfer a small amount of the trypan blue-cell suspension mixture to both chambers of the Neubauer haemocytometer.
- Starting with chamber 1 of the haemocytometer, all the cells in the centre square and the four 1 mm corner squares (see Fig 2.2) were counted. Viable cells appeared translucent whilst non-viable cells stained blue.
- The above step was repeated for chamber 2.

Analysis of results

The number of cells was determined by the following equation:

$$\text{Cell/ml} = n/v \times \text{dilution factor} \times 10^6$$

$$n = \text{number of cells counted}$$

$$v = \text{area (number of big squares counted)} \times \text{depth (0.1)}$$

$$\text{Dilution factor} = 2 \text{ (equal volume of cell suspension and trypan blue)}$$

The test was performed in duplicate using a Haemocytometer (Figure 2.20 counting chamber and counting the unstained cells using a light microscope. The lymphocytes and neutrophils were adjusted to 1×10^6 cells/ml by the addition of oxygenated PBS solution. This concentration was used for all subsequent experiments and a 99% cell viability was maintained.

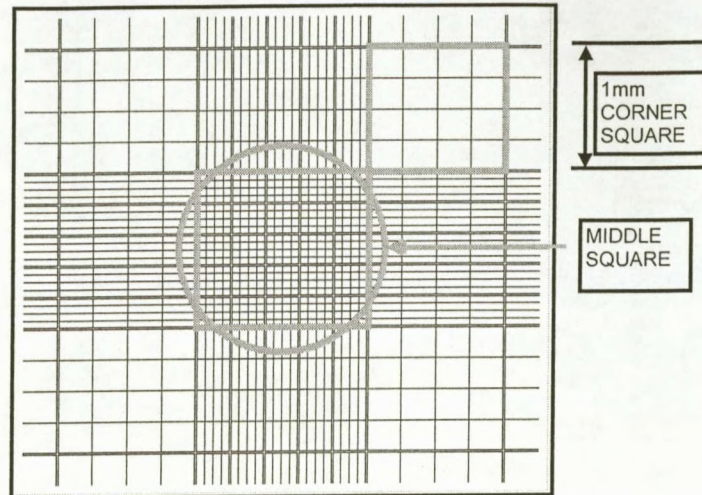


Fig. 2.2 Haemocytometer chamber

2.4 DOSE AND TIME RESPONSE OF FB₁ AND OTA ON LYMPHOCYTES AND NEUTROPHILS

In recent years there has been a great increase in the characterization and exploitation of *in vitro* assays for cytotoxicity (Williams *et al.*, 1983). The advantages of cytotoxicity tests are their suitability for the screening of numerous materials at a range of concentrations and the generation of an objective endpoint measurement using a scanning spectrophotometer (ELISA reader) or a dye.

In this study, the biological effect of FB₁ and OTA on lymphocytes and neutrophils (cytotoxicity) was determined by the Trypan blue dye exclusion test method and the MTT assay (Hanelt *et al.*, 1994). The dose and time at which 50% cell death (LD₅₀) occurred, was determined.

Preparation of fumonisin B₁

One milligram of FB₁ (Sigma, St Louis) was suspended in 1 ml of acetonitrile:water in the ratio 1:1 resulting in a concentration of 1 mg/ml FB₁. Hundred microlitre aliquots were placed in eppendorf tubes and resuspended in 900 µl of 1:1 acetonitrile:water, resulting in a final toxin concentration of 100 µg/ml (stock solution). The stock solution was stored at -70°C. The toxin was further diluted in oxygenated PBS to yield the following concentrations: 50 µg/ml, 30 µg/ml, 25 µg/ml, 20 µg/ml and 10 µg/ml.

Preparation of ochratoxin A

Ochratoxin A (Sigma, St. Louis) was prepared by dissolving 1 mg in 60 µl of ethanol, 340 µl dimethylsulfoxide (DMSO) and 19.6 ml complete culture medium (CCM) (RPMI medium supplemented with 10% fetal calf serum and 100 µl antibiotic) to give a final toxin concentration of 50 µg/ml (Reubel *et al.*, 1987). The toxin was further diluted in oxygenated PBS to yield 25 µg/ml and 20 µg/ml.

Protocol

Isolated lymphocytes and neutrophils were re-suspended in oxygenated PBS as described in section 2.3 and dispensed into eppendorf tubes for dose and time response measurements.

Two hundred microlitres each of a 100 µg/ml, 50 µg/ml, 30 µg/ml, 25 µg/ml, 20 µg/ml and 10 µg/ml concentrations of FB₁ was added to eppendorf tubes containing an equal volume of 10⁶ cells/ml cell suspension (neutrophils and lymphocytes). This was incubated at 37°C for various time intervals (Table 2.1). Controls were made up of lymphocytes and neutrophils in oxygenated PBS buffer omitting the mycotoxin. Each experiment was carried out in triplicate.

For OTA, dose and time response studies were conducted with 50 µg/ml, 30 µg/ml and 20 µg/ml concentrations of the toxin at 0 h, 2 h, 4 h and 23 h (overnight) as indicated in Table 2.1.

After each time interval had elapsed the eppendorf tubes were removed from the incubator and the viability of the cells was determined by the Trypan blue exclusion test method and the MTT assay.

Table 2.1 Dose and time of exposure of FB₁ and OTA

Toxin	Dose	Time (Hours)
Fumonisin B ₁	10 µg/ml	0, 1, 2, 3, 4
	20 µg/ml	
	25 µg/ml	
	30 µg/ml	
	50 µg/ml	
	100 µg/ml	
Ochratoxin A	20 µg/ml	0, 2, 4, 23
	25 µg/ml	
	30 µg/ml	
	50 µg/ml	

2.4.1 Trypan blue assay

After exposure, 50 µl of the 10⁶ cells/ml cell suspension was mixed gently with 50 µl of trypan blue solution (0.2%) in an eppendorf tube, as described in section 2.3, and counted.

2.4.2 MTT assay

Principle of test

The MTT assay was performed with the use of an MTT *in vitro* toxicology assay kit obtained from Sigma (St. Louis, USA). The kit is designed for determining cell number spectrophotometrically as a function of mitochondrial activity in living cells. This study makes use of a modification of the MTT assay, a colorimetric cell culture assay first employed by Mossman (1983). The MTT method is simple, accurate and yields reproducible results. The key component is (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) or MTT (Liu, 1981). Solutions of MTT, dissolved in medium or balanced salt solutions without phenol red, are yellowish in colour. Mitochondrial dehydrogenases, such as succinate dehydrogenase, of viable cells cleave the tetrazolium ring, yielding purple formazan crystals, which are insoluble in aqueous solutions (Alley *et al.*, 1988). The crystals are dissolved in acidified isopropanol.

The resulting purple solution is spectrophotometrically measured. The quantity of formazan generated by cells gave an indication of the number and/or metabolic activity of surviving cells (Swisher *et al.*, 1991). Therefore, an increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material (toxin).

Protocol

One hundred microlitres of the 10^6 cells/ml cell suspension and 100 μ l of mycotoxin (FB₁ or OTA) of various concentrations as indicated in Table 2.1 were used. Each sample was assayed in triplicate and the experiment was carried out in duplicate in 96-well microtitre

plates (Corning Cell Wells™, Corning, USA). These samples were incubated at 37°C. In the case of controls, the cells were incubated in oxygenated PBS. The plates were removed from the incubator at the appropriate time intervals (Table 2.1) and 20 µl (10% of the total cell suspension) of MTT (reconstituted with 3 ml RPMI medium) was added to each well. This was incubated for a further 2 h at 37°C. After the incubation period, plates were removed and the resulting formazan crystals were dissolved by adding 100 µl of MTT solubilization solution (10% Triton X-100 and 125 ml 0.1 N HCL in anhydrous isopropanol). The samples were triturated to completely dissolve the MTT formazan crystals. The absorbance was measured spectrophotometrically at 570 nm with a reference wavelength of 690 nm on a DAS Microplate Reader (modello: A2; Rome, Italy).

The percentage viability of the neutrophils and lymphocytes was calculated using the following equation:

$$\% \text{ viability} = \frac{\text{absorbance of sample}}{\text{absorbance of total cells (control)}} \times 100$$

2.5 EFFECT OF FB₁ AND OTA ON OESOPHAGEAL AND BREAST CANCER PATIENT'S NEUTROPHILS AND LYMPHOCYTES

Challenge tests were performed by exposing 10⁶ cells/ml of lymphocytes and neutrophils (isolated from volunteers and the two patient groups) to the LD₅₀ dose and time of FB₁ and OTA. Two hundred microlitres of cells (lymphocytes or neutrophils) and 200 µl of toxins (FB₁ or OTA) were added to eppendorf tubes and placed in an incubator at 37°C for the appropriate time *i.e.* 2 h for FB₁ and 23 h for OTA. Controls consisted of equal quantities of

cells and oxygenated PBS (no toxin) and placed in the incubator at 37°C. After 2 h and 23 h, viability studies, morphological studies, changes in cytokine receptors *i.e.* CK, G-CSF and TNF and analysis of cytokines (IL-1, IL-6, IL-8, IL-10 and TNF- α) were undertaken, as indicated in Figure 2.3 in order to demonstrate the effect of the toxins on the three population groups:

Ninety individuals participated in the various challenge tests - 30 in each group (Figure 2.3).

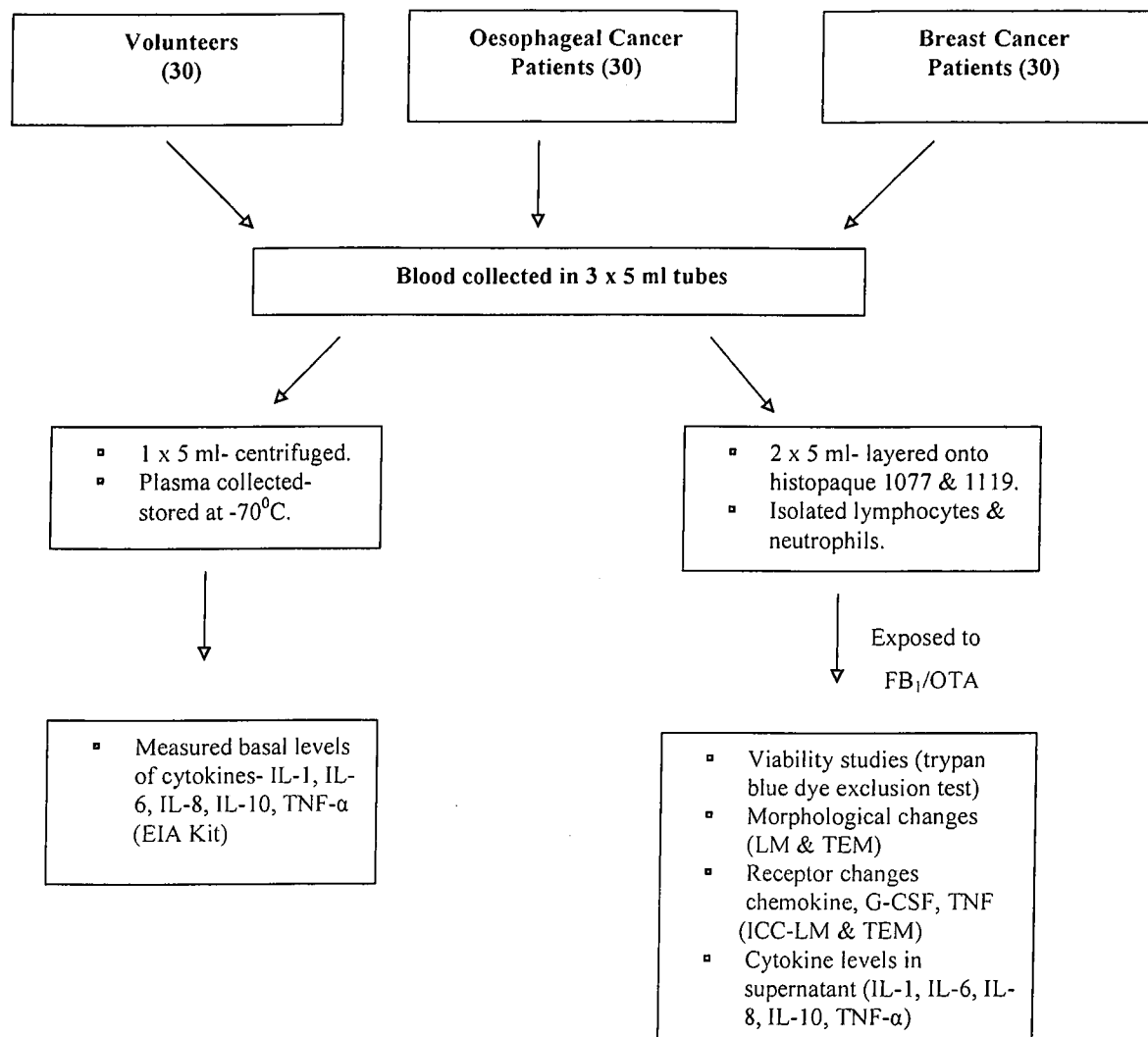


Fig. 2.3 Algorithm depicting tests conducted on lymphocytes and neutrophils of cancer patients and volunteers to evaluate effect of Fumonisin B₁ and Ochratoxin A

2.5.1 Effect of toxins on viability of neutrophils and lymphocytes

Lymphocytes and neutrophils were isolated from 30 individuals (10 volunteers, 10 oesophageal cancer patients and 10 breast cancer patients). After exposing them to FB₁ and OTA as described in section 2.5, the viability of cells was demonstrated via the Trypan blue dye exclusion test method (section 2.3).

2.5.2 Morphological effects of FB₁ and OTA

The effect of toxins on morphology of neutrophils and lymphocytes was studied by light microscopy and transmission electron microscopy. Thirty individuals participated in this study (10 volunteers, 10 oesophageal cancer patients and 10 other cancer patients *i.e.* breast cancer patients).

2.5.2.1 Light microscopy

Light microscopic changes were observed using the May-Grunwald-Giemsa's Stain. Neutrophils and lymphocytes (10^6 cells/ml) that were exposed to the toxins (25 µg/ml FB₁ for 2 h and 50 µg/ml OTA for 23 h) were smeared on to glass microscope slides and air-dried. These were subsequently fixed in methanol for 5 to 10 min. They were then transferred to May-Grunwald's stain (Appendix C), which was freshly diluted with an equal volume of buffered water (Appendix C). After films were allowed to stain for 5 min, they were transferred without washing to Giemsa's stain (Appendix C), which was freshly diluted with nine volumes of buffered water. Following staining for 15 min, the slides were then transferred to a jar containing buffered water and rapidly washed in three or four changes of water and finally allowed to stand undisturbed in water for a short time (2 to 5 min) for differentiation to take place. When differentiation was complete, the slides were allowed to dry. Films were covered by a rectangular No.1 cover-glass, using DPX mounting medium (Appendix C), which is miscible with xylol. Slides were viewed using a light microscope (Nikon 151961) attached to a Panasonic CCTV digital camera (Japan) coupled to an image analysis system (ATI).

2.5.2.2 Transmission electron microscopy

Two individuals were randomly selected from each of the three groups of individuals *i.e.* volunteers, oesophageal cancer patients and breast cancer patients in order to demonstrate the effects of the toxins on the ultrastructure of lymphocytes and neutrophils. The neutrophils and lymphocytes (10^6 cells/ml) were exposed to 25 µg/ml FB₁ for 2 h or 50 µg/ml OTA for 23 h. Controls were cells that were not exposed to the toxin. These cells (0.5 ml aliquots) were placed in eppendorf tubes. They were washed with PBS by centrifugation at 600 x g for 10 min at RT and fixed immediately with 1% glutaraldehyde (Appendix D). Eppendorf tubes were used because the samples were in suspension. The cells were prepared for transmission electron microscopy (TEM) by the procedure outlined in Table 2.2.

Table 2.2 Method for the processing of samples for TEM

Procedure	Volume (ml)	Time (min)	Temperature (°C)
1. Fixation: 1% glutaraldehyde in HBSS	0.5	30	37
2. Buffer rinse: 0.2M Sodium cacodylate (pH 7.4) x 2	0.5	10	25
3. Post fixation: 1% O ₃ O ₄ in cacodylate	0.5	60	25
4. Buffer rinse: 0.2M Sodium cacodylate (pH 7.4) x 2	0.5	15	25
5. Dehydration: 70%, 90%, 100% (x2) in EtOH	0.5	15	25
6. Clearing: Propylene oxide	0.5	30	25
7. Resin incorporation: Propylene oxide:Araldite 1:1	0.5	60	60
8. Embedding medium: Araldite x 2	0.5	90	60
9. Polymerisation: Araldite	0.5	1440	60

Because the cells were in suspension, each step of the procedure except for the embedding medium (48 h) required centrifugation of the sample at 250 x g for 2 min. The embedded samples were removed from the eppendorf tubes and sectioned with glass knives in a

Rechert-Jung ultramicrotome. The sections were cut to 60 nm thick and were stretched with xylene fumes. This was done by holding a piece of filter paper (Whatman No.1) soaked with xylene immediately over the sections.

The sections were then transferred to a 200 mesh copper viewing grids for TEM. The TEM sample grids were counterstained by first placing the grids on a droplet (10 μ l) of uranyl acetate (Appendix D) for 3 min. Thereafter the grids were transferred with fine forceps to a droplet of distilled water. This was followed by jet washing the grids 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 μ l) of lead citrate for 3 min. This was followed by another droplet (10 μ l) of distilled water and 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 sections were viewed using a Joel JEM 100 TEM.

2.5.3 Effect of toxins on cytokine receptors

Changes in the quantification or number of the cytokine receptors in lymphocytes and neutrophils was demonstrated by immunocytochemistry (ICC) using light microscopy and TEM. The method used was the avidin-biotin complex (ABC) method adapted from Polak and van Noorden (1986).

Principle of test

In ICC, labeled antibodies are used as reagents for the detection of specific substances or antigens *in situ*. Immunocytochemistry may therefore be defined as the identification and localization in a biological system of an antigen to which an antibody may be raised and

marked by a visible label (Snyman, 1993). Most currently used immunocytochemical methods rely on the detection of labelled secondary antibodies directed against the immunoglobulin of the species used for producing the primary antibody (Figure 2.4).

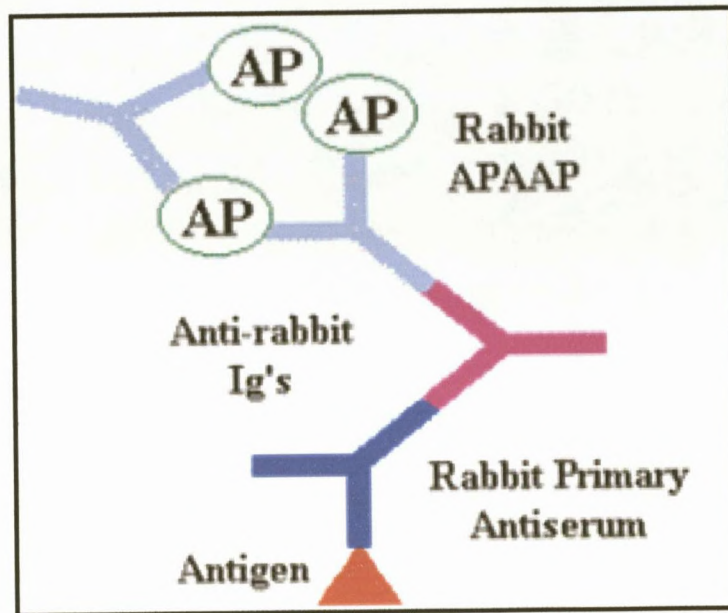


Fig. 2.4 A schematic presentation of the principle of the immunocytochemical immunolabelling protocol.

At light microscope level horseradish peroxide is often used to detect the probe and at electron microscope level gold is used. The choice of fixative for ICC generally requires a compromise between the preservation of good morphology and the retention of biological activity. Formaldehyde is generally recommended as a fixative in ICC.

For this study, the peroxidase-anti-peroxidase (PAP) method, adapted from Polak and van Noorden (1986), was used for light microscopy. Peroxidase was used as an enzymatic probe to localize the antigens. Endogenous peroxidases were blocked using 3% H_2O_2 while normal rabbit serum was used to block non-specific binding sites. The principle is that the non-immune serum from the same species as that donating the secondary antibody is applied at the

beginning of the procedure and sticks to the protein binding sites (Polak and van Noorden 1986). The serum was drained off before primary antibody was applied. Each primary antibody binds to an antigen, *i.e.* a specific cytokine receptor on the lymphocyte and neutrophils, forming a complex which is coupled to the secondary antibody which is biotin-conjugated. The third layer is the ABC (Figure 2.5). The avidin having been reacted with biotinylated peroxidase in such a proportion that three of the biotin-binding sites are taken up by the biotinylated peroxidase, leaving one site per molecule free to react with the biotin on the secondary antibody. A large amount of label is therefore localised over the original antigenic site.

The resulting complex is rendered visible using a colour dye *viz.* diaminobenzidine (DAB). Diaminobenzidine is identified by the presence of brown particles.

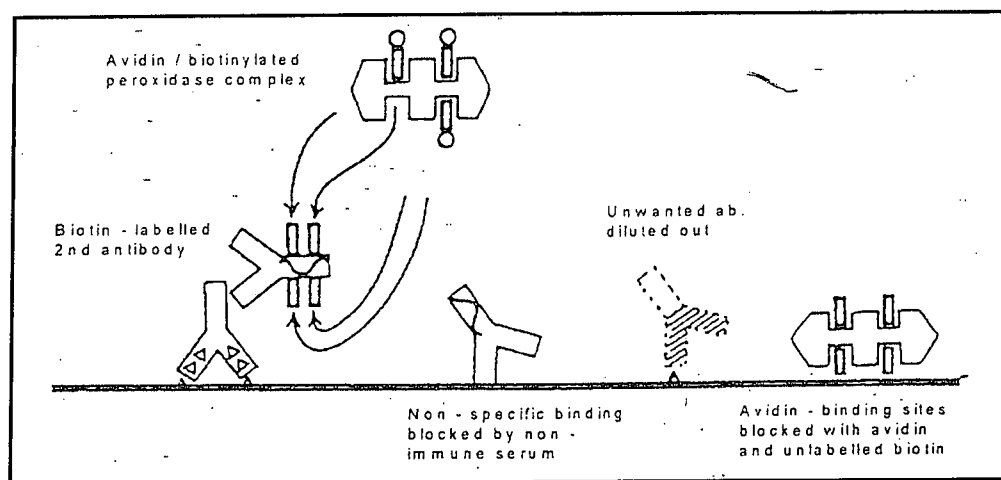


Fig. 2.5 A schematic representation of the ABC method (Polak and van Noorden, 1986)

Protocol

In this study the effect of FB₁ and OTA was demonstrated on three cytokine receptors (CSF, TNF and CK) on lymphocytes and neutrophils. For immunolabelling of the cytokine

receptors, three cytokine receptor antibodies were used, *i.e.* G-CSF receptor antibody, sTNF receptor antibody and C-X-X-X-C chemokine receptor antibody (obtained from Davies Diagnostics). The characteristics and the optimum dilution for each of the primary antibodies are provided in Table 2.3.

Table 2.3 Characteristics of receptor antibodies used in ICC

Receptor antibody	Species reactivity	Known applications	Format	Host	Quantity	Optimum dilution
C-X-X-X-C polyclonal	Human, Mouse & Rabbit	IB	Purified	Rabbit	100 µg	1:500
G-CSF monoclonal	Human	BLK, IB, Neut	Purified	Rabbit	100 µg	1:50
STNF polyclonal	Human	IB, EIA, Neut	Purified	Rabbit	50 µg	1:200

IB = immunoblotting (Western), BLK = function blocking assays

EIA = enzyme immunoassay (ELISA), Neut = neutralizing

Dilution curves for all three receptor antibodies were prepared by diluting the stock solutions from the manufacturer in ratios of 1 : 50, 1 : 100, 1 : 200, 1 : 300 and 1 : 500 with 0.01 M PBS (pH 7.4) as the diluent to determine their optimum dilutions for immunolabelling of the receptors (Table 2.3).

Fifty microlitres of 10^6 cells/ml isolated lymphocytes and neutrophils were separately incubated at 37°C with 50 µl mycotoxin (LD₅₀), *i.e.* 25 µg/ml FB₁ (at 0 time and 2 h incubation) and 50 µg/ml OTA (at 0 time and 23 h incubation).

2.5.3.1 Light microscopy

After the cells were exposed to the toxins they were pipetted onto poly-L-lysine (Sigma, St. Louis) coated slides at the appropriate times. This was done by dipping the slides in a 1:10 dilution of the poly-L-lysine solution for 5 min at RT, and drying in an oven at 60°C. The slides were air-dried and fixed with paraformaldehyde (4% in 0.01 M PBS, pH 7.4) for 10 min. Slides were stored at 4°C until immunolabelling. The immunolabelling protocol is outlined in Table 2.4.

After the slides had undergone the immunolabelling procedure, they were viewed and images obtained by using a Nikon 151961 light microscope mounted with a Panasonic CCTV digital camera (Japan) coupled to an image analysis system (ATI) and images obtained.

To assess and validate the immunolabelling procedure, controls were set up by omitting the primary antibody and replacing it with 0.01 M PBS (pH 7.4) buffer. The absence of labelling validated the specificity of the method.

Table 2.4 Protocol for immunolabelling of slides for light microscopy

Procedure	Time
1. Rehydrated fixed, stored slides Washed in 0.01M PBS (pH 7.4)	5 min
2. Quenching endogenous peroxidase 3% hydrogen peroxide in methanol Washed in 0.01M PBS (pH 7.4)	20 min 5 min
3. Blocking serum LSAB kit, DAKO, USA	20 min
4. Primary antibody Washed in 0.01M PBS (pH 7.4)	Overnight at 4°C 5 min
5. Probe development Biotin-conjugated 2 nd antibody at RT (LSAB kit) Washed in 0.01M PBS (pH 7.4) Avidin-Biotin complex (LSAB kit, DAKO, USA) Washed in 0.01M PBS (pH 7.4) DAB solution in the dark at RT (LSAB kit) Washed in tap water	10 min 5 min 10 min 5 min 10 min
6. Counterstained Mayers haematoxylin (Sigma, St. Louis)	30 seconds
7. Dehydration 70% ethanol 90% ethanol 100% ethanol Xylene	Dipped 4-5 times in each solution
8. Mounting Entellen mounting medium	

2.5.3.2 Electron microscopy

After exposing the cells to the toxins *i.e.* 25 µg/ml FB₁ (at 0 time and 2 h incubation) and 50 µg/ml OTA (at 0 time and 23 h incubation), they were fixed, dehydrated and embedded in eppendorf tubes using the procedure as outlined in Table 2.5.

Table 2.5 Protocol for the processing/preparation of tissues for electron microscopy

Procedure	Time
1. Fixation: Fixed in paraformaldehyde (4% in 0.01M PBS pH 7.4) Centrifuged at 2000 rpm	30 min 5 min
2. Buffer rinsed: Washed in 0.01M PBS (pH 7.4) Centrifuged at 2000 rpm	5 min 5 min
3 Dehydrated in graded series of alcohols: 70% 90% 100%	15 min 15 min 15 min
4. Clearing: Propylene oxide (1ml)	30 min
5. Resin impregnation: 1:1 propylene oxide and Spurr's resin (1 ml) Spurr's resin Replaced resin in a new tube	30 min 1 h at 37°C 1 h
6. Embedding: Embedded in new tube & cured	Overnight at 37°C

After each step of the processing procedure the samples were centrifuged at 500 x g for 2 min to spin the cells into a pellet. Eppendorf tubes were used as they were convenient for moulding the embedding resin to a tip which contained the pelleted cells, thus making sectioning easier.

Embedded samples were removed from eppendorf tubes and sectioned with glass knives. Sections were then transferred to nickel viewing grids and immunolabelled using the procedure as outlined in Table 2.6.

Table 2.6 Immunolabelling procedure for electron microscopy: colloidal gold method

Procedure	Time
1. Grids washed Washed in 0.01M PBS (pH 7.4)	
3. Blocking agent 5% maleic acid blocker	2 min
4. Primary antibody Washed in 0.01M PBS (pH 7.4)	3 h at RT
5. Probe development Colloid gold-conjugated 2 nd antibody (Amersham Bioscience) Washed in 0.01M PBS (pH 7.4)	30 min at RT
6. Counterstain Uranyl acetate (10 µl)	3 min

Grids were dried with Whatman No.1 filter paper and then viewed under the electron microscope and images were captured.

2.5.4 Effect of toxins on cytokine levels

An enzyme immunoassay (EIA) kit (ChemiKine) supplied by Chemicon was used for measuring the various cytokines (IL-1, IL-6, IL-8, IL-10 and TNF- α) present in the plasma of volunteers and the two patient groups, and in the supernatants when lymphocytes and neutrophils isolated from the three population groups were exposed to either FB₁ or OTA. This system measured natural and recombinant forms of the cytokine.

ChemiKine human cytokine kit measured the "total" (bound and free) amount of cytokine in serum, plasma, and serum-free biological fluids. The ChemiKine assay system is not hindered by autoantibodies, soluble receptors, or binding proteins that can interfere with most

commercial sandwich assays. The result is that nanogram quantities of cytokine can often be detected using the ChemKine system. ChemiKine also accurately measures cytokine concentration in cell culture supernatants provided the concentration is within the dynamic range of the standard curve.

Principle of test

With the ChemiKine assay system, precoated goat anti-rabbit antibody plates are used to capture a specific cytokine complex in each sample consisting of the cytokine antibody, standard or unknown, and biotinylated cytokine. Biotinylated cytokine conjugate (competitive ligand), and sample or standard compete for cytokine specific antibody binding sites. Therefore, as the concentration of the cytokine in the sample increases, the amount of biotinylated cytokine captured by the antibody decreases. The assay is visualised using a streptavidin alkaline phosphatase conjugate and an ensuing chromogenic substrate reaction (Figure 2.6).

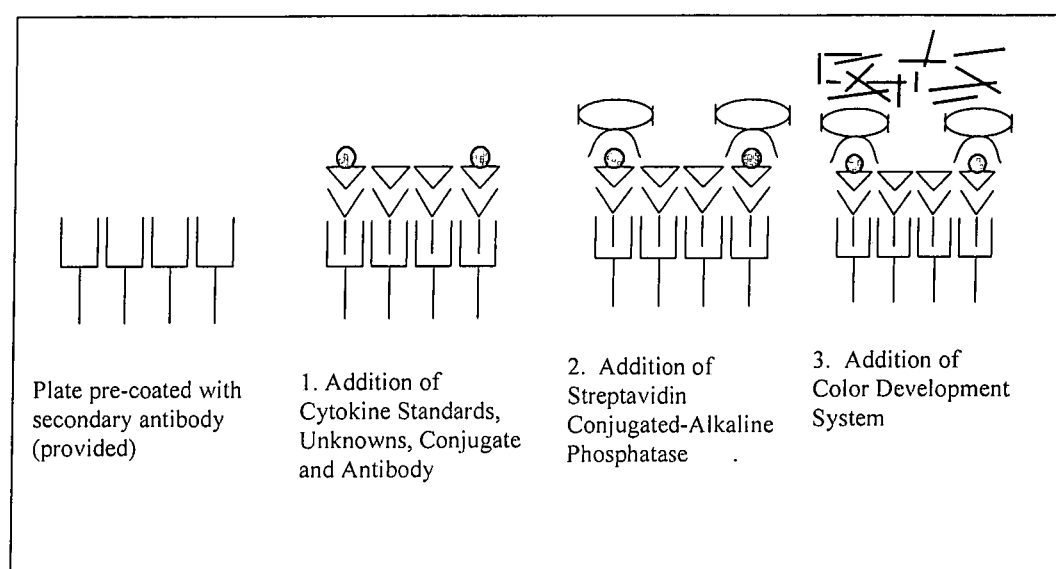


Fig. 2.6 Schematic representation of chemikine's competitive enzyme immunoassay

The amount of cytokine detected in each sample is compared to a cytokine standard curve which demonstrates an inverse relationship between Optical Density (OD) and cytokine concentration, *i.e.* the higher the OD, the lower the cytokine concentration in the sample.

Preparation of reagents

Rabbit Anti-Human Cytokine Antibody

The lyophilised cytokine antibody was reconstituted with 3.5 ml of the diluent provided and vortexed.

Recombinant Cytokine Standard

The cytokine standard vial was reconstituted with the diluent provided. The diluent was further used to dilute the standards as follows:

- a) Six 12 x 75 test tubes were labelled from 2-6 and "0-dose." The diluent (750 μ l) was added to the six standard tubes.
- b) The cytokine standard was reconstituted in 1000 μ l of the diluent. This solution was standard # 1 and had a concentration of 200 ng/ml.
- c) Standards # 2-6 were then prepared by performing a 1:4 dilution of the preceding standard. For example, to make standard # 2, 250 μ l of standard #1 was added to tube # 2 and vortexed and so on. The "0 Dose" standard tube had no cytokine.

Human Cytokine Conjugate

The lyophilized cytokine conjugate was reconstituted with 3.5 ml of diluent and vortexed.

Diluting Wash Buffer

The contents of concentrated wash buffer was diluted to 1.0 L with deionised water and stirred to homogeneity.

Streptavidin-Alkaline Phosphatase

The lyophilized streptavidin-alkaline phosphatase was reconstituted in 6.0 ml of diluent and vortexed.

Colour reagents

Colour reagent A and colour reagent B were allowed to come to RT.

Protocol

1. 100 μ l of the standards # 0-6 (of each cytokine) were dispensed into their designated wells in duplicate (Table 2.7). A standard curve was run for each cytokine.

Table 2.7 Serial dilution of cytokine standards (IL-1, IL-6, IL-8, IL-10 and TNF- α)

Lyophilized Standard	250 μ l	250 μ l	250 μ l	250 μ l	250 μ l	250 μ l	250 μ l
Initial Volume (μ l)	1000	750	750	750	750	750	750
Concentration (ng/ml)	200	50	12.5	3.125	0.781	0.195	0.0
Standard Number	#1	#2	#3	#4	#5	#6	0 dose

2. Test Sample:

The test sample comprised of (a) plasma that was obtained from the three groups of individuals and stored at -70°C in cocktail medium as explained in section 2.2 and (b) supernatant that was obtained after exposing 10^6 cells/cm² of lymphocytes and neutrophils (isolated from volunteers and the two patient groups) to the LD₅₀ dose and time of FB₁ and OTA as explained in section 2.7. The supernatant was also stored at -70°C in cocktail medium.

For each individual sample, 100 μ l of sample + 200 μ l of diluent (1) + 100 μ l of Diluent (2) was added to a 12 x 75 test-tube and vortexed. To each of the designated wells (precoated with secondary antibody), 100 μ l of each diluted sample preparation was dispensed (in duplicate).

3. Twenty five microlitres of reconstituted rabbit anti-human polyclonal antibody (for the specific cytokine) was dispensed into each well. Plates were covered with the acetate plate sealer to prevent evaporation and incubated for 3 h at RT.
4. The plate sealer was gently removed, 25 μ l of reconstituted cytokine conjugate was dispensed into each well, and plate was resealed and incubated for 30 min at RT.

5. Important wash step:

The plate sealer was gently removed and the plate was washed five times. A thorough washing of the plate was extremely important to reduce background. A multi-channel pipette was used to fill each well with 250 μ l of diluted wash buffer. Fluid removal from the wells was best accomplished by inverting the plate over a sink and flicking the fluid out of the wells and then blotting the plate on clean paper towels. Using the multichannel pipette 250 μ l of wash buffer was added to each well; the plate was flicked and blotted. This procedure was repeated for a total of four times. Two hundred and fifty microlitres of diluted wash buffer was dispensed a fifth time and the plate soaked for 10 min. After 10 min of soaking, each well was blotted and aspirated to remove any excess fluid.
6. Colour reagent A and colour reagent B were allowed to come to RT (refer to Appendix for reagent preparation). Fifty microlitres of the diluted streptavidin-alkaline phosphatase was dispensed into each well. The plate was resealed with the plate sealer and incubated for 30 min at RT.
7. The plate sealer was gently removed. The plate was washed 5 times using the wash method described above making sure to soak plate with the wash buffer for 10 min on wash cycle 5 before final fluid removal and aspiration of each well.
8. Two hundred microlitres of the prepared colour reagent solution was dispensed into each well. The plate was incubated at RT for 15 min.

9. The colour generation was monitored very closely. The plate was read at 490 nm using a DAS Microplate Reader (modello: A2; Rome, Italy) during the 25 min incubation period to monitor the speed at which colour was generated.
When the OD for "0 Dose" had reached 1.6, a reading was taken and saved.
10. Fifty microlitres of stop solution was added into each well in the same order that the colour reagent solution was added.
11. The plate was read a final time at 490 nm.

After reading the optical densities for the various test samples, the concentration of IL-1, IL-6, IL-8, IL-10 and TNF- α present in the plasma of volunteers and the 2 patient groups, and in the supernatant of cells exposed to the toxins, was obtained from the standard curves for IL-1, IL-6, IL-8, IL-10 and TNF- α .

2.6 STATISTICAL ANALYSIS

Statistical analysis was carried out by the SPSS 12 computer programme and reviewed by a statistician. Results are presented as the mean and standard error of the mean (SEM). Significance was calculated by a two-tailed, unpaired Student's t-test, the Mann-Whitney test and the Kruskal-Wallis test. Levels of significance were determined using a 95% confidence interval; a p-value < 0.05 was taken to be statistically significant.

CHAPTER THREE: RESULTS

3.1 EFFECT OF FB₁ AND OTA ON LYMPHOCYTES AND NEUTROPHILS FROM HEALTHY VOLUNTEERS

3.1.1 Effect on viability

Initial experiments on the effect of FB₁ and OTA on purified lymphocytes and neutrophils from healthy volunteers showed that both toxins decreased the number of viable cells with increasing concentration of toxin. With FB₁ the effect was rapid and was evident over a 4 h period and with OTA this effect was observed over a 24 h period. With 100 µg/ml FB₁ exposure both lymphocyte and neutrophil cell death occurred immediately. With 10 µg/ml FB₁ exposure both lymphocytes and neutrophils remained viable for over the time period. Fumonisin B₁ had a more rapid toxicological effect than OTA since OTA required twice the dosage (50 µg/ml) over a longer time frame *i.e.* 23 h to obtain 50% cell death (Figures 3.1 to 3.8). The dosage required to kill 50% of neutrophils and 50% of lymphocytes was determined to be 25 µg/ml FB₁ (after 2 h exposure) and 50 µg/ml OTA (after 23 h exposure) as shown in Tables 3.1 and 3.2.

A comparison of the trypan blue dye exclusion test method and the MTT assay to measure viability of lymphocytes and neutrophils exposed to FB₁ and OTA showed no significant differences ($p \geq 0.05$). The LD50 and the viability of the immunocytes at the different toxin concentrations tested follow similar patterns regardless of testing method as shown in Figures 3.1 - 3.8. Subsequent experiments were conducted using the trypan blue dye exclusion test since it is more rapid and cost effective.

Table 3.1 Lethal dose for lymphocytes and neutrophils exposed to FB₁

FB₁ [µg/ml]	Lymphocytes LD₅₀ @ time	Neutrophils LD₅₀ @ time
20	3	2
25	2	2
30	1	1
50	1	0

Table 3.2 Lethal dose for lymphocytes and neutrophils exposed to OTA

OTA [µg/ml]	Lymphocytes LD₅₀ @ time	Neutrophils LD₅₀ @ time
20	0	0
25	0	0
30	0	0
50	23	23

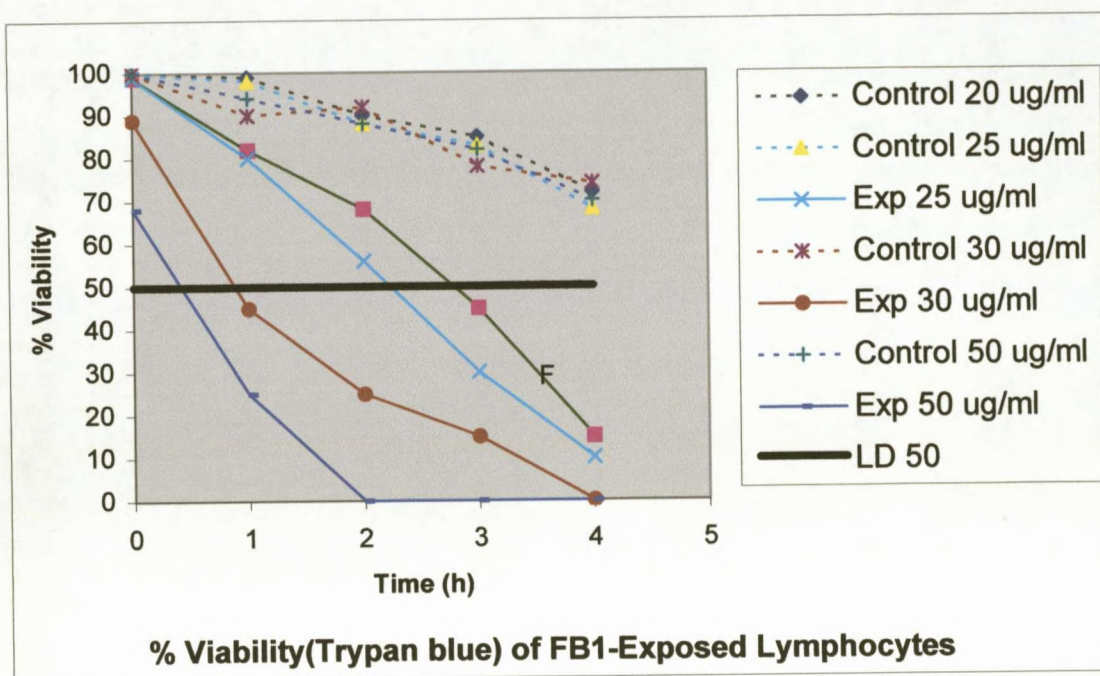


Fig. 3.1 Time-response of lymphocytes to different concentrations of FB₁ (Trypan blue dye)

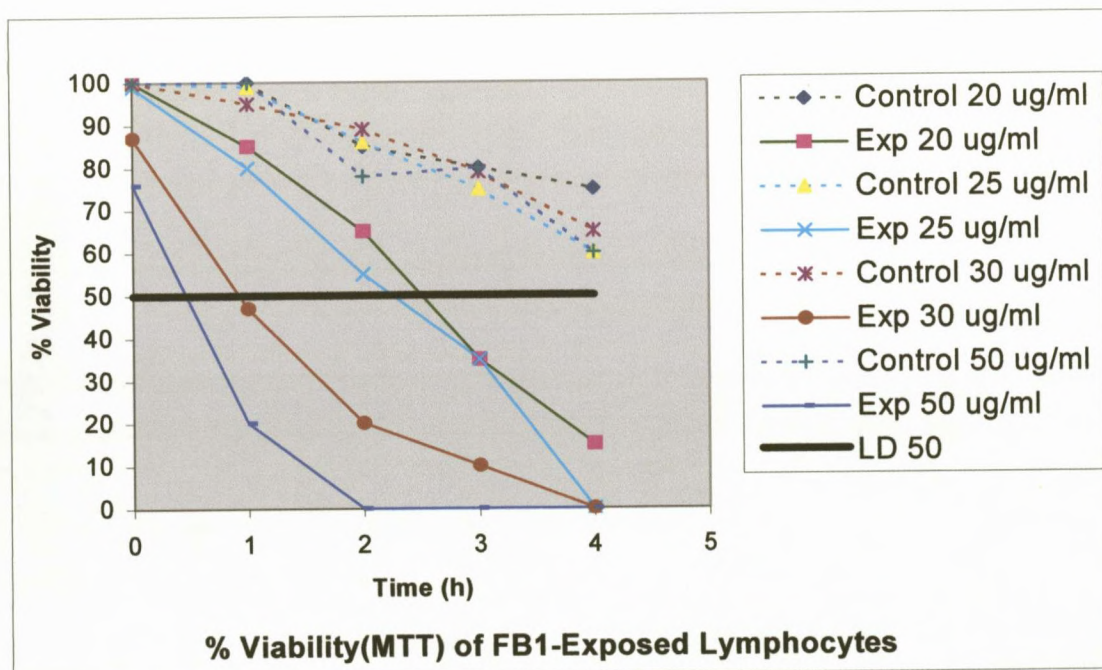


Fig. 3.2 Time-response of lymphocytes to different concentrations of FB₁ (MTT assay)

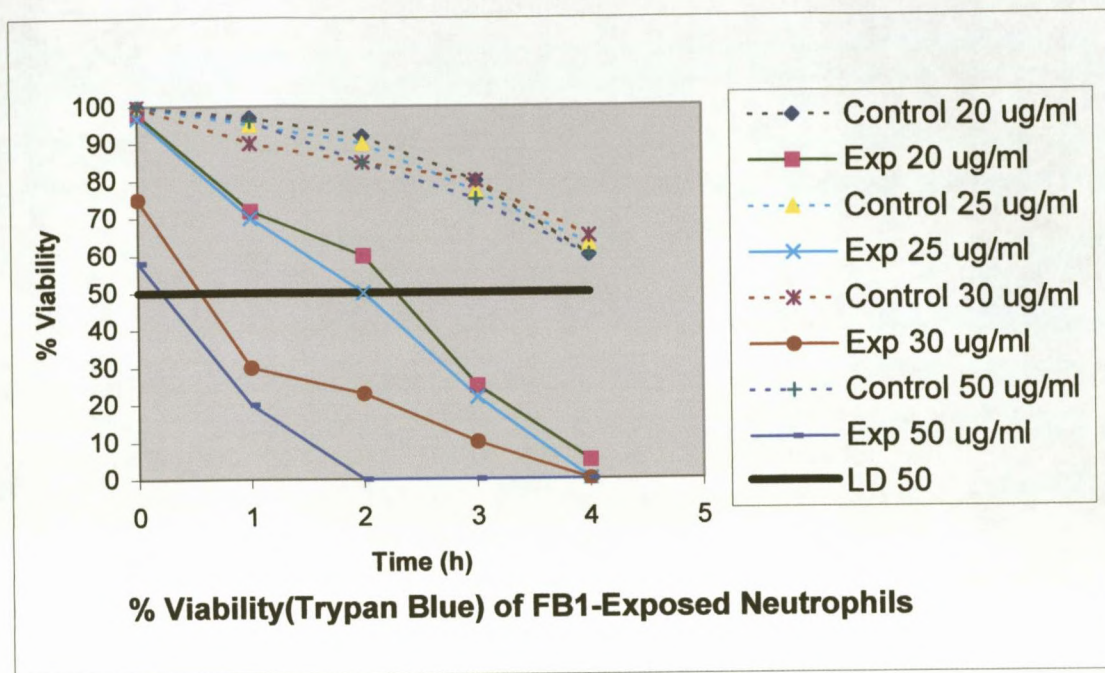


Fig. 3.3 Time-response of neutrophils to different concentrations of FB₁ (Trypan blue dye)

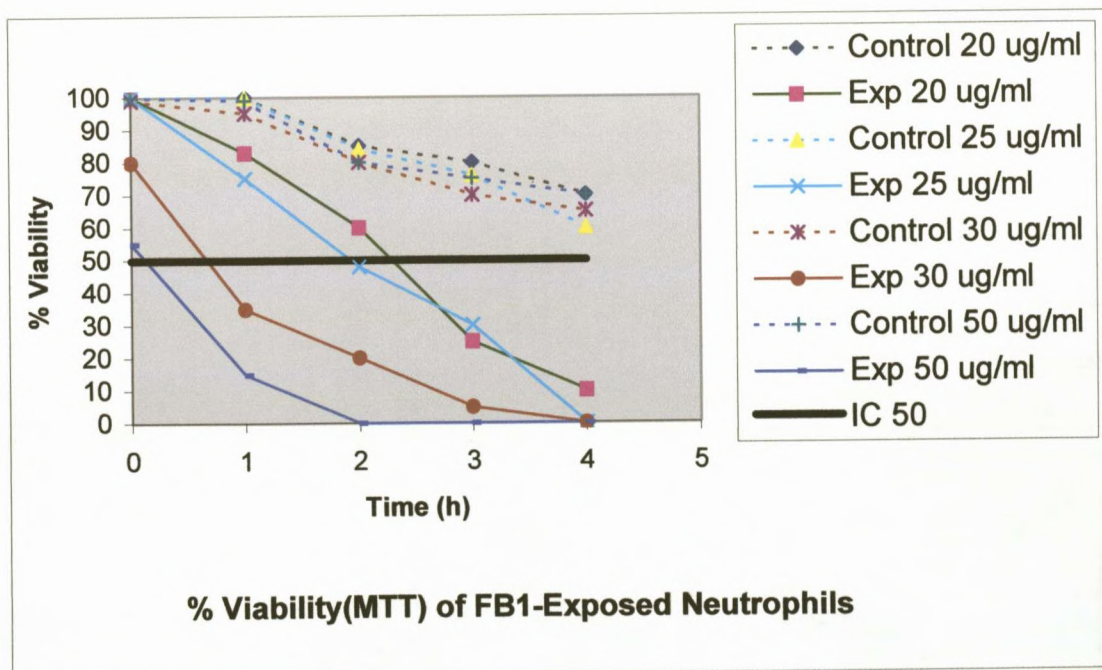


Fig. 3.4 Time-response of neutrophils to different concentrations of FB₁ (MTT assay).

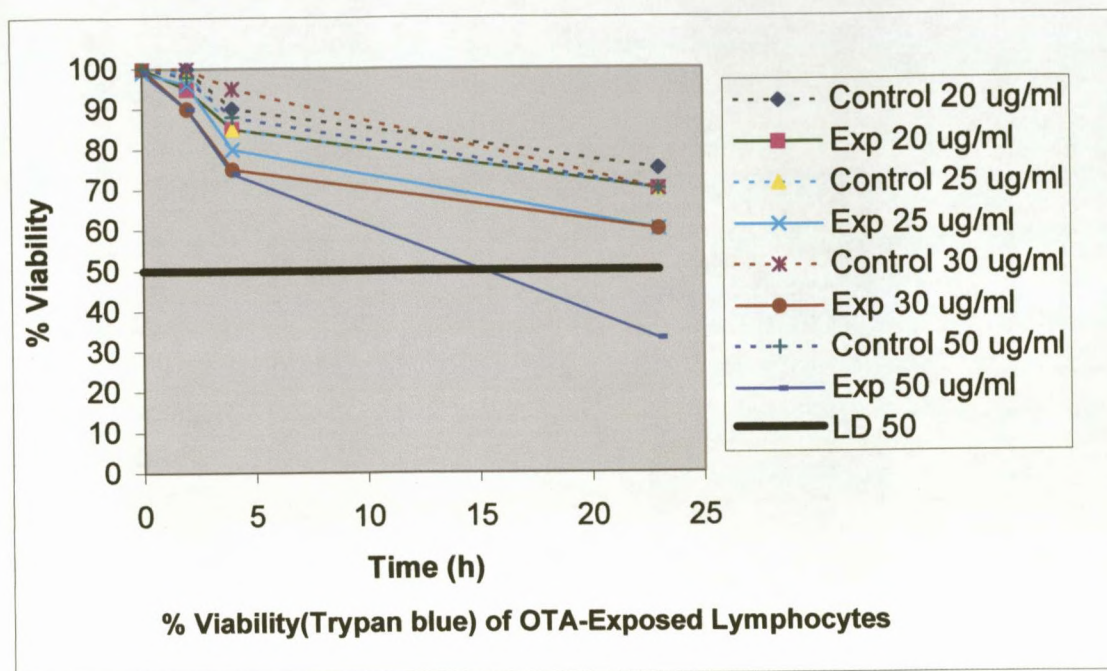


Fig. 3.5 Time-response of lymphocytes to different concentrations of OTA (Trypan blue dye)

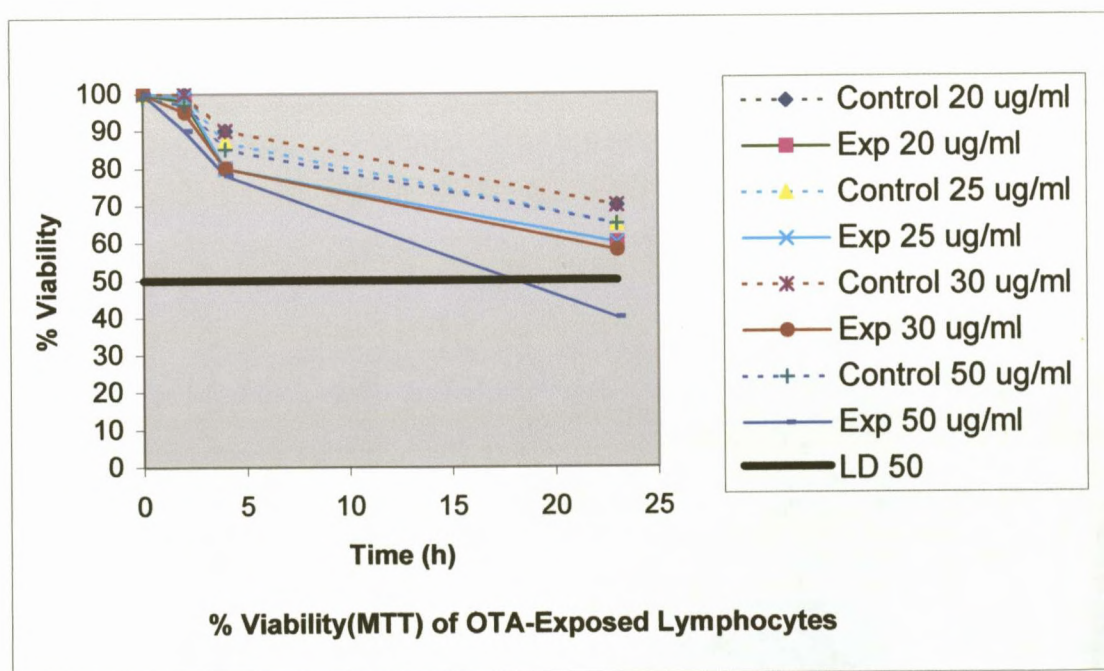


Fig. 3.6 Time-response of lymphocytes to different concentrations of OTA (MTT assay).

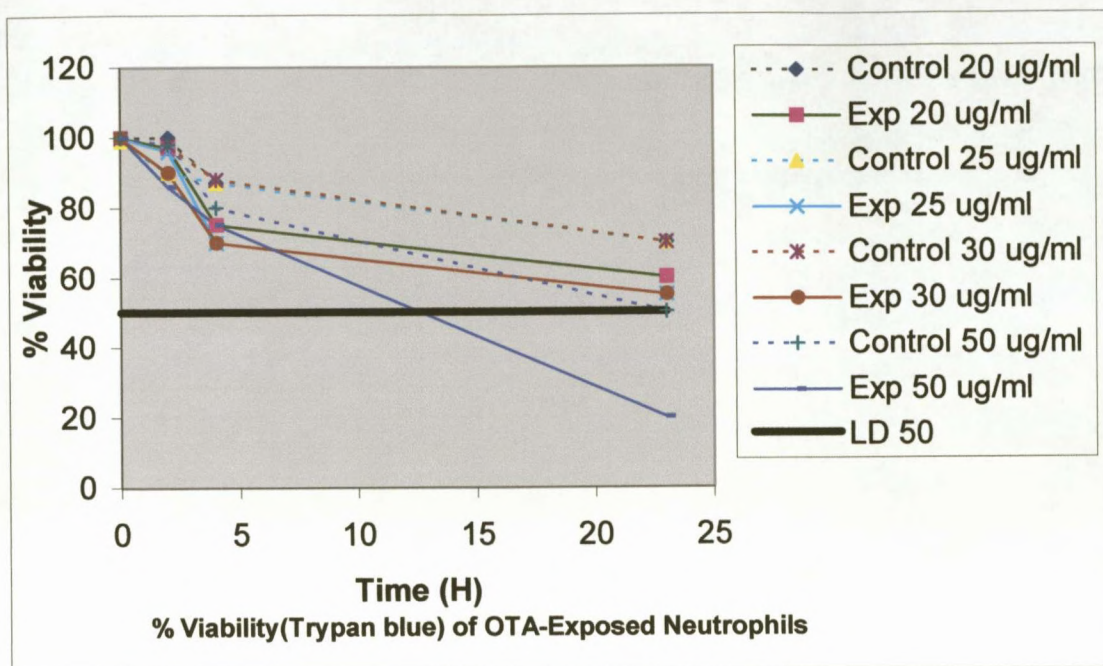


Fig. 3.7 Time-response of neutrophils to different concentrations of OTA (Trypan blue dye)

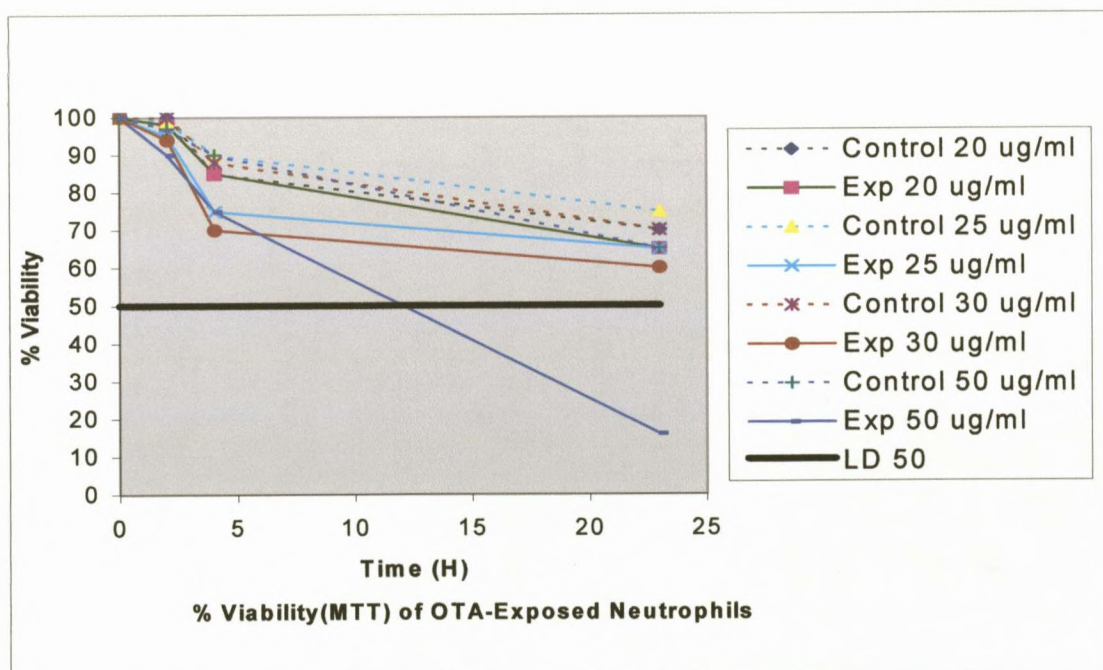


Fig. 3.8 Time-response of neutrophils to different concentrations of OTA (MTT assay)

.1.2 Effect on morphology

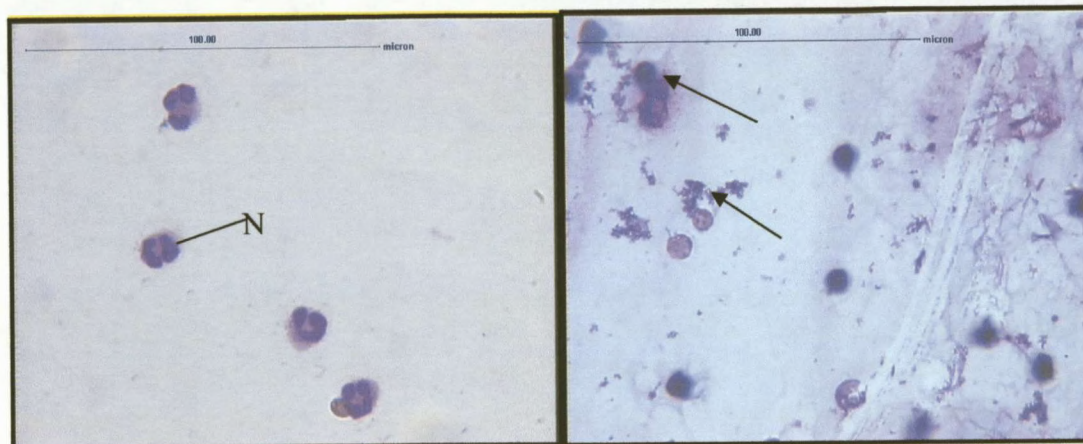
3.1.2.1 Gross morphological changes

Neutrophils exposed to FB₁ and OTA

Light microscopic observation of the effect of the two toxins on neutrophils showed that neutrophils unexposed to toxins appeared healthy with no indication of cell damage and had distinct polymorphonuclear morphology with a granular appearance exhibiting a distinct three to five lobed nucleus (Figure 3.9a). Exposure of neutrophils to 25 µg/ml of FB₁ for 2 h showed cell lysis and loss of cell membrane integrity. The nucleus lost its lobed appearance and appeared to be undifferentiated from the cytoplasm (Figure 3.9b). Exposure to 50 µg/ml to OTA showed most of the neutrophils to be lysed. In the few remaining cells further disintegration of the nucleus was evident which appeared to be undifferentiated from the cytoplasm; however, these cells had no cell membrane (Figure 3.9c).

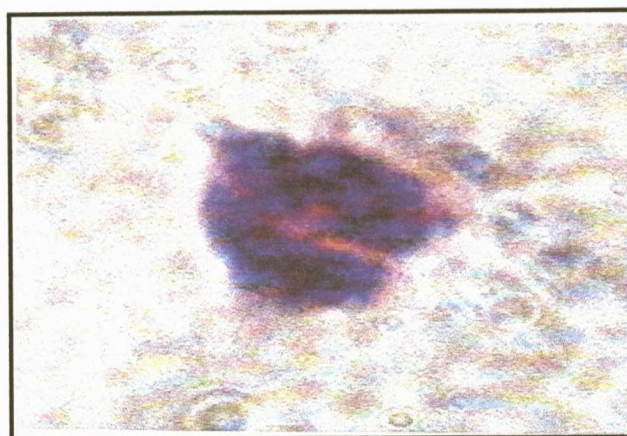
Lymphocytes exposed to FB₁ and OTA

Lymphocytes unexposed to toxins (control) appeared healthy with distinct mononucleus, a thin cytoplasmic region and intact cell membrane (Figure 3.10a). On exposure to 25 µg/ml FB₁ for 2 h the lymphocytes showed signs of membrane disruption. Cytoplasm expulsion and nuclear damage was also observed (Figure 3.10b). Similar results were obtained when lymphocytes were exposed to 50 µg/ml OTA for 23 h (Figure 3.10c).



(a) X40

(b) X40

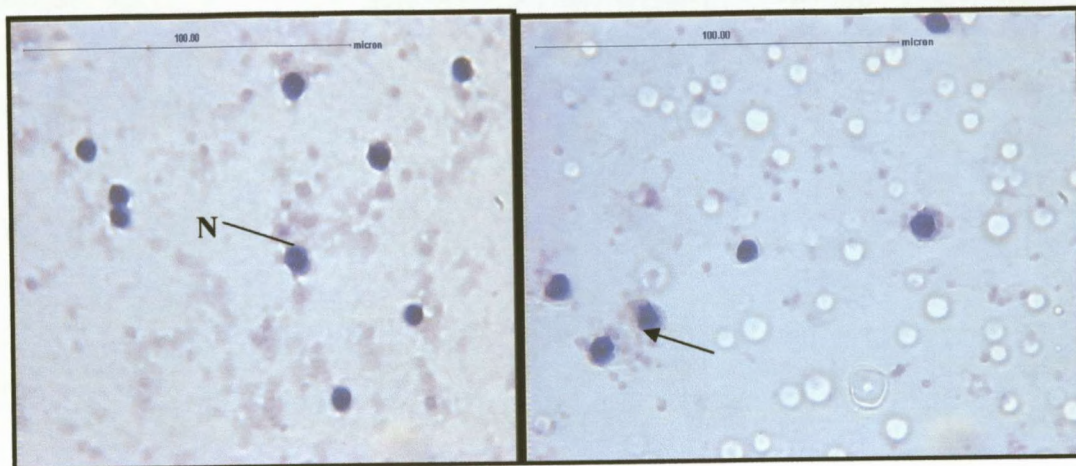


(c) X100

Fig. 3.9

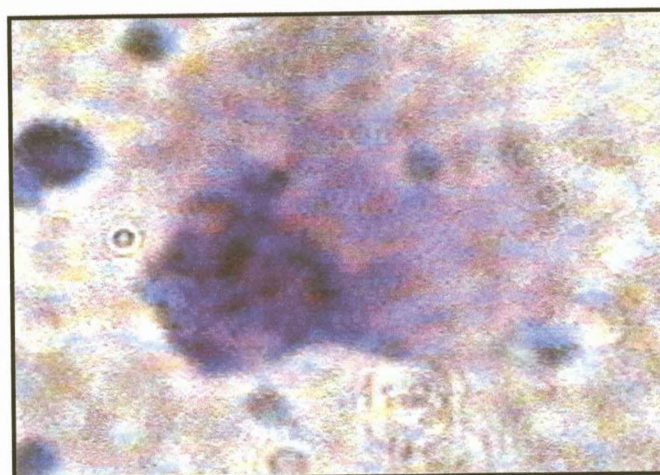
Morphology of neutrophils.

(a) control neutrophils with lobed nucleus (N), **(b)** neutrophils exposed to 25 $\mu\text{g/ml}$ of FB_1 for 2 h, arrows represents cell lysis, and **(c)** neutrophils exposed to 50 $\mu\text{g/ml}$ OTA for 23 h



(a) X40

(b) X40



(c) X100

Fig. 3.10 Morphology of lymphocytes.

(a) control lymphocytes with round nucleus (N), (b) lymphocytes exposed to 25 µg/ml of FB₁ for 2 h, arrow represents cytoplasmic expulsion, and (c) lymphocytes exposed to 50 µg/ml OTA for 23 h

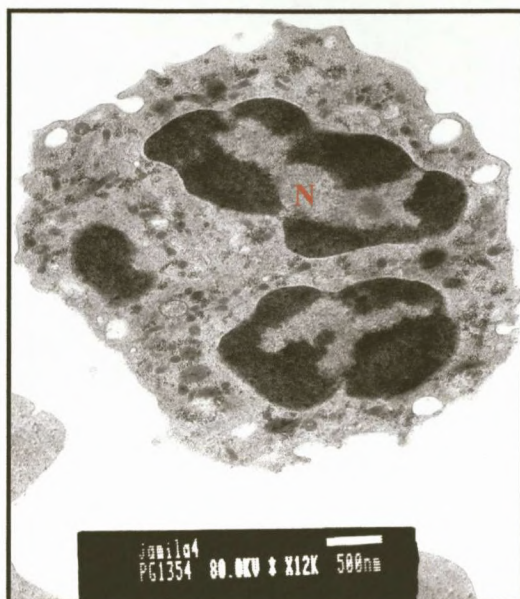
3.1.2.1 Ultrastructural changes

Neutrophils exposed to FB₁ and OTA

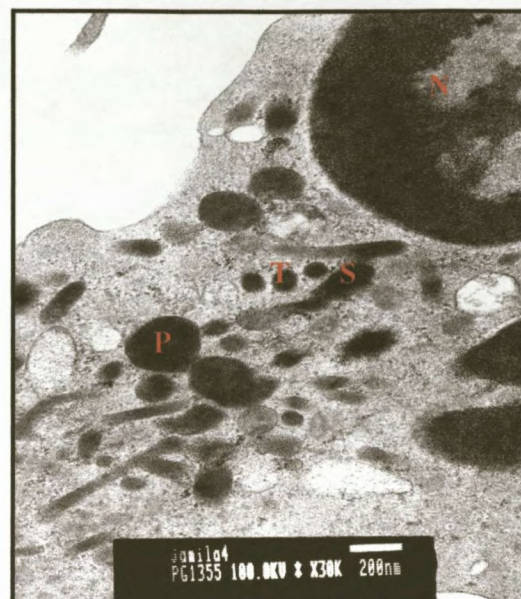
Neutrophils unexposed to toxins (control) appeared healthy, characterized by distinct organelles, which included lobed nuclei (Figure 3.11a), primary, secondary and tertiary granules (Figure 3.11b), and an intact cell membrane (Figure 3.11c).

Neutrophils exposed to 25 µg/ml FB₁ for 2 h showed neutrophil injury which was indicative of apoptosis, represented by the loss of cellular integrity (Figure 3.12a), loss of cellular organization (Figure 3.12b) with a large number of vacuoles, lipid granules and leakage of nucleoplasm (Figure 3.12c). Some of the toxin appeared to be encapsulated in phagosome vesicles.

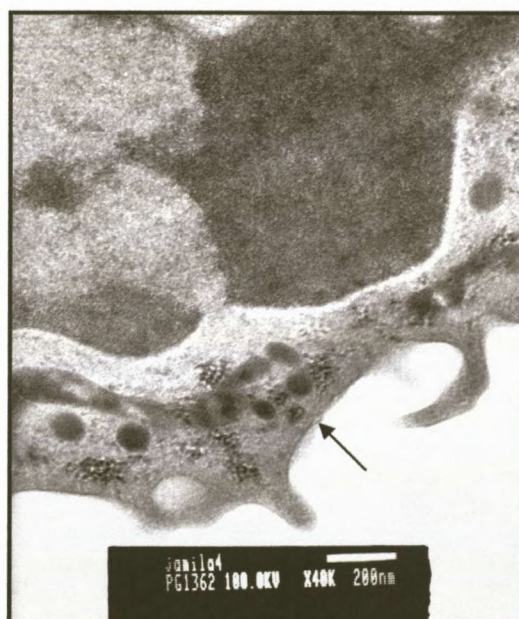
Neutrophils exposed to 50 µg/ml OTA for 23 h (Figure 3.13) displayed loss of cellular organization and integrity. Large numbers of vacuoles were present and the plasma membrane was damaged (Figure 3.13a). Leakage of nucleoplasm was observed, as well as phagosome encapsulation of toxin (Figure 3.13b).



(a) X 14 000



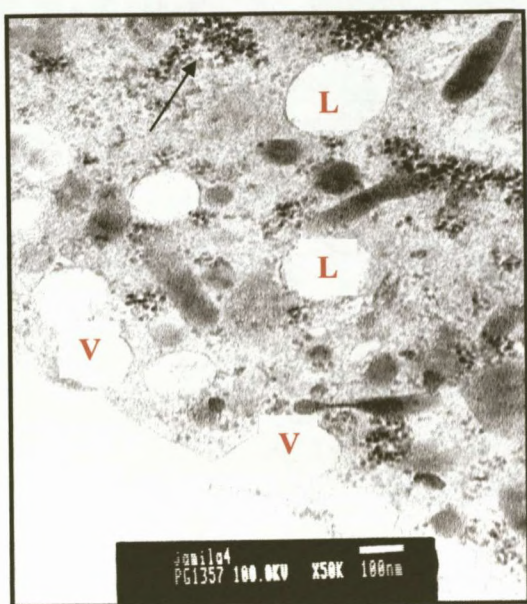
(b) X 35 000



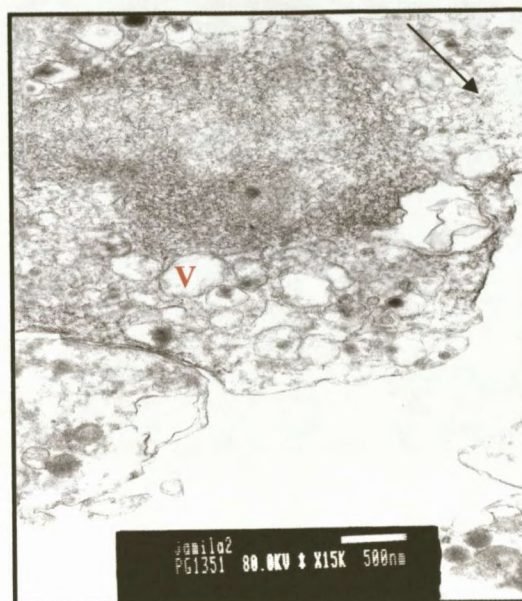
(c) X 55 000

Fig.3.11 Electron micrographs of neutrophils unexposed to toxins.

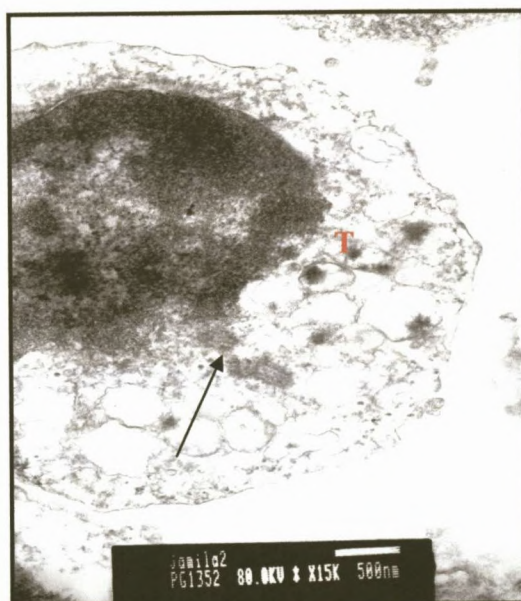
(a) lobed nucleus (N), (b) primary granules (P), secondary granules (S) and tertiary granules (T), and (c) intact cell membrane (arrow).



(a) X 70 000



(b) X 20 000



(c) X 20 000

Fig. 3.12 Electron micrographs of neutrophils exposed to 25 $\mu\text{g/ml}$ FB_1 for 2 h.
 (a) loss of cellular integrity (arrow), lipid granules (L) and vacuoles (V),
 (b) loss of cellular organization (arrow) and vacuolization (V), and
 (c) nucleoplasm leakage (arrow) and phagosome encapsulation of toxin (T).

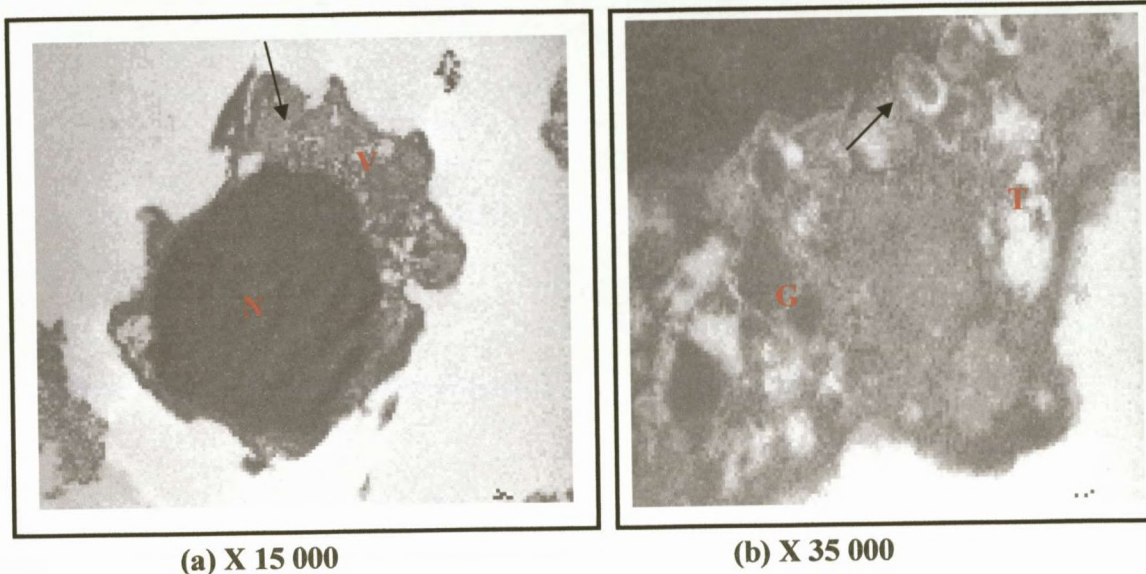


Fig. 3.13 Electron micrographs showing neutrophils exposed to 50 $\mu\text{g/ml}$ OTA for 23 h. **(a)** dense nucleus (N), loss of cellular organization and cytoplasmic organelles (arrow), intracytoplasmic vacuoles (V) and invaginations in plasma membrane, and **(b)** nucleoplasm leakage (arrow), phagosome encapsulation of toxin (T) and intracytoplasmic inclusions / granules (G).

Lymphocytes exposed to FB₁ and OTA

Lymphocytes unexposed (control) appeared healthy, indicated by a mononucleated, intact cell with distinct cellular structures (Figure 3.14a), a regular cell membrane and a large number of enzymatic vesicles within the cytoplasm (Figure 3.14b). On exposure to 25 µg/ml FB₁ for 2 h the lymphocytes had shrunk slightly and the cell membrane had started to degrade. The nuclear region appeared more condensed and vacuoles had started to form in the cytoplasm. Although the mitochondrial cristae appeared intact, the mitochondria were swollen (Figure 3.15a). An increased number of lipid granules and autophagic vesicles with enzymatic breakage of cellular structure deposits were seen within the cytoplasm (Figure 3.15b).

Lymphocytes exposed to 50 µg/ml OTA for 23 h showed signs of apoptotic cell death indicated by fragmentation of the nucleus (Figure 3.16a), a loss of cellular organization, an increased number of vacuoles and degradation of the cell membrane (Figure 3.16b).

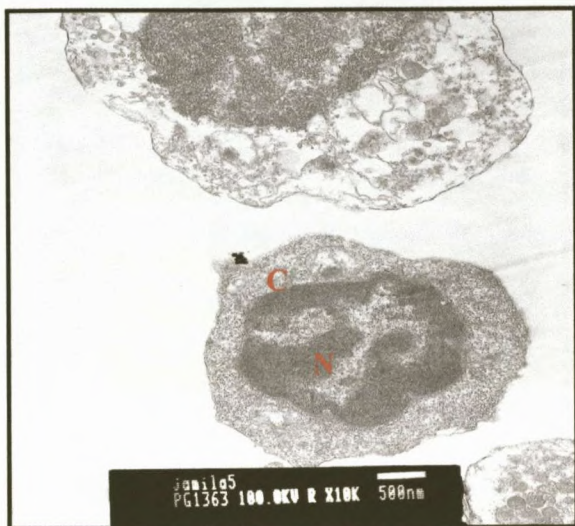


Fig. 3.14 (a) X 14 000 (b) X 30 000
Electron micrographs of control lymphocytes.

(a) mononucleated intact cell with rounded nucleus (N) and thin rim of cytoplasm (C), and (b) intact cell membrane (arrow) in the presence of enzymatic vesicles within the cytoplasm (Ve).

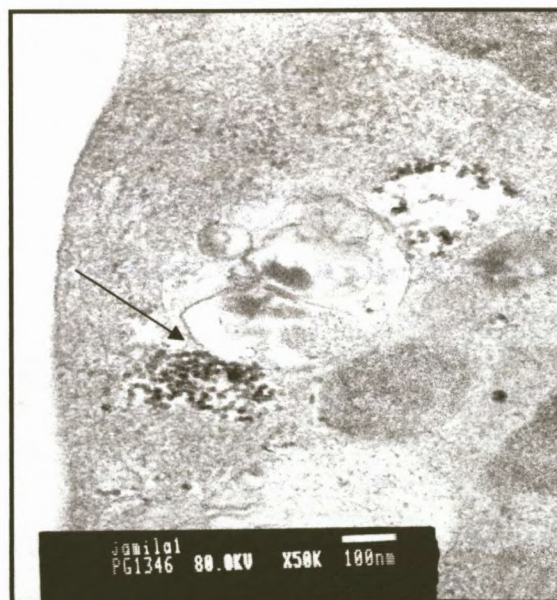
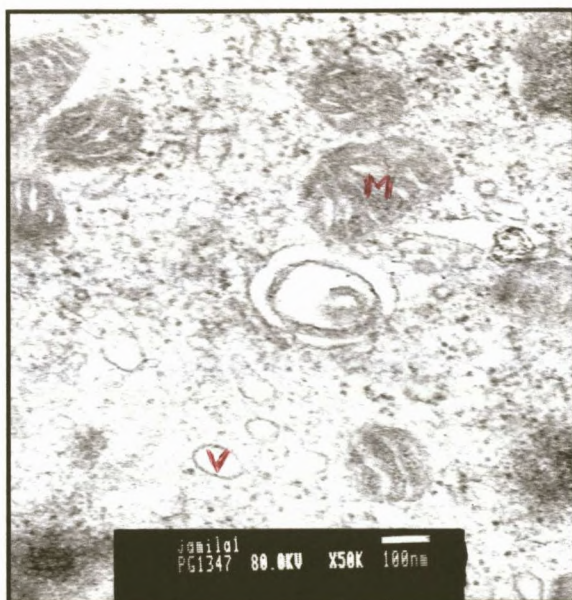
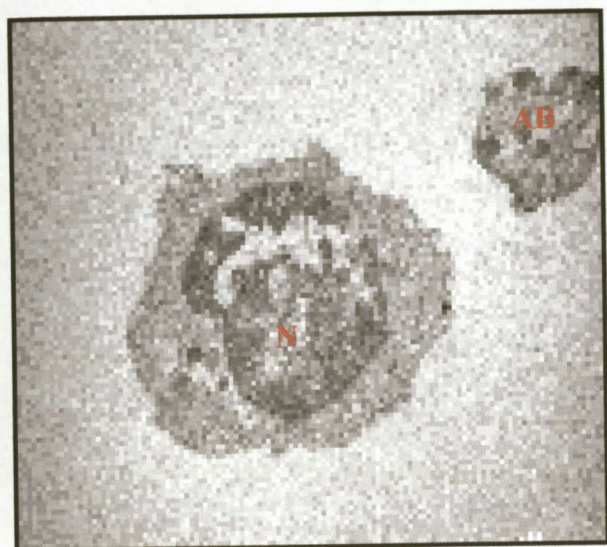
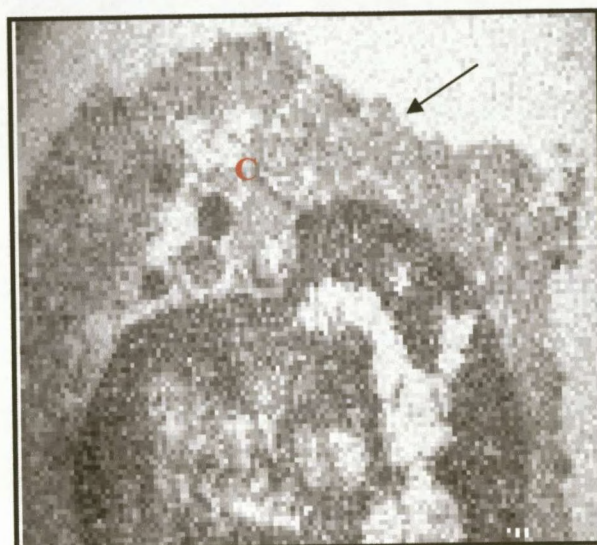


Fig. 3.15 (a) X 60 000 (b) X 70 000
Electron micrographs of lymphocytes exposed to 25 µg/ml FB₁ for 2 h.

(a) swollen mitochondria (M) and vacuolation (V), and (b) presence of autophagic vesicles (Ve) with enzymatic breakage of cellular deposits (arrow).



(a) X 14 000



(b) X 35 000

Fig. 3.16 Electron micrographs of lymphocytes exposed to 50 $\mu\text{g/ml}$ OTA at 23 h.

(a) fragmentation of the nucleus (N) and presence of apoptotic body (AB),
 and **(b)** loss of cytoplasmic structures (C) and degradation of cell membrane
 (arrow).

3.2 EFFECT OF FB₁ AND OTA ON LYMPHOCYTES AND NEUTROPHILS FROM PATIENTS WITH OESOPHAGEAL AND BREAST CANCER

Lymphocytes and neutrophils isolated from 30 healthy volunteers, 30 oesophageal cancer patients and 30 breast cancer patients were separately exposed to 25 µg/ml FB₁ for 2 h and 50 µg/ml OTA for 23 h.

3.2.1 Viability

Healthy volunteers control

In the healthy population, lymphocytes unexposed to FB₁ showed an average of 99% viability at 0 h and 94% viability after 2 h, whereas lymphocytes exposed to 25 µg/ml FB₁ showed an average of 91% viability at 0 h and 65% viability after 2 h (Table 3.3; Appendix E). Neutrophils unexposed to FB₁ showed an average of 98% viability at 0 h and 90% viability after 2 h, whereas neutrophils exposed to 25 µg/ml FB₁ showed an average of 89% viability at 0 h and 65% viability after 2 h exposure (Table 3.3; Appendix E). Lymphocytes unexposed to OTA showed an average of 99% viability at 0-time and 82% viability after 23 h, whereas lymphocytes exposed to 50 µg/ml OTA showed an average of 91% viability at 0 h and 66% viability after 23 h exposure (Table 3.4; Appendix E). Neutrophils unexposed to OTA showed an average of 98% viability at 0 h and 86% viability after 23 h, whereas neutrophils exposed to 50 µg/ml OTA showed an average of 92% viability at 0 h and 75% viability after 23 h exposure (Table 3.4; Appendix E).

The toxin-exposed immunocytes from healthy volunteers showed an average reduction of 18.2% and 22.7% in viability of lymphocytes and neutrophils respectively after exposure to 25 µg/ml FB₁ (Table 3.3; Appendix E) and an average reduction of 11.1% and 15.1% in viability of lymphocytes and neutrophils respectively after exposure to 50 µg/ml OTA (Table 3.4; Appendix E) on healthy individuals.

Oesophageal cancer patients

In the oesophageal cancer patients, lymphocytes unexposed to FB₁ showed an average of 99% viability at 0 h and 98.5% viability after 2 h, whereas lymphocytes exposed to 25 µg/ml FB₁ showed an average of 98% viability at 0 h and 79% viability after 2h exposure (Table 3.5; Appendix E). Neutrophils unexposed to FB₁ showed an average of 99% viability at 0-time and 95% viability after 2 h, whereas neutrophils exposed to 25 µg/ml FB₁ showed an average of 95% viability at 0 h and 74% viability after 2 h exposure (Table 3.5; Appendix E). Lymphocytes unexposed to OTA showed an average of 99% viability at 0 h and 90% viability after 23 h, whereas lymphocytes exposed to 50 µg/ml OTA showed an average of 99% viability at 0 h and 71% viability after 23 h exposure (Table 3.6; Appendix E). Neutrophils unexposed to OTA showed an average of 99% viability at 0 h and 95% viability after 23 h, whereas neutrophils exposed to 50 µg/ml OTA showed an average of 97% viability at 0 h and 69% viability after 23 h exposure (Table 3.6; Appendix E).

The toxin-exposed immunocytes showed an average reduction of 19.1% and 17.8% in viability of lymphocytes and neutrophils respectively after exposure to 25 µg/ml FB₁ (Table

3.5 Appendix E) and an average reduction of 22.2% and 18.2% in viability of lymphocytes and neutrophils respectively after exposure to 50 µg/ml OTA (Table 3.6 Appendix E) on oesophageal cancer patients.

Breast cancer patients

In the breast cancer patients, lymphocytes unexposed to FB₁ showed an average of 98% viability at 0 h and 89% viability after 2 h, whereas lymphocytes exposed to FB₁ showed an average of 92% viability at 0 h and 70% viability after 2 h exposure (Table 3.7; Appendix E). Neutrophils unexposed to FB₁ showed an average of 98% viability at 0 h and 90% viability after 2 h, whereas neutrophils exposed to 25 µg/ml FB₁ showed an average of 95% viability at 0 h and 67% viability after 2 h exposure (Table 3.7; Appendix E). Lymphocytes unexposed to OTA showed an average of 99% viability at 0 h and 91% viability after 23 h, whereas lymphocytes exposed to 50 µg/ml OTA showed an average of 97% viability at 0 h and 73% viability after 23 h exposure (Table 3.8; Appendix E). Neutrophils unexposed to OTA showed an average of 99% viability at 0 h and 90% viability after 23 h, whereas neutrophils exposed to 50 µg/ml OTA showed an average of 93% viability at 0 h and 71% viability after 23 h exposure (Table 3.8; Appendix E).

The toxin-exposed immunocytes showed an average reduction of 14.4% and 20.7% in viability of lymphocytes and neutrophils respectively after exposure to 25 µg/ml FB₁ (Table 3.7; Appendix E) and an average reduction of 16.1% and 14.3% in viability of lymphocytes and neutrophils respectively after exposure to 50 µg/ml OTA (Table 3.8; Appendix E) on breast cancer patients.

Comparison of FB₁ and OTA responses

A comparison of the effects of FB₁ and OTA on neutrophils and lymphocytes isolated from the three groups are summarized in Figures 3.17 and 3.18. The highest percentage reduction in viability of neutrophils, was with breast cancer patient-derived neutrophils exposed to 25 µg/ml FB₁ for 2 h (Figure 3.17) and in lymphocytes, was with those derived from oesophageal cancer patients exposed to 50 µg/ml OTA (Figure 3.18).

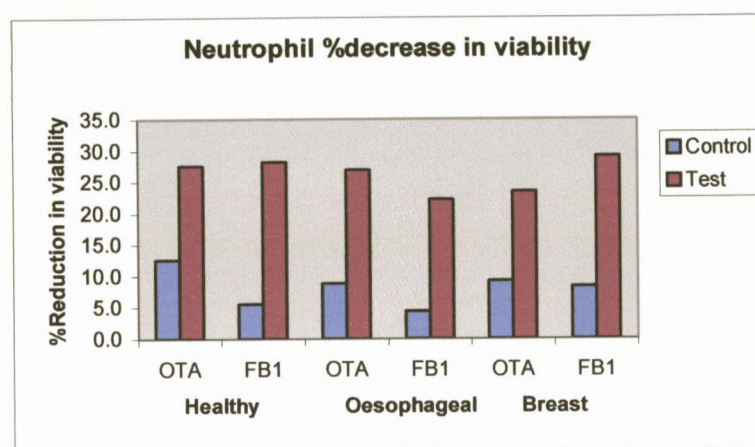


Fig. 3.17 Percentage viability of neutrophils exposed to 25 µg/ml FB₁ for 2 h or 50 µg/ml OTA for 23 h

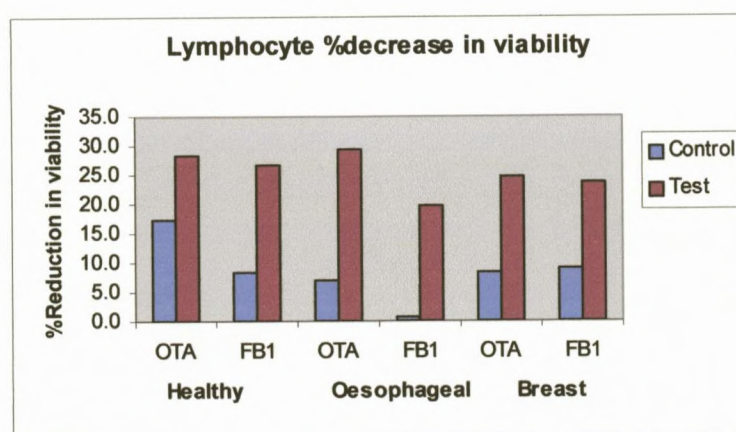


Fig. 3.18 Percentage viability of lymphocytes exposed to 25 µg/ml FB₁ for 2 h or 50 µg/ml OTA for 23 h

3.2.2 Toxin effect on morphology

3.2.2.1 Oesophageal cancer patients

Unexposed lymphocytes

Unexposed oesophageal cancer patient-derived lymphocytes appeared rounded with a large, sometimes slightly invaginated, densely stained nucleus surrounded by a thin peripheral layer of cytoplasm (Figure 3.19a). There was a paucity of organelles within the cytoplasm. A normal dispersion of chromatin was noted (Figure 3.19c). Single nucleoli could be seen in some cells. The heterochromatin appeared crenated and condensed on the periphery of the nucleolemma (Figure 3.19b). The mitochondria were of ovoid shape and contained visible cristae. Golgi apparatus, endoplasmic reticulum (ER) and free ribosomes were occasionally noticed. The plasma membrane exhibited finger-like projections (Figure 3.19c). Unexposed lymphocytes observed at 0 h, 2 h and 23 h retained normal morphology and resembled healthy lymphocytes.

Lymphocytes exposed to FB₁

At a low magnification, cellular debris and platelet cells (Figure 3.20a) were observed. Only a few viable lymphocytes were observed. Nucleoli were not seen. Dense heterochromatin was observed (Figure 3.20b). Higher magnification (X 55 000) revealed some intact and some disrupted mitochondrial cristae. Swollen ER was frequently noticed. Vacuolation was prominent (Figure 3.20c). The plasma membranes exhibited fewer projections. The nucleolemma appeared swollen (Figure 3.20d).

Lymphocytes exposed to OTA

Cells in various stages of degeneration were noticed. Cellular population contained platelet-like cells (apoptotic bodies) and necrotic cells (Figure 3.21a). Vacuolation (Figure 3.21c) and lipid inclusions were noted (Figure 3.21a). The plasma membrane was deeply invaginated (Figure 3.21b). Pycnotic nuclei exhibiting crescentic apolar aggregation of chromatin were observed (Figure 3.21b), characteristic of apoptosis.

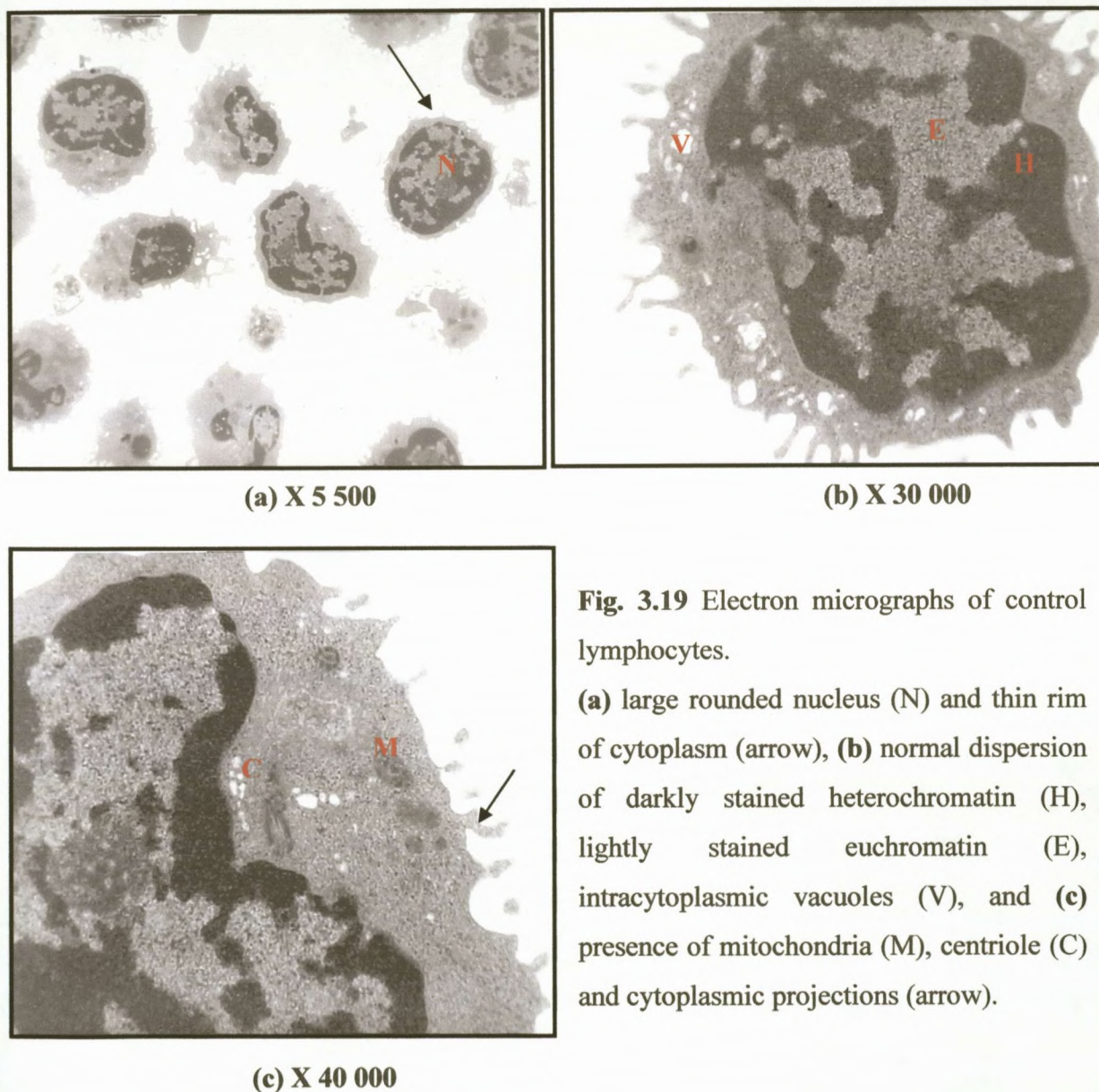
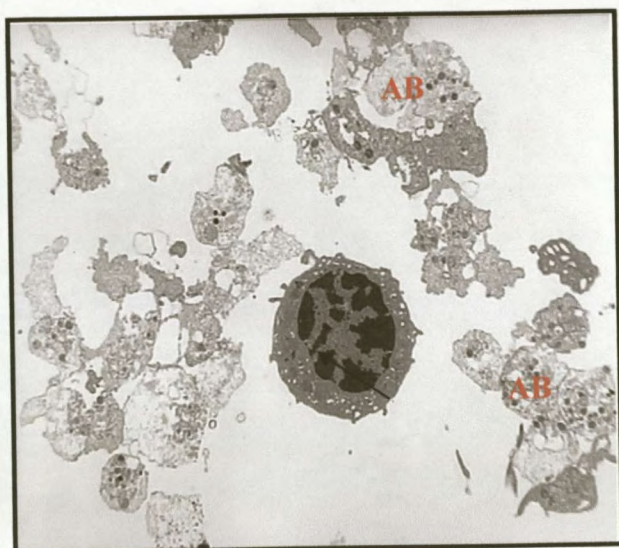
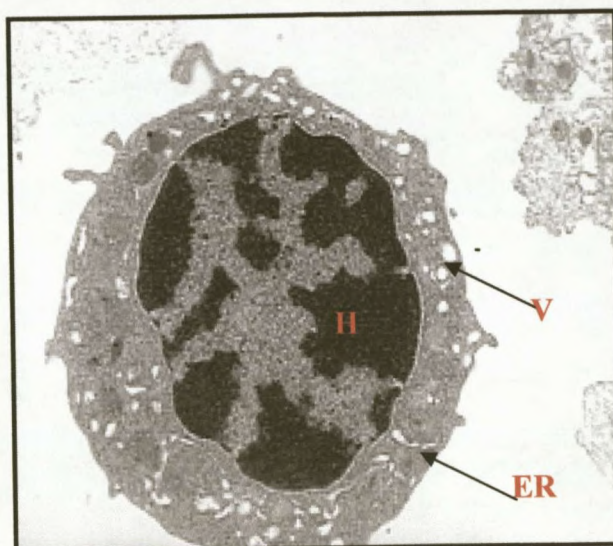


Fig. 3.19 Electron micrographs of control lymphocytes.

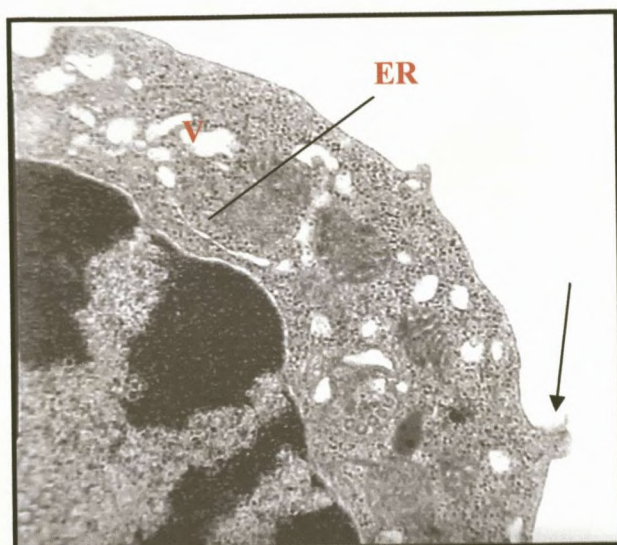
(a) large rounded nucleus (N) and thin rim of cytoplasm (arrow), (b) normal dispersion of darkly stained heterochromatin (H), lightly stained euchromatin (E), intracytoplasmic vacuoles (V), and (c) presence of mitochondria (M), centriole (C) and cytoplasmic projections (arrow).



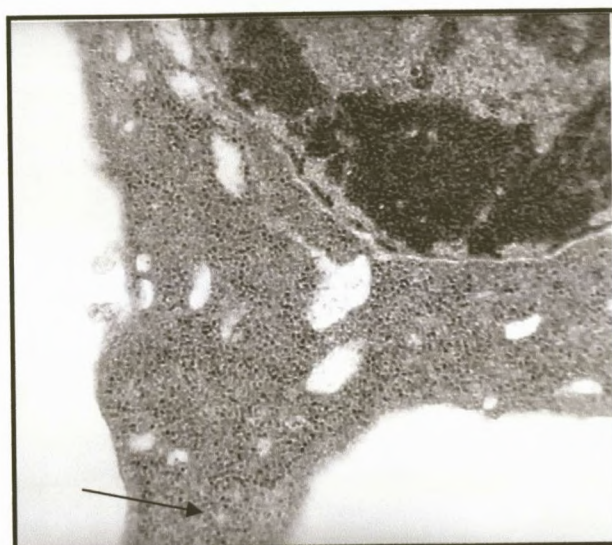
(a) X 6 000



(b) X 15 000



(c) X 55 000



(d) X 65 000

Fig. 3.20 Electron micrographs of lymphocytes exposed to 25 $\mu\text{g/ml}$ FB_1 at 2 h.

(a) a single lymphocyte amongst cellular debris and apoptotic bodies (AB), (b) a lymphocyte with dense heterochromatin (H), intracytoplasmic vacuolation (V) and endoplasmic reticulum oedema (ER), (c) swollen endoplasmic reticulum (ER), cytoplasmic vacuolation (V) and fewer plasma membrane projections (arrow), and (d) folded plasma membrane projection (arrow).

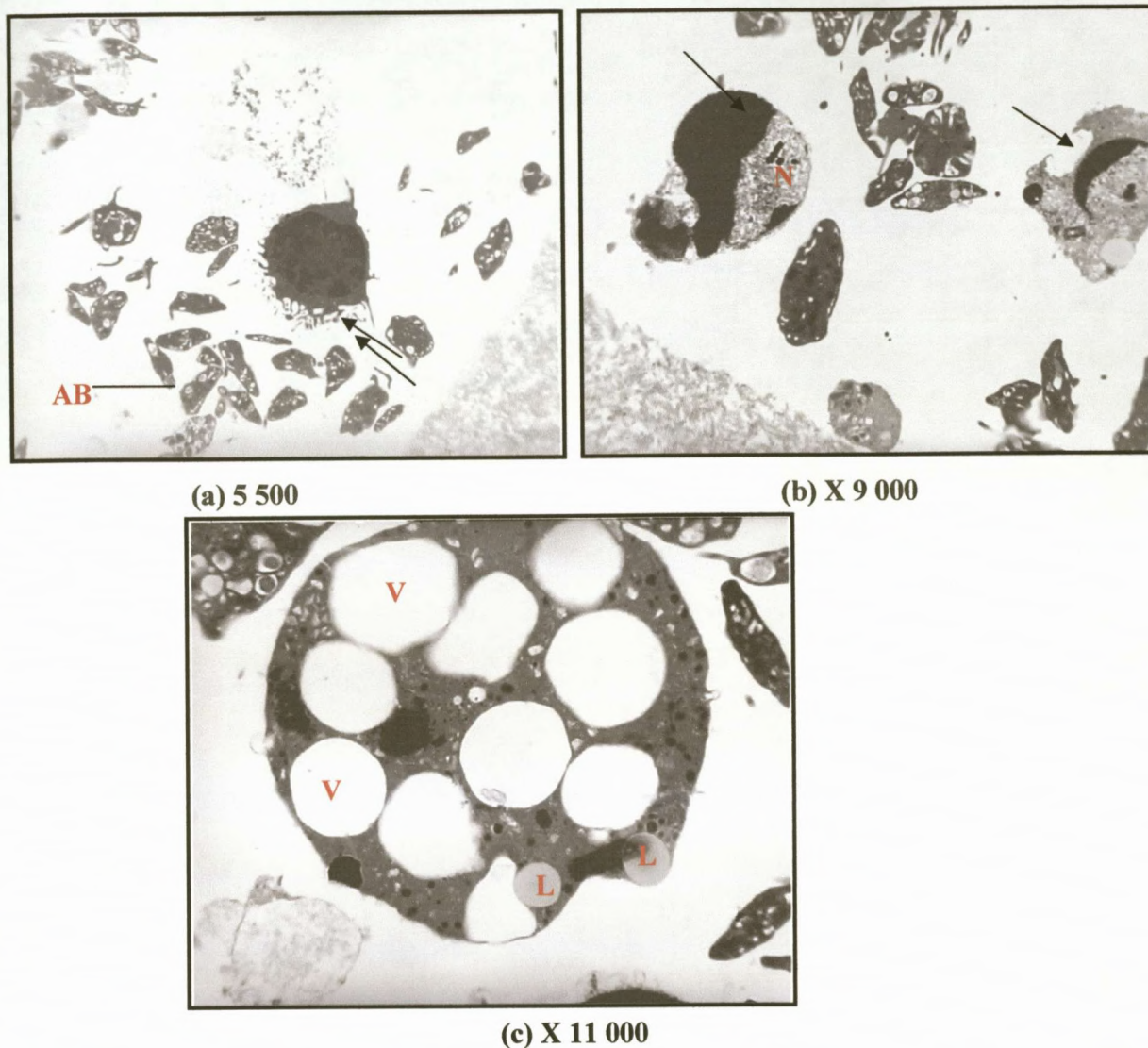


Fig. 3.21 Electron micrographs of lymphocytes exposed to 50 µg/ml OTA for 23 h. (a) cells in various stages of degeneration and plasma lemma exhibiting microvilli-like projections (arrows), (b) Pycnotic nucleus (N) exhibiting crescentic apolar aggregation of chromatin (arrows), and (c) intracytoplasmic vacuolation (V) and some lipid granules (L).

Unexposed neutrophils

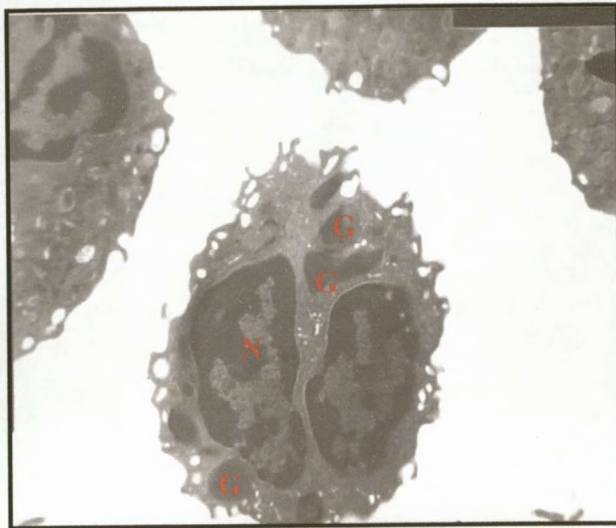
Neutrophils appeared normal with bilobed nuclei. (Figure 3.22a). The plasmalemma appeared to have many processes. Vesicles and vacuoles were observed beneath the plasmalemma (Figure 3.22b). Intracytoplasmic granules, mitochondria and ribosomes were observed. The heterochromatin was distributed peripherally (Figure 3.22c).

Neutrophils exposed to FB₁

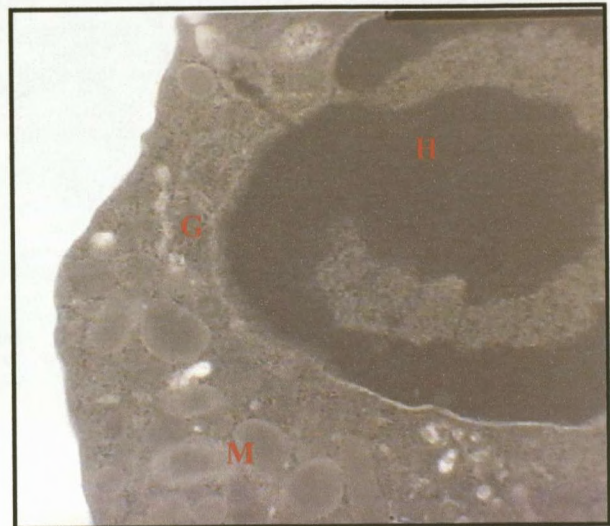
Cells in various stages of degeneration were observed (Figure 3.23a). Neutrophils contained bilobed nuclei, with dense heterochromatin surrounding small areas of euchromatin. The cytoplasmic inclusions appeared normal (Figure 3.23b), but cytoplasmic vacuolation and nucleolemmal widening was evident (Figure 3.23c).

Neutrophils exposed to OTA

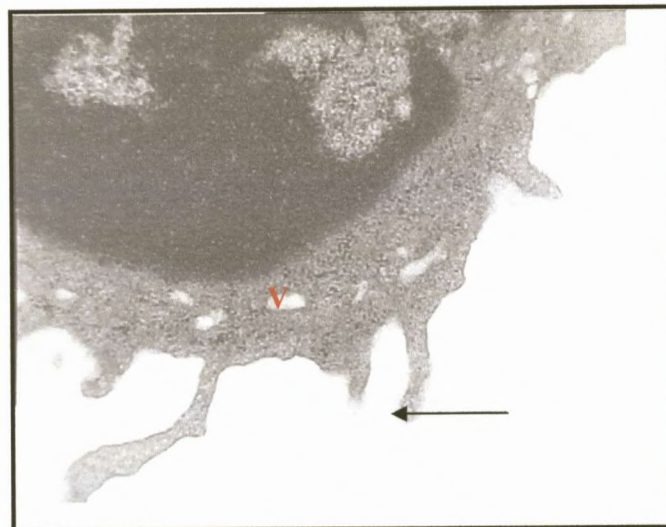
The sample consisted predominantly of degenerative cells. Cells with various degrees of degeneration were observed and are illustrated: cell containing a large nucleus with dense peripherally located heterochromatin, and many vacuoles (Figure 3.24a and b); at high magnification (Figure 3.24c) inclusion bodies were observed.



(a) X 15 000



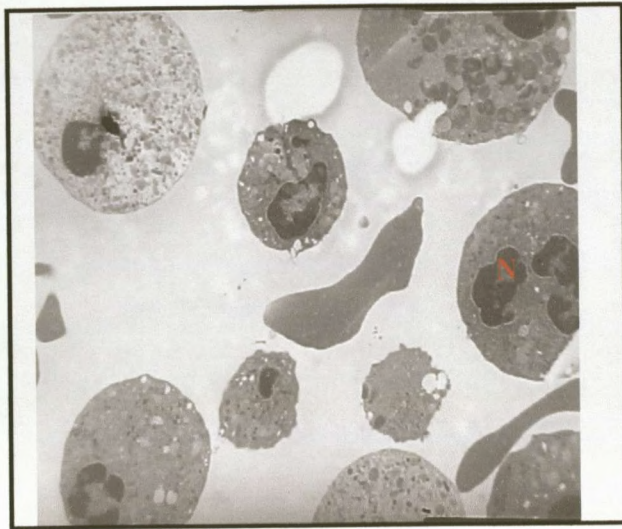
(b) X 35 000



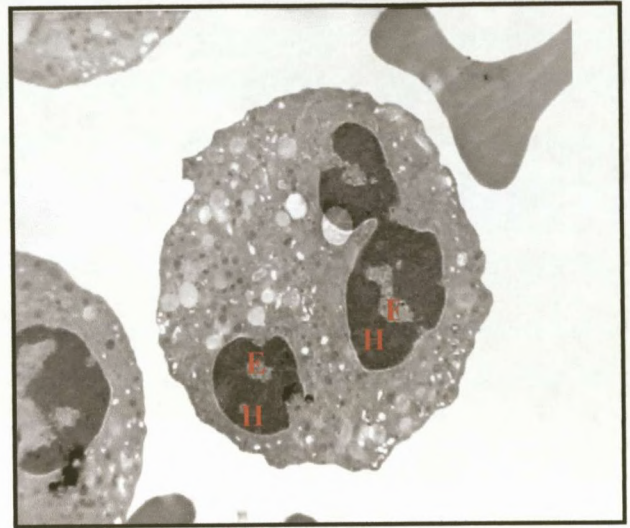
(c) X 45 000

Fig.3.22 Electron micrographs of unexposed neutrophils.

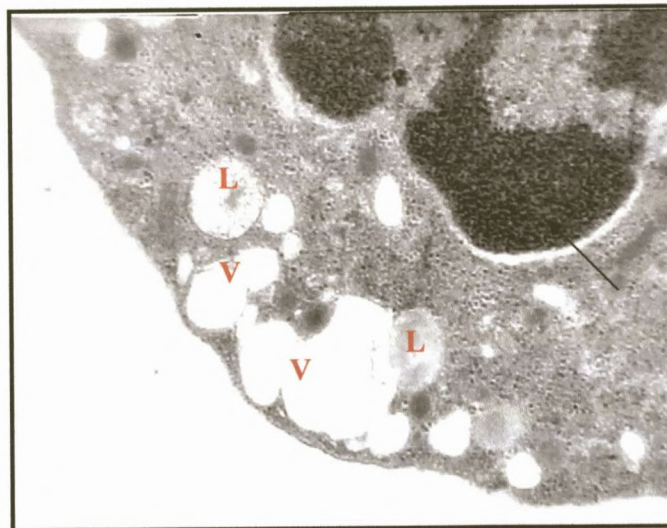
- (a) large bi-nucleated nuclear region (N) and intracytoplasmic granules (G),
- (b) peripheral location of heterochromatin (H) and mitochondria (M), and
- (c) presence of intracytoplasmic vacuoles (V) and cytoplasmic projections (arrow).



(a) X 5 000



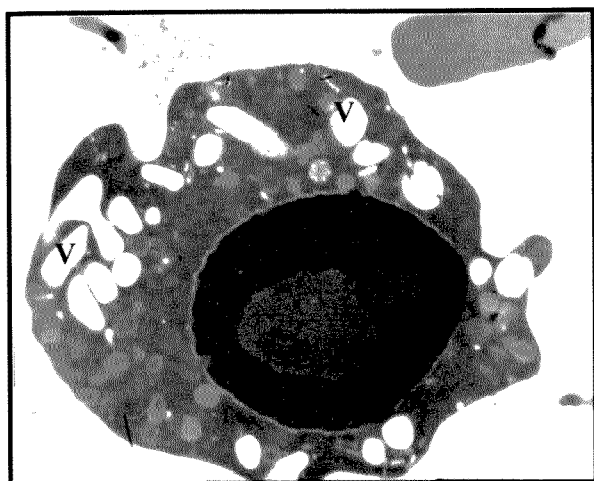
(b) X 11 000



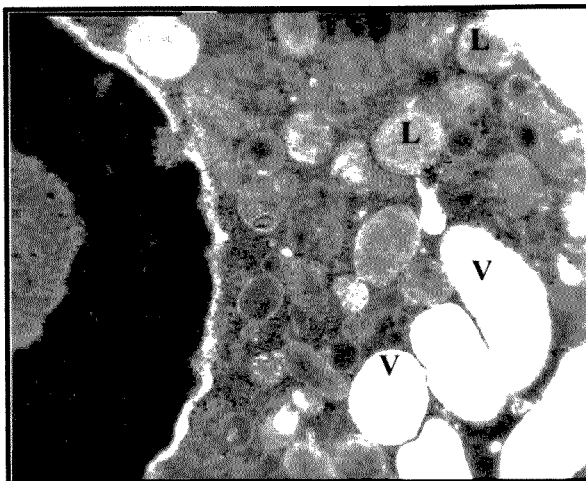
(c) 45 000

Fig. 3.23 Electron micrographs of neutrophils exposed to 25 $\mu\text{g/ml}$ FB_1 for 2 h.

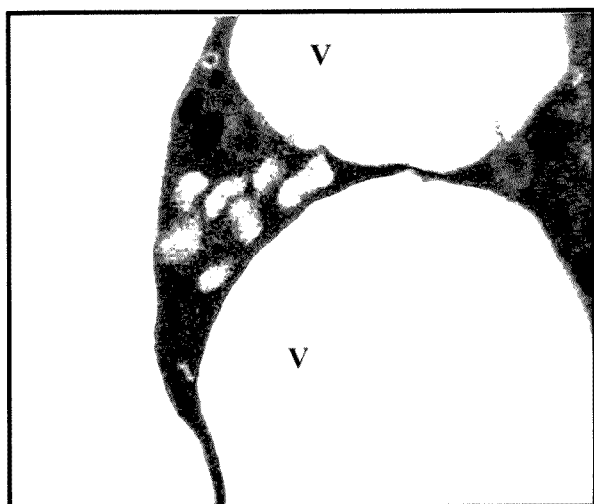
- (a) cells in various stages of degeneration, neutrophil with bilobed nucleus (N),
- (b) dense heterochromatin (H), surrounding small areas of euchromatin (E), and
- (c) intracytoplasmic vacuolation (V), some lipid granules (L) and widening of nucleolemma (arrow).



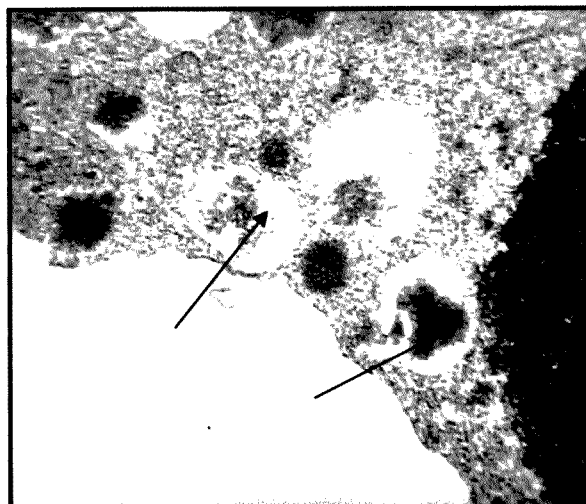
(a) X 18 000



(b) X 55 000



(c) X 45 000



(d) X 75 000

Fig. 3.24 Electron micrographs of neutrophils exposed to 50 $\mu\text{g/ml}$ OTA for 23 h.

(a) dense and peripheral distribution of heterochromatin (H) and vacuolation (V),
 (b) vacuolation (V) , lipid bodies (L), intracytoplasmic granules (G) and
 nucleolemma widening (arrow), (c) intracytoplasmic vacuoles (V),
 intracytoplasmic granules (G), and (d) inclusions within vacuoles (arrows).

3.2.2.2 Breast Cancer Patients

Unexposed lymphocytes

The unexposed lymphocytes appeared as expected with a large densely stained nucleus surrounded by a thin rim of cytoplasm. In some cells the nucleus was slightly invaginated (Figure 3.25a). Very few organelles were visible in the cytoplasm. The mitochondria were ovoid in shape and contained visible cristae (Figure 3.25b). The plasma membrane exhibited finger-like projections.

Lymphocytes exposed to FB₁

At low magnification numerous cellular debris were observed together with platelet-like cells, a feature of apoptosis (Figure 3.26a). Very few viable lymphocytes were observed. Plasma membrane of the exposed lymphocytes contained fewer projections compared to that of the unexposed lymphocyte. Intracytoplasmic vacuolation as well as disintegration of plasma membrane were observed. Higher magnification revealed loss of cytoplasm integrity and organization (Figure 3.26b). Cytoplasm organelles and nucleoli were not visible. Disintegration of chromatin and cytoplasm, leakage of neoplasm into the cytoplasm and damage to mitochondria were observed. Hyperchromatin could not be differentiated from the euchromatin.

Lymphocytes exposed to OTA

Exposure to 50 $\mu\text{g/ml}$ OTA for 23 h showed that the lymphocytes had undergone further cell injury. As a result, few lymphocytes were observed which at low magnification showed various stages of degeneration. The cell population comprised mainly necrotic cells and plate-like cells. Fragmentation of chromatin material and cytoplasm was noted (Figure 3.27a). Cytoplasmic vacuolation was dominant (Figure 3.27b). The plasma membrane had ruptured and no projections were visible in the plasma membrane. The nuclear pore seemed to have increased in size resulting in leakage of nucleoplasm.

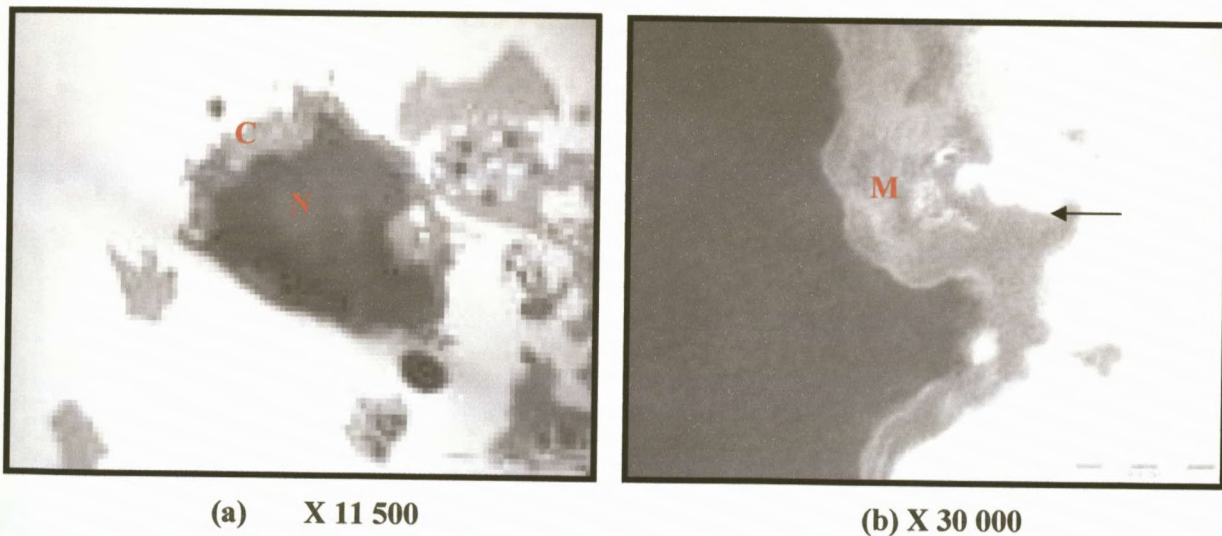
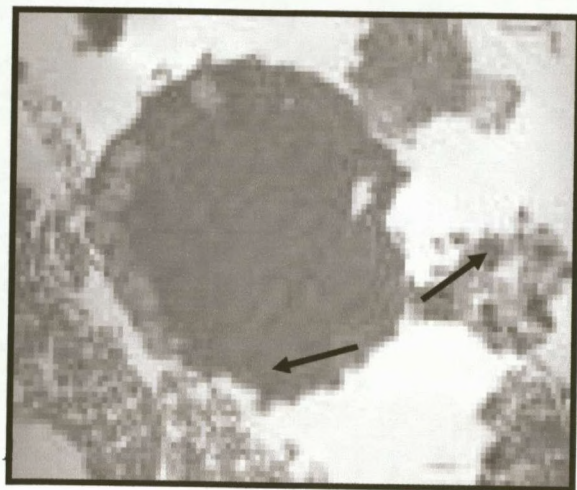
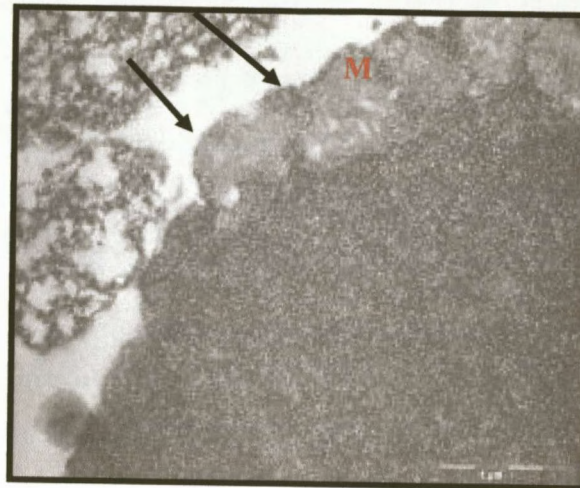


Fig. 3.25 Electron micrographs of control lymphocytes.

(a) slightly invaginated and dense nucleus (N) with a thin rim of cytoplasm (C), and (b) ovoid shaped mitochondria (M) and cytoplasmic projection (arrow).



(a) X 11 500



(b) X 30 000

Fig. 3.26 Electron micrographs of lymphocytes exposed to 25 $\mu\text{g/ml}$ FB_1 for 2 h
(a) debris and plate-like cells in the background (arrows), and **(b)** disintegration of the plasma membrane (arrow), nucleoplasm leakage (arrow) and damaged mitochondria (M).

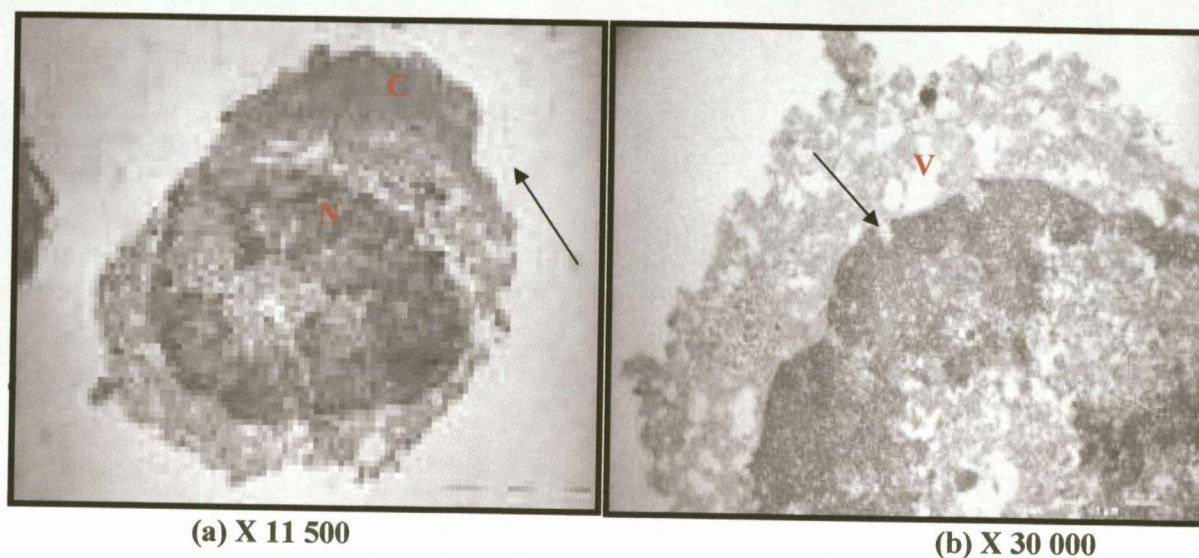


Fig. 3.27 Electron micrographs of lymphocytes exposed to 50 µg/ml OTA.
(a) fragmentation of nuclear (N) and cytoplasmic (C) structures and rupture of plasma membrane (arrow), and **(b)** cytoplasmic vacuolization (V) and increase in size of nuclear pore (arrow).

Unexposed neutrophils

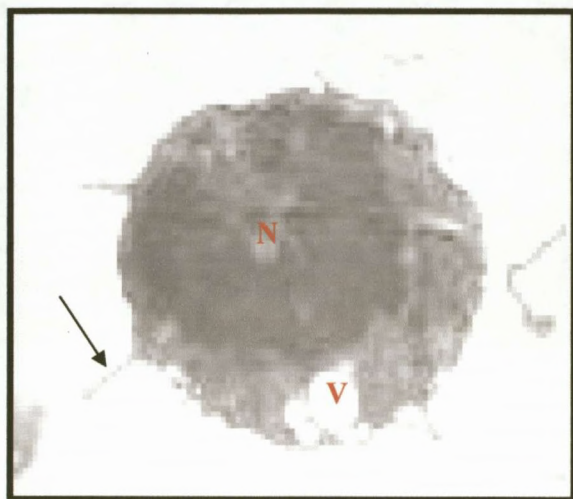
At low magnification the neutrophils appeared normal with lobed nuclei. The plasmalemma had many processes (Figure 3.28a). The nucleus contained dense peripherally located heterochromatin and no nucleolus was visible. The cytoplasm contained dense granules and vesicles. At high magnification intracytoplasmic granules, mitochondria and ribosomal content of the cytoplasm were observed. Intracytoplasmic vesicles were observed beneath the plasmalemma (Figure 3.28b).

Neutrophils exposed to FB₁

At low magnification the cellular population consisted of debris, degenerative cells and neutrophils. The nucleus contained dense chromatin material. Nucleolemma invagination was evident with nucleoplasm leakage (Figure 3.29b). The cytoplasm contained intracytoplasmic granules and vacuoles. Fewer finger-like projections were visible on the plasmalemma (Figure 3.29a). However, they appeared to be thickened with the presence of vesicles at the base (Figure 3.29b). Invaginations in the plasma membrane were also evident.

Neutrophils exposed to OTA

The sample consisted of mainly degenerative cells, where various stages of degeneration were present. Loss of cellular organization was prominent (Figure 3.30a). The nucleus had completely disintegrated and nuclear material had become packaged into apoptotic bodies within the cytoplasm. The cytoplasm was highly vacuolated and the plasmalemma had disintegrated completely (Figure 3.30b).



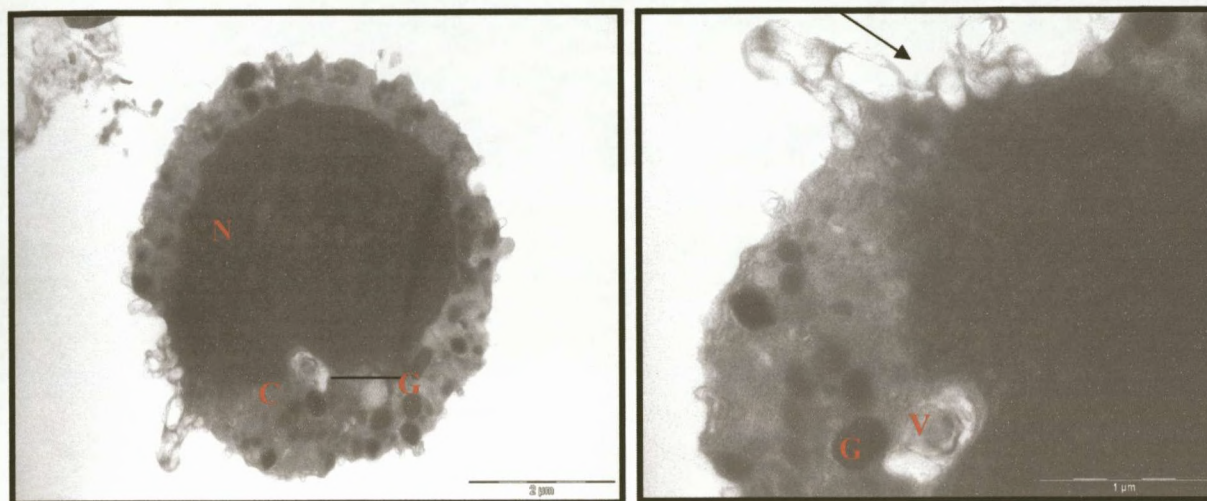
(a) X 11 500



(b) X 30 000

Fig. 3.28 Electron micrographs of unexposed neutrophils.

(a) lobed nuclei (N), intracytoplasmic vesicles (V) and finger-like projections in plasma membrane (arrow), and (b) numerous intracytoplasmic granules (G) and some intracytoplasmic vesicles (V).

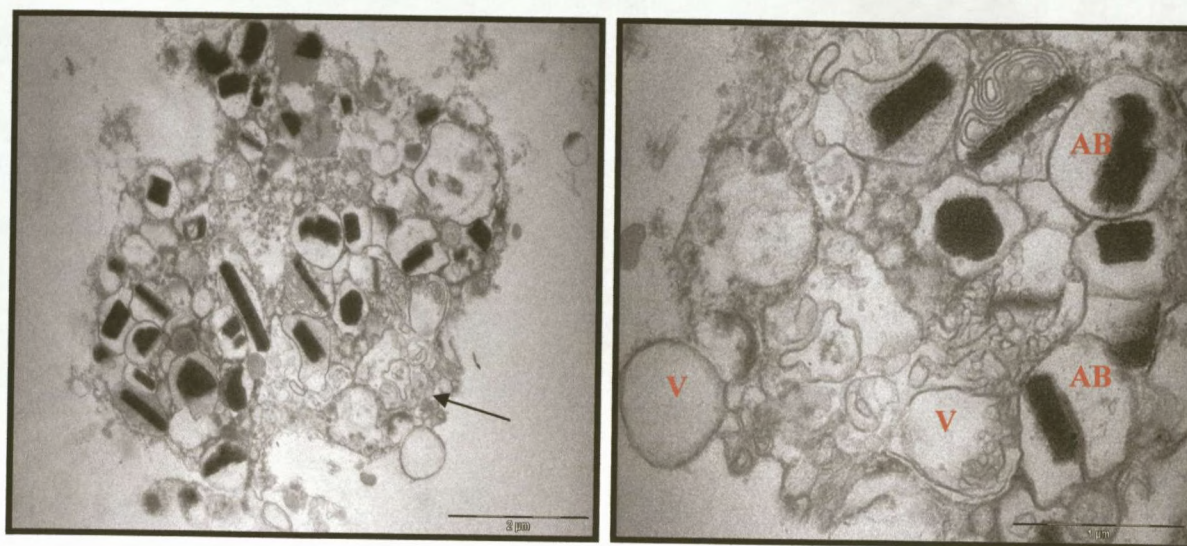


(a) X 11 500

(b) X 30 000

Fig. 3.29 Electron micrographs of neutrophils exposed to 25 $\mu\text{g/ml}$ FB_1 for 2 h.

(a) Nucleus (N) containing dense chromatin material, Cytoplasm (C) containing numerous intracytoplasmic granules (G) and disintegration of plasma membrane (arrow), and (b) intracytoplasmic vesicles (V), intracytoplasmic granules (G) and disintegration of cytoplasm (arrow).



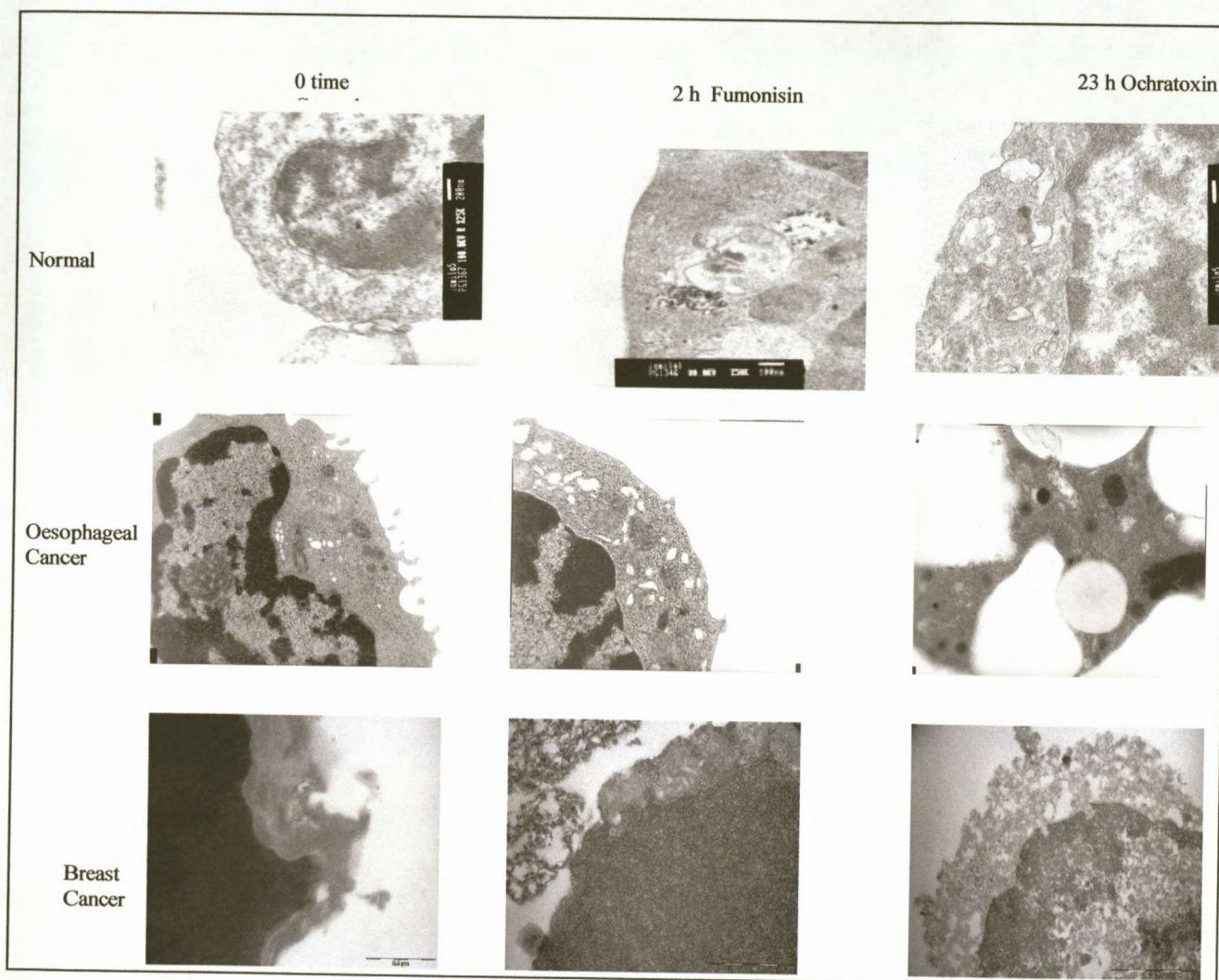
(a) X 35 000

(b) X 55 000

Fig. 3.30 Electron micrographs of neutrophils exposed to 50 µg/ml OTA.

- (a) loss of cellular structures, disintegration of plasma membrane (arrow) and
- (b) cytoplasmic vacuolation (V) and nuclear material in apoptotic bodies (AB).

Electron microscope observations of FB₁ treated lymphocytes and neutrophils from the three population groups (Figures 3.31b and 3.32b) showed morphological features consistent with apoptosis *i.e.* cytoplasmic and chromatin condensation, chromatin margination, increased vacuolation and cell shrinkage. The changes observed when lymphocytes and neutrophils were exposed to FB₁ and OTA appeared to be most severe in breast cancer patients followed by oesophageal cancer patients. Pyknosis or karyorrhexis, cytoplasmic vacuolation, chromatin margination, increased intracytoplasmic granules, relatively high numbers of nuclear pores, fragmentation of cell membrane and disintegration of cytoplasm was seen in OTA exposed cells.

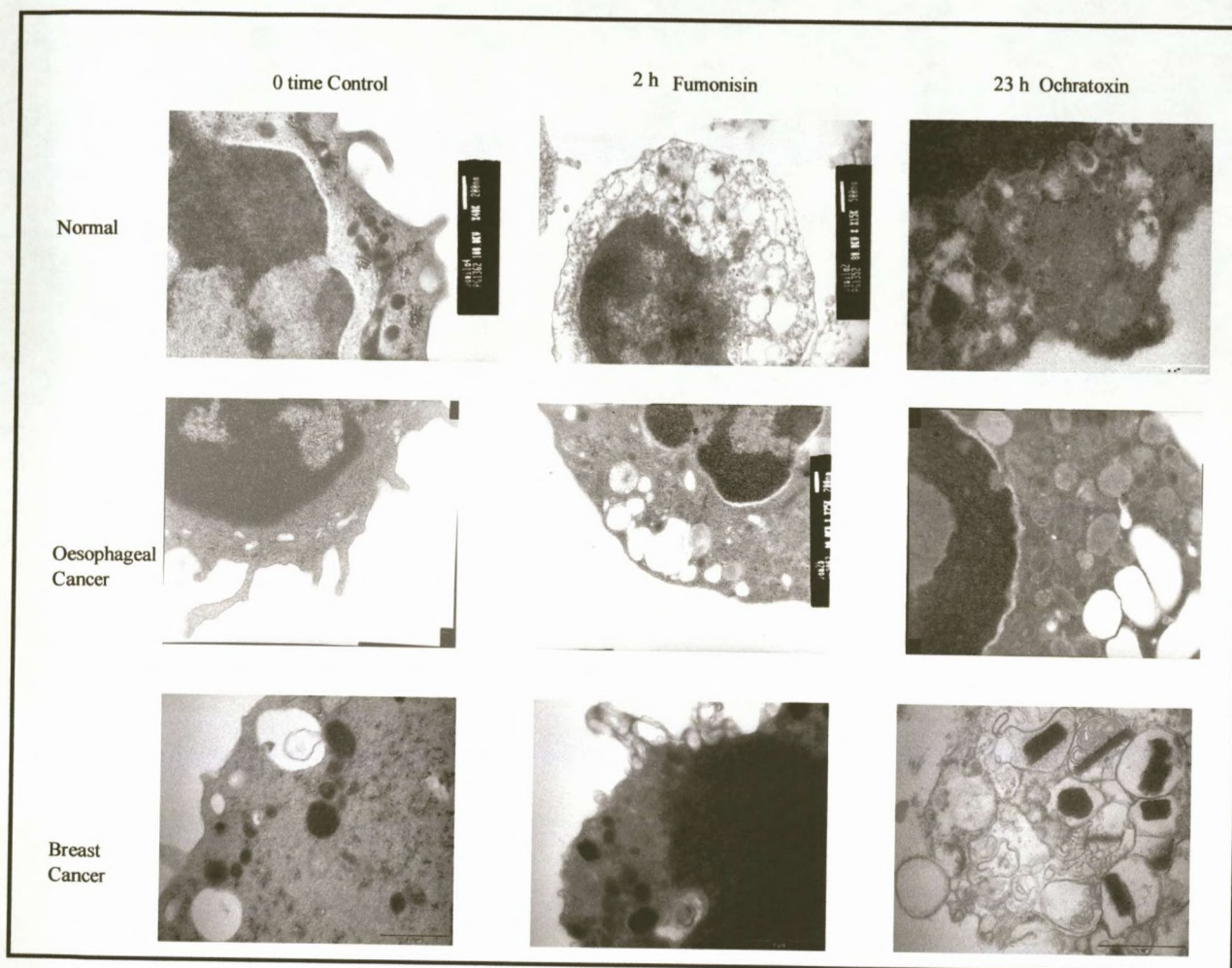


(a)

(b)

(c)

Fig. 3.31 Electron micrographs of lymphocytes at time zero and 2 h after exposure to 25 $\mu\text{g/ml}$ FB₁ or 23 h after exposure to 50 $\mu\text{g/ml}$ OTA



(a)

(b)

(c)

Fig. 3.32 Electron micrographs of neutrophils at time zero and after exposure to 25 $\mu\text{g ml}$ FB_1 or 50 $\mu\text{g/ml}$ OTA for respective time periods

3.2.3 Receptor localization

3.2.3.1 CK Receptor localization by light microscopy

Following exposure to 25 µg/ml FB₁ or 50 µg/ml OTA, isolated lymphocytes and neutrophils, from normal healthy volunteers and the two patient groups, oesophageal and breast cancer, were immunolabelled to localize the CK receptors following incubation with toxins in all three groups. There was a reduction in the labelling intensity of receptors (represented by the dark brown coating on the membrane surfaces surrounding the lymphocytes and neutrophils), and therefore a reduction in receptor numbers.

Figure 3.33 and Figure 3.34 show immunolabelled CK receptors on lymphocytes and neutrophils exposed to either 25 µg/ml FB₁ or 50 µg/ml OTA at 0 h (no toxin exposure) and after the respective incubation periods. A decrease in the labelling intensity of the receptors was observed after 2h incubation with 25 µg/ml fumonisin B₁ and 23 h incubation with 50 µg/ml OTA in contrast to the 0 h control cells.

CK Receptor

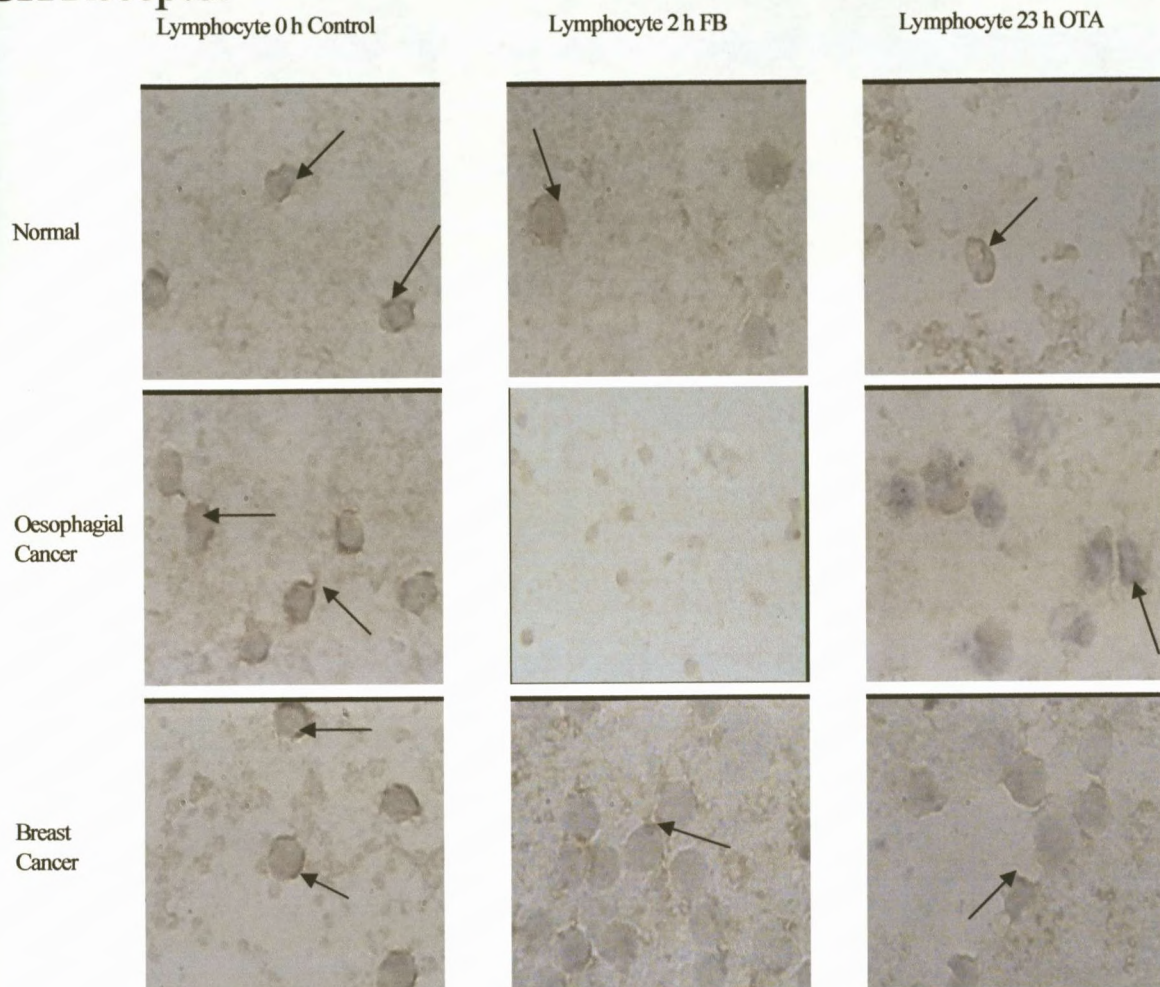


Fig. 3.33 Light microscopic (LM) images of labelled (arrows) CK receptors on surfaces of lymphocytes from healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 $\mu\text{g/ml}$ FB_1 for 2 h incubation or 50 $\mu\text{g/ml}$ OTA for 23 h incubation. The lymphocytes at 0 h were not exposed to any toxin.

CK Receptor

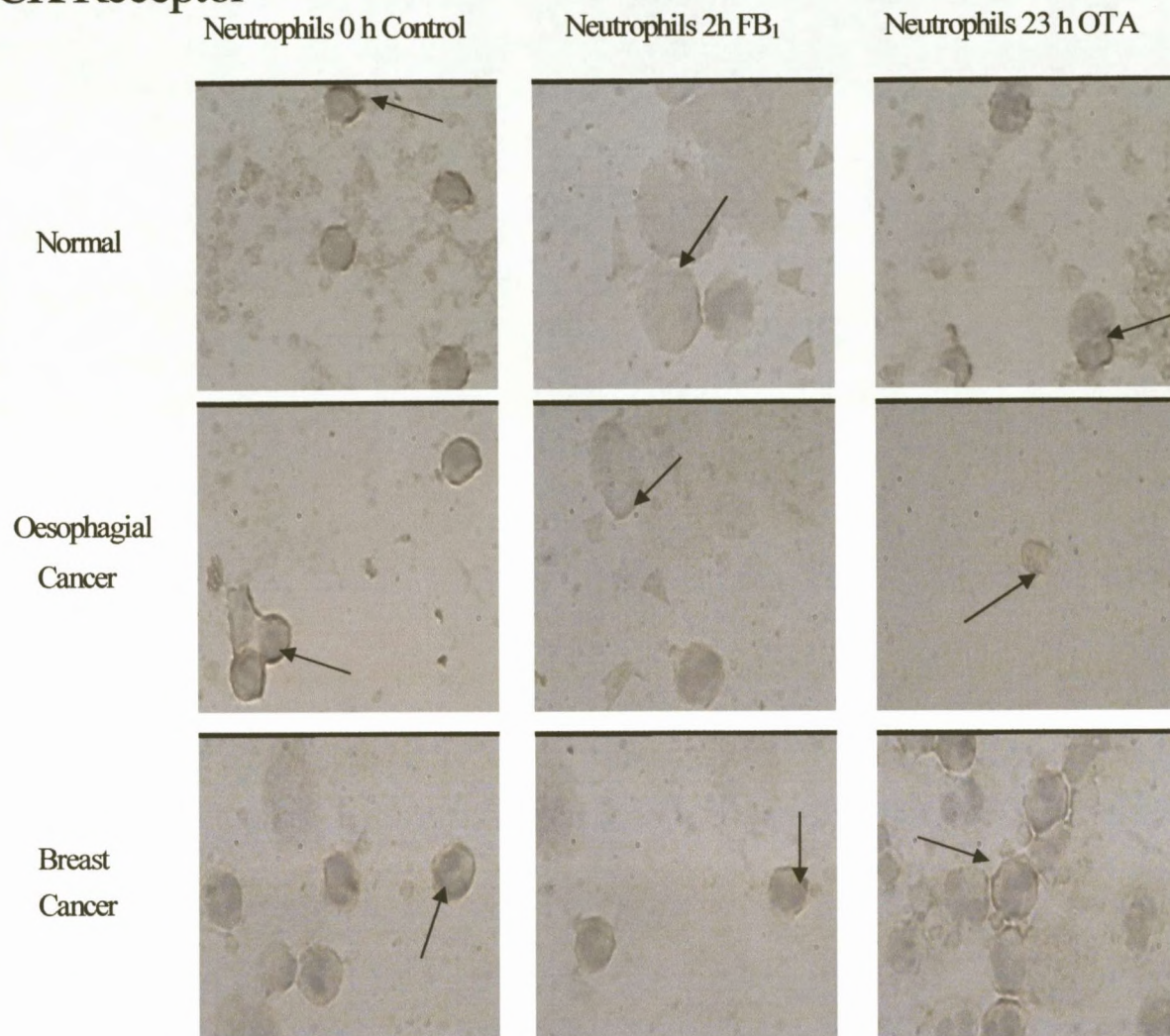


Fig. 3.34 Light microscopic images of labelled (arrows) CK receptors on surfaces of neutrophils from healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 $\mu\text{g/ml}$ FB₁ for 2 h incubation or 50 $\mu\text{g/ml}$ OTA for 23 h incubation. The neutrophils at 0 h were not exposed to any toxin.

3.2.3.2 CK, TNF and CSF Receptor localization by electron microscopy

After exposure either to 25 µg/ml FB₁ for 2 h and 50 µg/ml OTA for 23 h, isolated lymphocytes and neutrophils were immunolabelled to localize the cytokine receptors (CK, TNF and CSF) on their cell surface membranes. Our objective was to localize the antigen so as to preserve antigenicity. Tissue preservation was compromised, more in the 2 h and 23 h incubations than at time 0. Tissue preservation compromise could be observed to various degrees with regards to loss of cellular membrane structure and deterioration of cytoplasmic organelle structures. These changes could be the result of poor fixation due to the nature of the procedure, for example, the long incubation periods between blood sampling and eventual fixation. Every effort was made to maintain homeostasis of the cells by adding oxygenated PBS (95% O₂ and 5% CO₂ mixture) to the sampling tubes, even during toxin incubation.

Figures 3.35 to 3.40 were taken at 60 000 x magnification to give an indication of the localization of toxin-exposed receptors by ICC using the electron microscope. Table 3.9 summarises the findings by providing a scale ranging from 0.5 (representing "very low label") to 4 (representing "good labelling"). The immunolabelling results observed indicated a definite reduction in receptor numbers (characterized by the decrease in tiny black dots) after incubation with the toxins. Best results were observed in the CK labelling of lymphocytes obtained from the 3 population groups at time 0 (Figure 3.35). Although there was a decrease in numbers, labels were present even after toxin exposure. Good labelling was also observed at 0 time in the CK receptors of neutrophils isolated from volunteers and in the CSF receptors of lymphocytes isolated from breast cancer patients.

Overall, in the TNF and CSF receptor localization, where label was present, it was generally low (Figures 3.37 to 3.40).

Table 3.9 Immunolabelling results of TNF, CSF and CK receptor localization on neutrophils and lymphocytes

RECEPTORS	LYMPHOCYTES			NEUTROPHILS		
(a) HEALTHY VOLUNTEERS						
	CONT	FB ₁	OTA	CONT	FB ₁	OTA
CK	++++	++	++	++++	++	+
CSF	++	±	+	++	+	+
TNF	++	+	+	++	+	+
(b) OESOPHAGEAL CANCER PATIENTS						
CK	++++	++	+	+	±	±
CSF	+	±	±	+	±	±
TNF	+	+	±	+	+	±
(c) BREAST CANCER PATIENTS						
CK	++++	+++	++	++	+	+
CSF	++++	+	±	+	±	±
TNF	+	+	+	+	+	+

CONT = 0 h unexposed cells, FB₁ = 2 h fumonisin B₁ exposure, and OTA = 23 h ochratoxin A exposure

SCALE

- 0,5 ± represents very low labelling
- 1 + represents low labelling
- 2 ++ represents moderate labelling
- 3 +++ represents good labelling
- 4 ++++ represents very good labeling

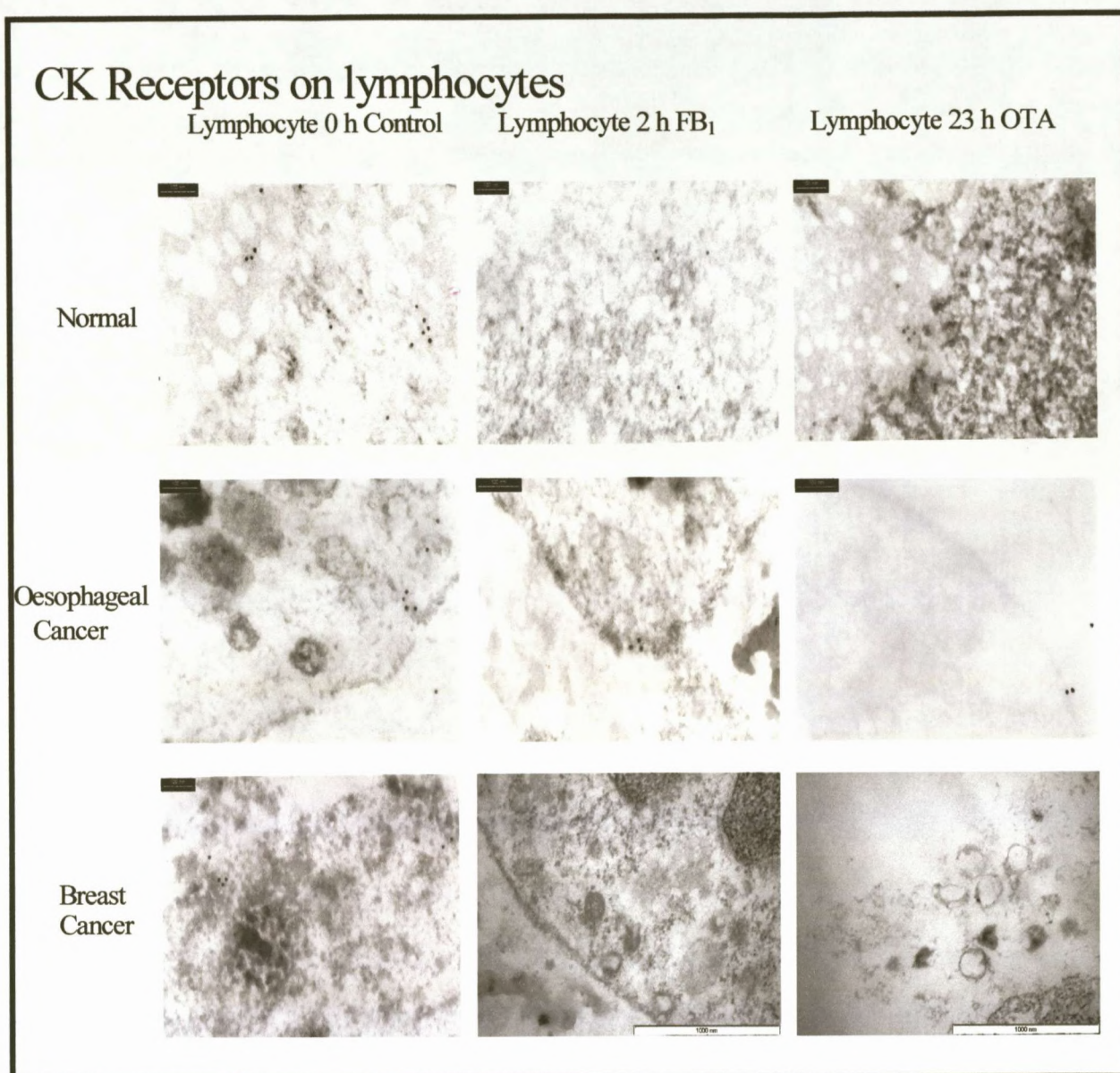


Fig. 3.35 Electron microscopic (EM) images of labelled (dots) CK receptors on surfaces of lymphocytes from healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 µg/ml FB₁ for 2 h incubation or 50 µg/ml OTA for 23 h incubation. The lymphocytes at 0 h were not exposed to any toxin.

CK Receptors on neutrophils

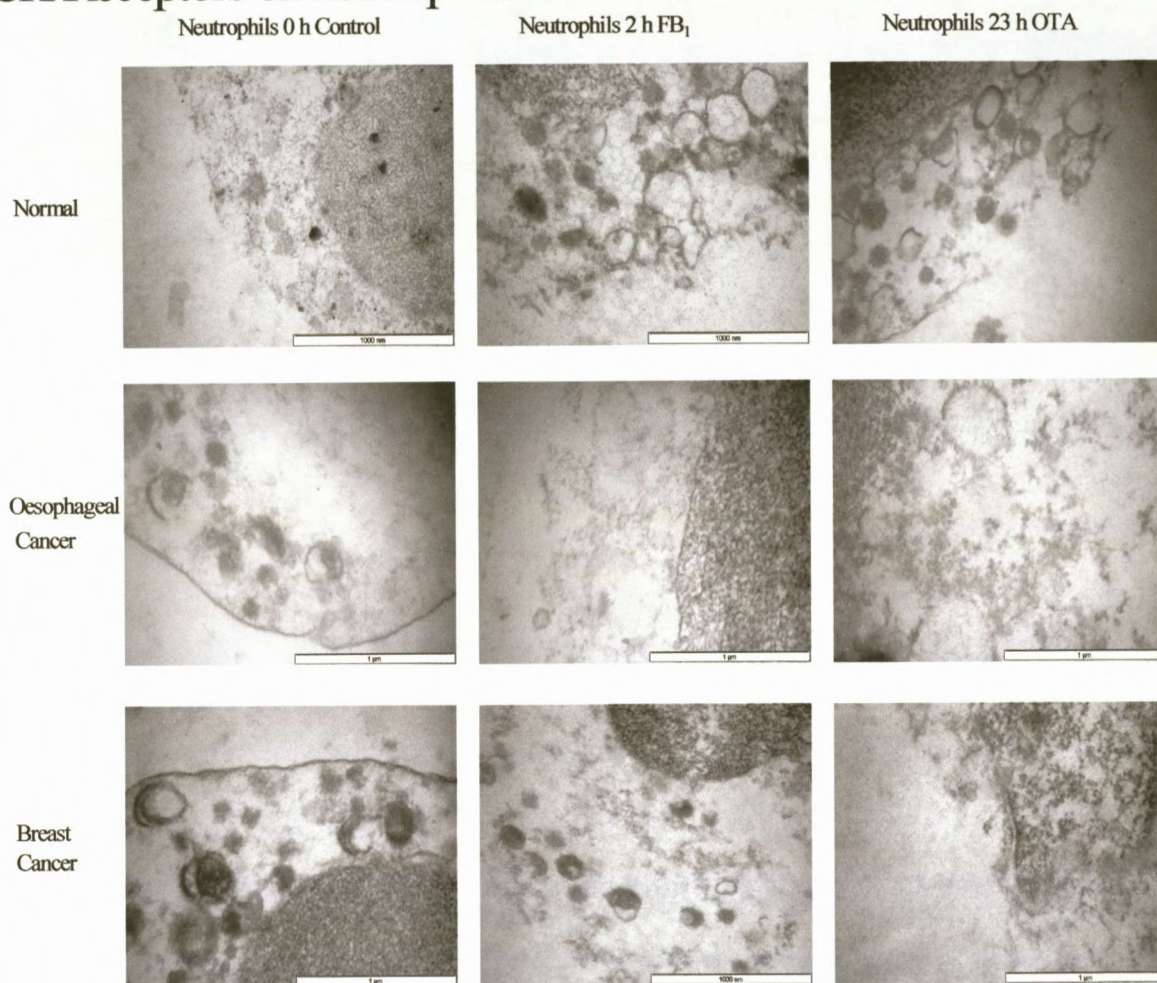


Fig. 3.36 EM images of labelled (dots) CK receptors on surfaces of neutrophils from healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 $\mu\text{g/ml}$ FB₁ for 2 h incubation or 50 $\mu\text{g/ml}$ OTA for 23 h incubation. The neutrophils at 0 h were not exposed to any toxin.

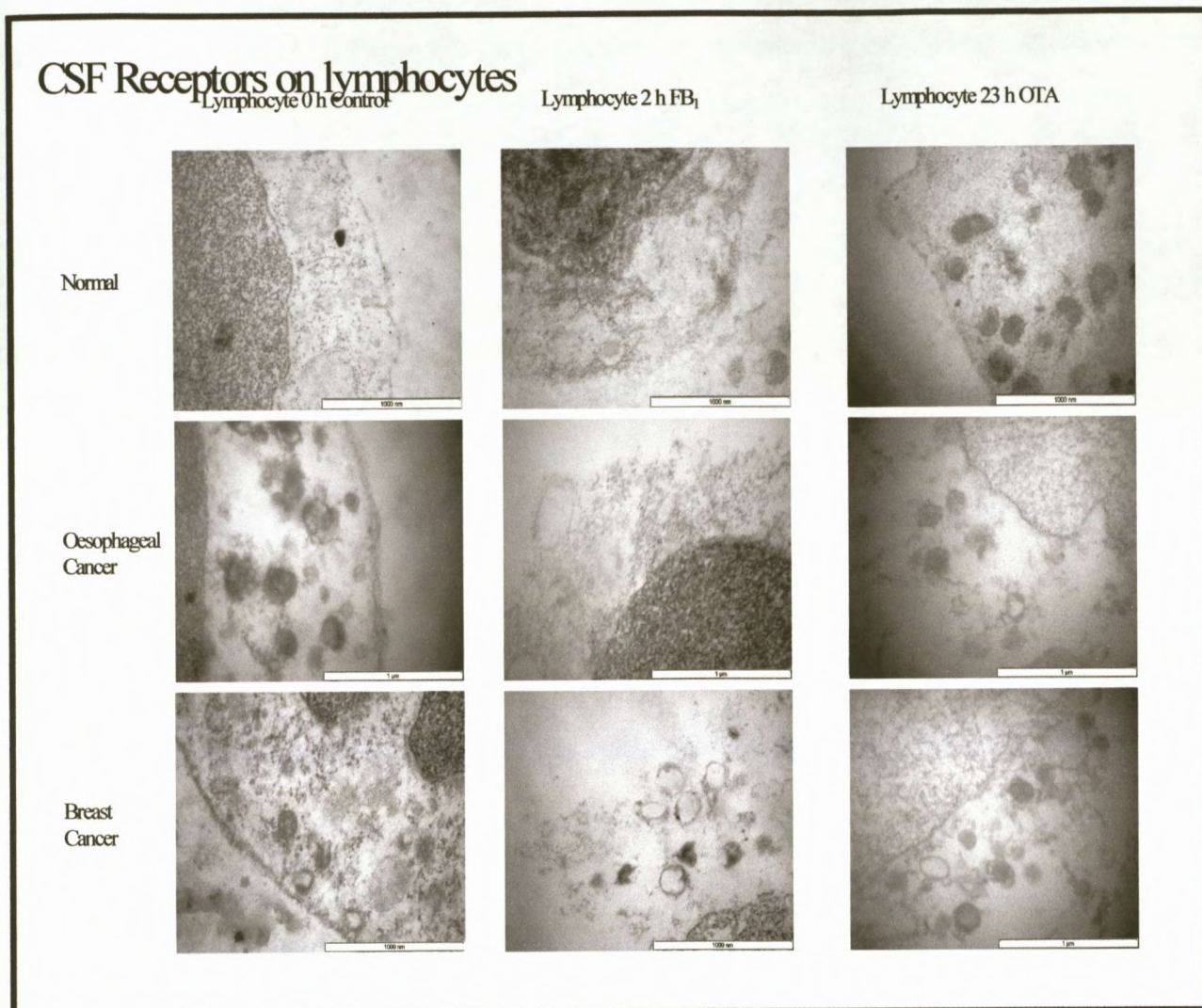


Fig. 3.37 EM images of labelled (dots) CSF receptors on surfaces of lymphocytes from healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 µg/ml FB₁ for 2 h incubation or 50 µg/ml OTA for 23 h incubation. The lymphocytes at 0 h were not exposed to any toxin.

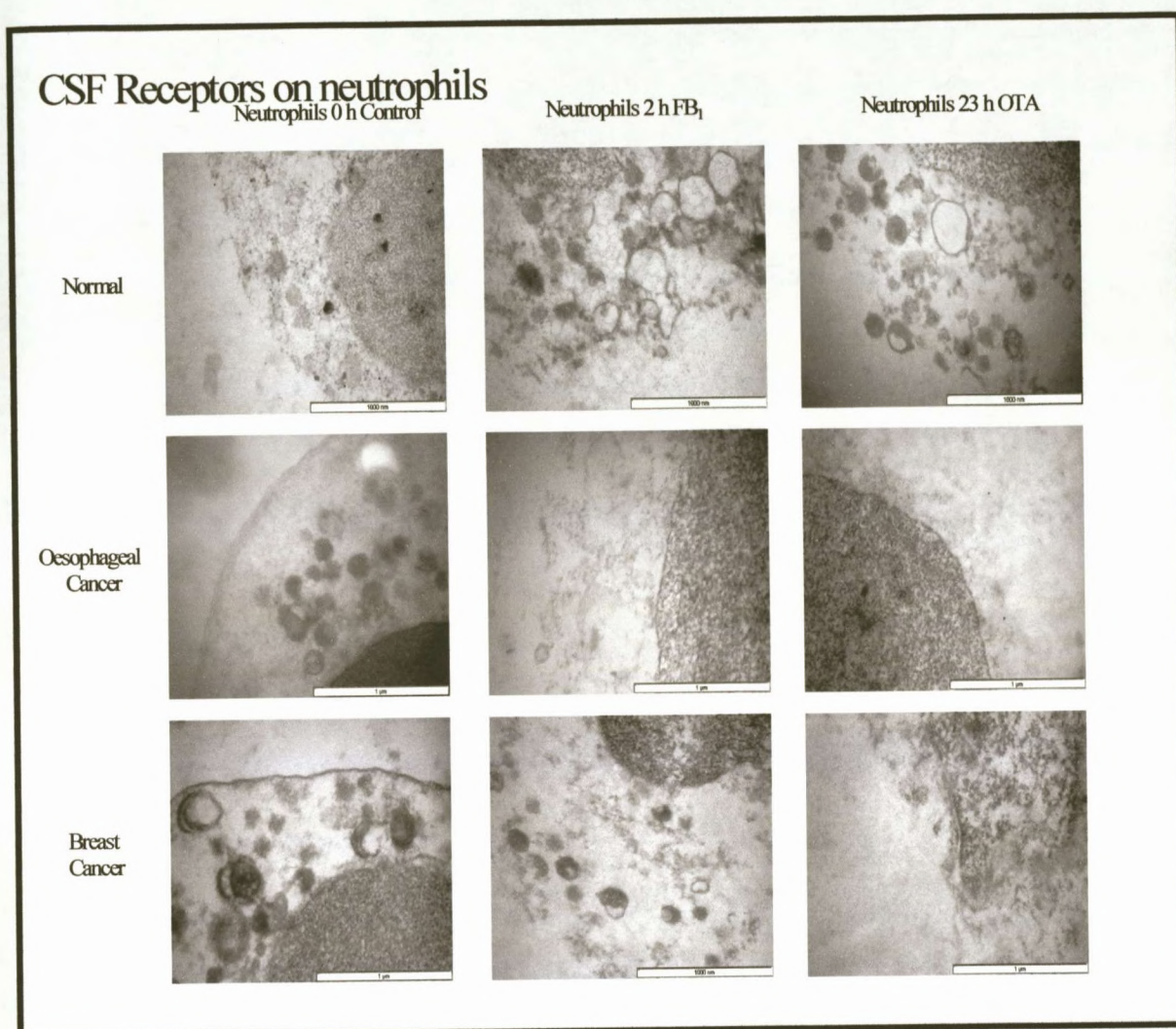


Fig. 3.38 EM images of labelled (dots) CSF receptors on surfaces of neutrophils of healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 $\mu\text{g/ml}$ FB₁ for 2 h incubation or 50 $\mu\text{g/ml}$ OTA for 23 h incubation. The neutrophils at 0 h were not exposed to any toxin.

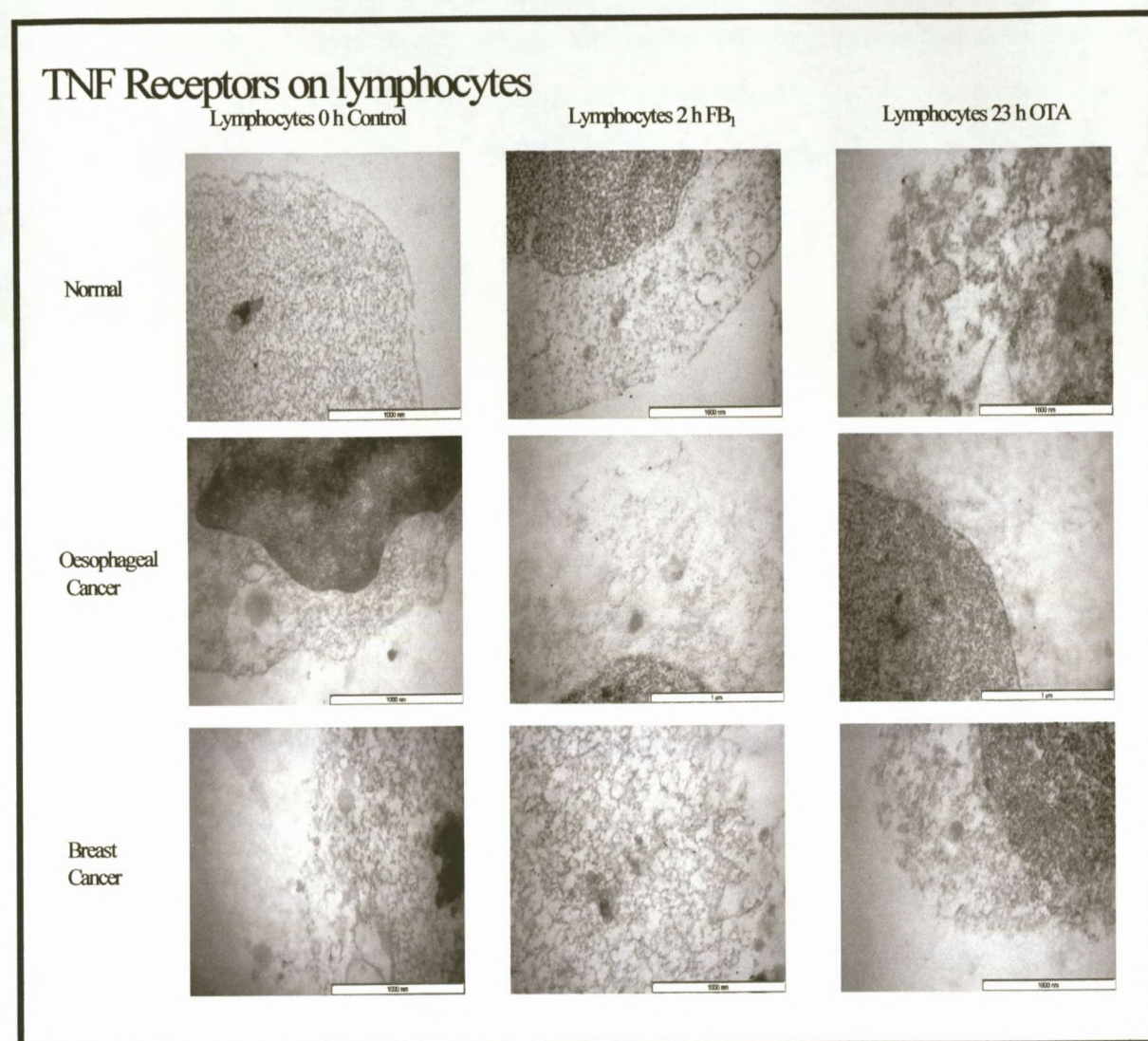


Fig. 3.39 EM images of labelled (dots) TNF receptors on surfaces of lymphocytes of healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 µg/ml FB₁ for 2 h incubation or 50 µg/ml OTA for 23 h incubation. The lymphocytes at 0 h were not exposed to any toxin.

TNF Receptors on neutrophils

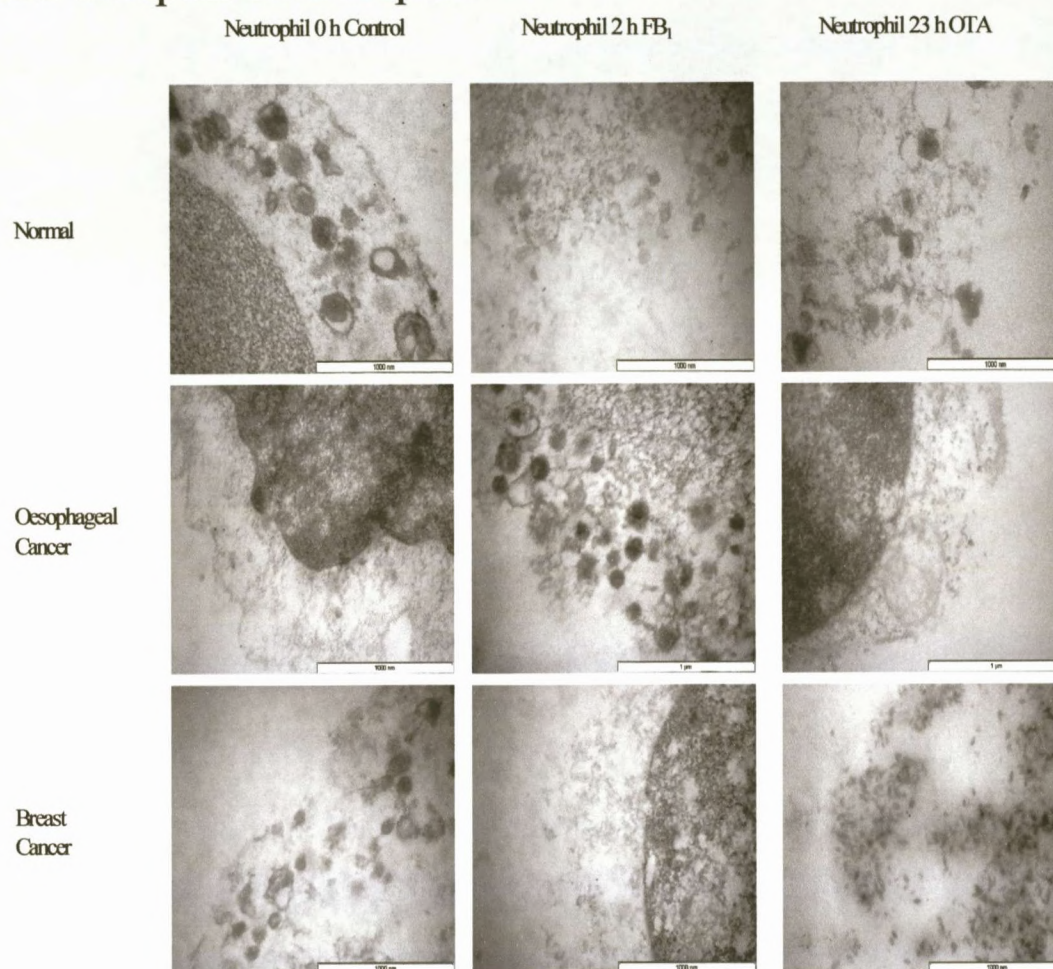


Fig. 3.40 EM images of labelled (dots) TNF receptors on surfaces of neutrophils of healthy individuals, oesophageal cancer and breast cancer patients exposed to 25 µg/ml FB₁ for 2 h incubation or 50 µg/ml OTA for 23 h incubation. The neutrophils at 0 h were not exposed to any toxin.

3.2.4 Cytokine levels (IL-1, IL-6, IL-8, IL-10 and TNF- α)

A standard curve was initially plotted for each of the interleukins. The standard curves for each of the cytokines had a sigmoid shape (Figure 3.41 to Figure 3.45, Appendix F) that showed an inverse relationship between the interleukin concentrations and the corresponding optical densities (OD's or absorbancies). In other words the greater the concentration of the interleukin in the sample, the lower the OD, or less red colour. A power series trend line was fitted to the standard values.

3.2.4.1 Concentrations of IL-1, IL-6, IL-8, IL-10 and TNF- α in plasma samples

Basal levels of IL-1, IL-6, IL-8, IL-10 and TNF- α were measured in the plasma samples collected from 10 healthy volunteers, 10 oesophageal cancer patients and 10 breast cancer patients. The samples were randomly selected. The concentrations of IL-1, IL-6, IL-8, IL-10 and TNF- α in the plasma samples of volunteers, oesophageal cancer patients and breast cancer patients were obtained from the respective standard curves. The mean concentrations are represented in Table 3.10.

Table 3.10 Mean concentrations of IL-1, IL-6, IL-8, IL-10 and TNF- α in plasma samples of volunteers, oesophageal cancer patients and breast cancer patients

Cytokine concentrations	Volunteers	Oesophageal cancer patients	Breast cancer patients
IL-1 (ng/ml)	0.416 \pm 0.497	0.183 \pm 0.326	0.052 \pm 0.068
IL-6 (ng/ml)	0.088 \pm 0.430	0.593 \pm 0.022	0.058 \pm 0.013
IL-8 (ng/ml)	0.029 \pm 0.014	0.027 \pm 0.012	0.024 \pm 0.009
IL-10 (ng/ml)	0.473 \pm 0.521	0.190 \pm 0.081	0.175 \pm 0.061
TNF- α (ng/ml)	0.020 \pm 0.011	0.164 \pm 0.009	0.007 \pm 0.004

No significant difference was noted in the concentrations of IL-1, IL-6, IL-8, IL-10 and TNF- α in the plasma samples obtained from the three groups of individuals as indicated by the following statistical analysis:

IL-1

Since normality was violated a non-parametric Kruskal Wallis test was done to compare the groups. A p-value of 0.245 was obtained showing that there was no significant difference between the groups, as the p-value is not \pm 0.05, where there is a significant difference.

IL-6, IL-8, IL-10 and TNF- α

For IL-6, IL-8, IL-10 and TNF- α , one- way Anova tests were carried out, where the p-values are all greater than 0.05, showing a statistically insignificant difference. The p-values are 0.136, 0.776, 0.152 and 0.006 respectively.

3.2.4.2 Concentration of IL-1, IL-6, IL-8, IL-10 and TNF- α in the supernatant

The concentrations of IL-1, IL-6, IL-8, IL-10 and TNF- α were measured in the supernatant of lymphocytes and neutrophils isolated from the three groups at 0 h, and at 2 h FB₁ exposure or 23 h OTA exposure. Five samples were randomly selected from each experimental group. The results are represented in Figures 3.46 to 3.50.

The concentrations of IL-1, IL-6, IL-8 and TNF- α either decreased or remained the same in the supernatant of both lymphocytes and neutrophils after FB₁ and OTA exposure in all three population groups *i.e.* healthy volunteers, oesophageal cancer patients and breast cancer patients. A difference was observed in IL-1 concentration present in the supernatant of lymphocytes and neutrophils of breast cancer patients (Figure 3.46), and IL-6 concentration in the supernatant of lymphocytes and neutrophils of healthy volunteers (Figure 3.47). For TNF- α the detection limit may have been a factor that shows 0 in the cancer patient groups (Figure 3.50). Standard curve range between 0.25 and 0.10 (Appendix E) – below detectable limit. Interleukin-10 concentration decreased in the supernatant of lymphocytes and neutrophils from both the patient groups and increased in healthy volunteers particularly in the supernatant of lymphocytes (Figure 3.49).

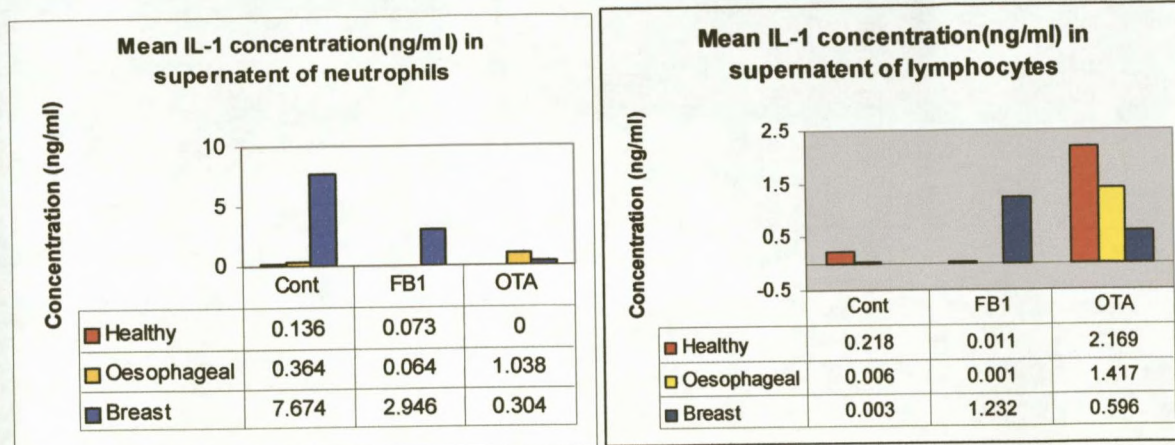


Fig. 3.46 Mean IL-1 concentration (ng/ml) in supernatant of lymphocytes and neutrophils at time zero and at 2 h exposure to 25 μ g/ml FB₁ exposure or 23 h exposure to 50 μ g/ml OTA

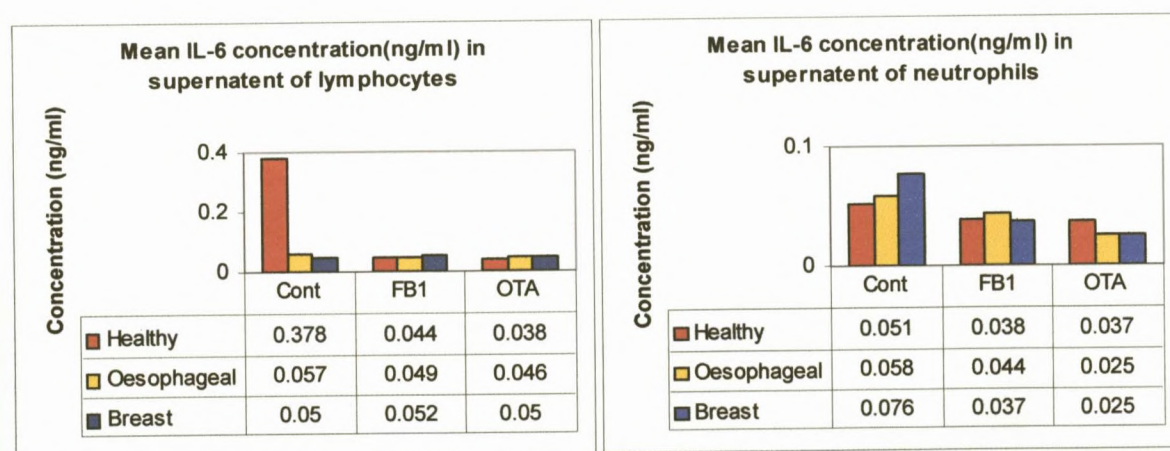


Fig. 3.47 Mean IL-6 concentration (ng/ml) in supernatant of lymphocytes and neutrophils at time zero and at 2 h exposure to 25 μ g/ml FB₁ exposure or 23 h exposure to 50 μ g/ml OTA

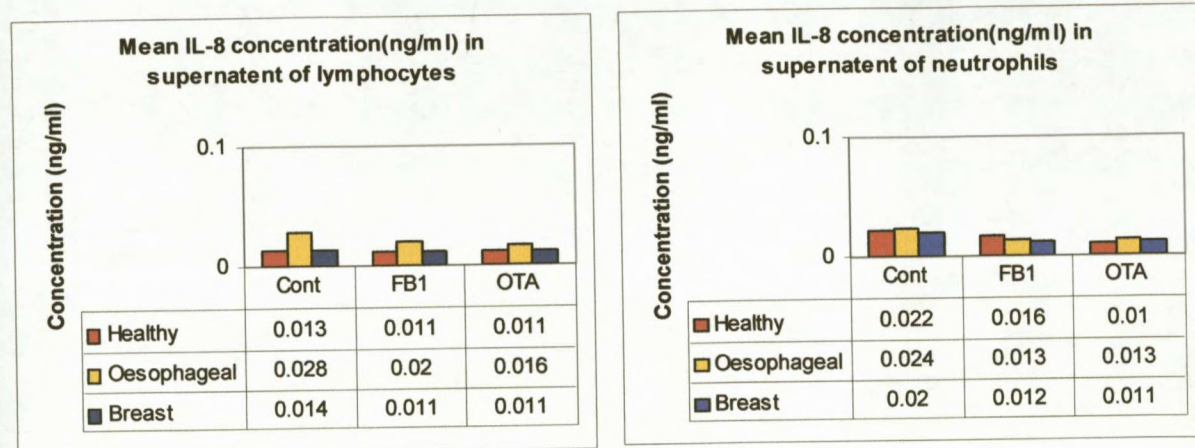


Fig. 3.48 Mean IL-8 concentration (ng/ml) in supernatant of lymphocytes and neutrophils at time zero (Control) and at 2 h exposure to 25 μ g/ml FB₁ exposure or 23 h exposure to 50 μ g/ml OTA

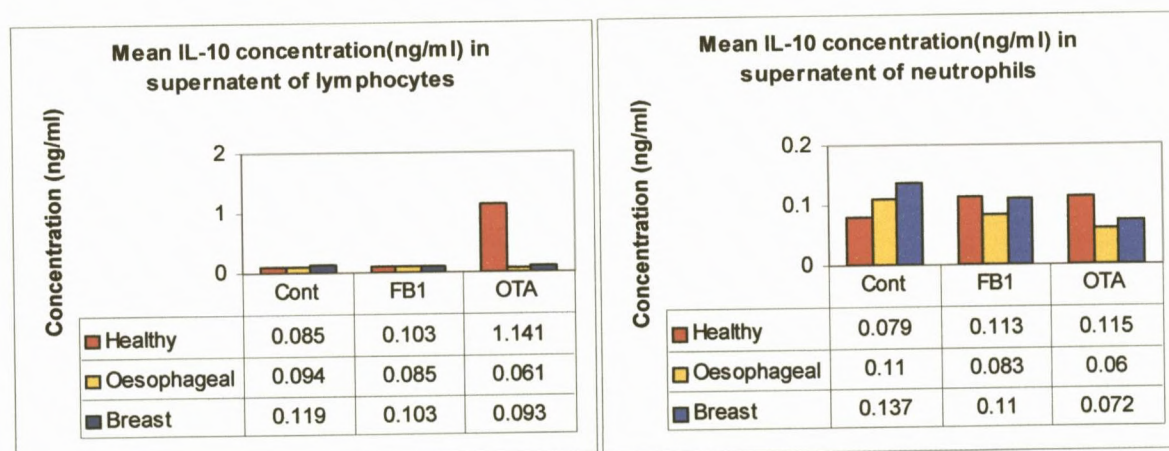


Fig. 3.49 Mean IL-10 concentration (ng/ml) in supernatant of lymphocytes and neutrophils at time zero (Control) and at 2 h exposure to 25 μ g/ml FB₁ exposure or 23 h exposure to 50 μ g/ml OTA

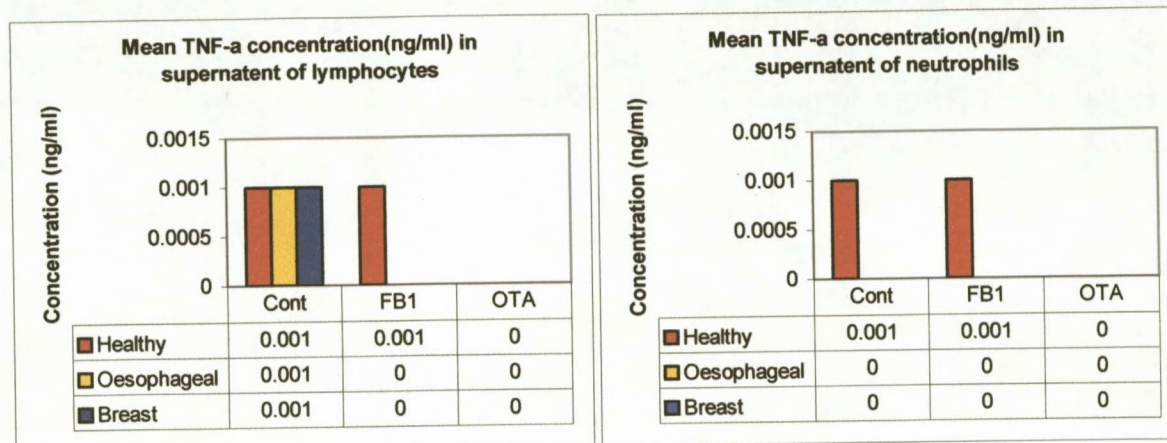


Fig. 3.50 Mean TNF- α concentration (ng/ml) in supernatant of lymphocytes and neutrophils at time zero (Control) and at 2 h exposure to 25 μ g/ml FB₁ exposure or 23 h exposure to 50 μ g/ml OTA

CHAPTER FOUR: DISCUSSION

Fumonisin B₁ and OTA exert adverse effects on humans, animals, and crops that result in illnesses and economic losses. Thus, they represent examples of mycotoxins of greatest public health and agro-economic significance. In particular, fumonisins (B₁ and B₂) are cancer-promoting metabolites of *Fusarium proliferatum* and *F. verticillioides*, (formerly *moniliforme*) that have a long-chain hydrocarbon unit (similar to that of sphingosine and sphinganine) which plays a role in their toxicity (Wang *et al.*, 1992). Fumonisin B₁ is the most toxic and has been shown to promote tumour in rats (Gelderblom *et al.*, 1988; Abel and Gelderblom, 1998) and cause equine leukoencephalomalacia (Marasas *et al.*, 1988) and porcine pulmonary oedema (Harrison *et al.*, 1990). It is suggested that FB₁ exerts toxic effects due to its structural analogy to sphingoid bases (Humpf *et al.*, 1988). This mycotoxin is cytotoxic and inhibits both protein and DNA synthesis. Moreover, it promotes oxidative stress, induces DNA fragmentation and cell cycle arrest (Abado-Becognee *et al.*, 1998; Abel and Gelderblom, 1998; Mobio *et al.*, 2000; Mobio *et al.*, 2003). Although the pathologic and toxic effects of FB₁ are well established, there is very little known about its effect on the immune cells. The available data on immunotoxicity indicate that fumonisin-induced changes in immune function are not species specific, and appear to involve aspects of humoral, cellular and innate immunity (Bondy and Pestka, 2000).

Ochratoxins are metabolites of both *Aspergillus* and *Penicillium* species. These compounds are known for their nephrotoxic effects in all animal species (Carlton and Tuite, 1977; Krogh *et al.*, 1974; Krogh, 1978; Lanza *et al.*, 1980; Manning and Wyatt, 1984; Wannemacher *et al.*, 1991), and may promote tumours in humans (Krogh, 1992; Petkova-Bocharova *et al.*, 1988; Plestina, 1992). Ochratoxin A is a structural analogue of phenylalanine and contains a

chlorinated dihydroisocoumarin moiety. It inhibits protein synthesis by competition with phenylalanine in the reaction catalysed by phenylalanyl-t-RNA synthetase (Creppy *et al.*, 1979;). In addition to nephrotoxicity, ochratoxin A impairs blood coagulation (Galtier *et al.*, 1979; Gupta *et al.*, 1979), glucose metabolism (Pitout, 1968), is immunosuppressive (Creppy *et al.*, 1983), teratogenic (Arora and Frolen, 1981; Mayura *et al.*, 1984), genotoxic (Creppy *et al.*, 1985; Pfohl-Leszkowicz *et al.*, 1991) and carcinogenic (Kanisawa and Suzuki, 1978; NTP, 1989). It is a known renal and hepatic carcinogen in rodents and also induces oxidative damage by enhancing lipid peroxidation (Omar *et al.*, 1991). The toxicology of OTA has been already reviewed (Boorman, 1989; DFG, 1990; Kuiper-Goodman, 1996; Petzinger and Ziegler, 2000), but some recent developments should be considered. In man OTA exhibits unusual toxicokinetics, with a half-life in blood of 840 h (35 days) after oral ingestion.

Homeostasis of the immune system is normally tightly regulated and any manipulation of this system might be expected to have potentially undesirable side effects, both with respect to cancer and other conditions. Suppression of innate immunity might decrease the ability to fight established cancers, whereas suppression of adaptive immunity may decrease cancer surveillance. Suppression of either arms of the immune system would be expected to increase susceptibility to toxin-induced cancers. Enhancement of innate immunity may result in chronic inflammation, which can lead to carcinogenesis. The modern Western diet, high in processed foods and relatively low in fruit and vegetables, has been described as pro-inflammatory and has been linked to the development of many cancers.

Both the neutrophils and lymphocytes play a significant role as a line of defense against invading organisms. If these cells were weakened by their exposure to toxins this may subsequently render the host susceptible to disease and infection.

Initial experiments were designed to determine the effect of the toxins on lymphocytes and neutrophils from normal subjects. Subsequently, experiments were performed on cells harvested from cancer patients. The effect was evaluated by measuring viability, morphology, expression of cytokine receptors (CK, TNF and CSF) and cytokines (IL-1, IL-6, IL-8, IL-10 and TNF- α).

4.1 Effect of FB₁ and OTA on cell viability of lymphocytes and neutrophils

The trypan blue dye exclusion test and the MTT cytotoxicity test were evaluated to establish an assay procedure to measure viability of lymphocytes and neutrophils isolated from volunteers exposed to varying concentrations of FB₁ and OTA. The Trypan blue dye exclusion test was found to be a better test as it was easier to perform, and could also assist in the determination of the extent of cellular damage by their appearance under the light microscope, since live, healthy cells are usually round, refractile and relatively small when compared to dead cells, which appear larger, crenated and non-refractile when in suspension (Doyle and Griffiths, 1998). Results obtained with the MTT assay were comparatively close to the counts achieved with the trypan blue dye exclusion test.

In our study there was a significant difference ($p < 0.005$) in cell viability with increasing toxin concentrations. Similar findings were observed with FB₁ on the viability of human lung adenocarcinoma cells and monkey kidney cells (Naicker, 2001). Whilst with a concentration of 20 $\mu\text{g/ml}$ FB₁ exposure a cell viability of 65% after 2 h was obtained, at 30 $\mu\text{g/ml}$ the level of cell viability dropped considerably to 15% after 2 h. With 30 $\mu\text{g/ml}$ OTA exposure for 2 h 100% of the cells remained viable. Short exposure (0 h) caused a less

pronounced effect, close to that of the unexposed controls with both mycotoxins in all three groups. A pronounced effect was seen after 2 h and 23 h incubation with 25 µg/ml FB₁ and 50 µg/ml OTA respectively. This was an indication that toxicity is dependent on time and dosage. Fumonisin B₁ and OTA are immunotoxic agents, but the rate of cell death by FB₁ was 2 h as compared to OTA, which was 23 h. The LD₅₀ for FB₁ was found to be 25 µg/ml FB₁ following 2 h incubation at 37°C and the LD₅₀ for OTA was 50 µg/ml OTA for 23 h at 37°C.

The findings from the viability studies clearly show that both FB₁ and OTA cause a reduction in the number of lymphocytes and neutrophils in the immune system, irrespective of whether or not carcinoma of the oesophagus was present. In all three experimental groups exposure to 25 µg/ml FB₁ for 2 h resulted in an average reduction of 20% in the number of lymphocytes. Neutrophils from oesophageal cancer patients, which were exposed to FB₁ had a average reduction in survival rate of 22%, while that of non-oesophageal (breast) cancer patients was 23%. A drop of 21% in viable neutrophils from healthy individuals was recorded.

Blood cells were also incubated with 50 µg/ml OTA for periods of 0 h and 23 h. This exposure resulted in an average reduction in viability of lymphocytes of 14% for oesophageal cancer patients as compared to the 20% for breast cancer patients. Healthy individuals showed a reduction of 24% in viability. The decline in viability of neutrophils was 15% for oesophageal cancer patients and 19% for breast cancer patients. Healthy individuals had a 23% reduction in cell viability.

It was interesting to learn that the results of the viability studies were similar in all three groups, especially where FB₁ was concerned. An explanation to this could be the fact that in all three groups the lymphocytes and neutrophils were only given a flush incubation (0 time

period) with the mycotoxins which resulted in a similar pattern in the reduction in viability. As for extended incubation (2 h and 23h), this type of experiment could not shed light on what happens over a long period of exposure (months or even years) to mycotoxins, as is the case where oesophageal cancer develops. The experiment cannot be used as a repetition of what happens during the etiology of the cancer, it can only serve to indicate whether or not the cell numbers are affected, by flush or extended incubations with mycotoxins.

4.2 Effect of FB₁ and OTA on morphology of lymphocytes and neutrophils

Changes in morphology assessed by light microscopy and transmission electron microscopy of lymphocytes and neutrophils exposed to FB₁ or OTA showed that cells exposed to either FB₁ or OTA lost their shape, which could be a result of damage to the cell membrane upon exposure to the toxin. These results were in keeping with results obtained by Dombrink-Kurtzman *et al.* (1994).

The presence of a large number of dead cells under the light microscope was probably due to the toxin causing cellular disintegration and possibly leakage of the contents to the exterior. Transmission electron microscopy (TEM) was ideal for visualization of intracellular structures. In this study TEM was used in order to assess the degree of cellular damage upon exposure to 25 µg/ml FB₁ incubation at 37°C for 2 h and 50 µg/ml OTA exposure at 37°C for 23 h at 37°C. It has already been established that both neutrophils and lymphocytes are primarily responsible for defense against invading organisms (Pestka and Bondy, 1990). The human neutrophil is frequently implicated as a mediator of tissue destructive events in inflammatory diseases. This study found that exposure to FB₁ toxin and OTA resulted in injury to neutrophils. The extent of injury was similar in all three

groups of individuals. This was illustrated by the results obtained via electron microscopy, which gave a clear indication of a loss of cell membrane integrity and loss of cellular organisation. The formation of a large number of vacuoles was also prevalent.

Lymphocytes also showed signs of cellular injury characterized by results obtained by TEM, which demonstrated the presence of swollen mitochondria as well as autophagic vesicles with enzymatic breakage of cellular structure deposits. It could be noted that the common mechanism of action of FB₁ and OTA on both neutrophils and lymphocytes included cytoplasmic vacuolization and nucleoplasm leakage. These observations were similar to those obtained by (Dombrink-Kurtzman *et al.*, 1994; Dwivedi *et al.*, 1984). The results of this study indicate that both the neutrophils and lymphocytes were susceptible to cellular damage by FB₁ and OTA in all three population groups, illustrated by distinctive morphological changes. This could consequently have negative effects on the immune system as a whole and in turn may render the host susceptible to infections. These results re-affirm the implications that FB₁ and OTA are immunosuppressive (Johnson *et al.*, 2003; Martinova and Merrill, 1995).

Morphological changes seen in lymphocytes and neutrophils exposed to FB₁ and OTA are changes normally seen in dying cells. Pyknosis, karyorrhexis and cytoplasmic vacuolation are seen in normal culture (Ghadially Feroze, 1984; 1985), and an increase in their incidence reflects a cytotoxic effect of FB₁ and OTA resulting in cell death and a decrease in the number of cells. Similar changes were observed by Odhav (1996) both *in vitro* and *in vivo* in keratinocytes exposed to FB₁. Tolleson *et al.* (1996) examined the effect of FB₁ on keratinocytes, fibroblasts, oesophageal epithelial cells and hepatoma cells. The principle effect of FB₁ on these cells was anti-proliferative, resulting from increased apoptotic cell

death, as opposed to decreased cellular replication. Anti-proliferative of FB₁ have also been reported in a panel of rat hepatome cell lines in culture (H4TG, H4-11-E, H4-11-E-C3, Fao, MH1C1, McA-RH8994), LLC-PK1 porcine kidney epithelial cells and MDCK canine epithelial cells, with IC₅₀ values ranging between 2.5 and 50 X 10⁻⁶M FB₁ (Abbas *et al.*, 1993; Shier *et al.*, 1991; Yoo *et al.*, 1992).

Quereshi and Hagler (1992) reported significant cytotoxicity and morphological features consistent with apoptosis, such as nuclear fragmentation and membrane blebbing, in primary cultures of chicken peritoneal macrophages exposed to 2.8 X 10⁻⁶ M FB₁ for 2 to 4 h. This observation suggests that FB₁ perhaps affects a common target in each of these cell types. Ceramide synthesis represents an obvious target candidate based on observations that FB₁ exposure results in accumulation of the sphingoid base substrate for that enzyme, sphinganine and sphingosine (Schroeder *et al.*, 1994; Yoo *et al.*, 1992; Wang *et al.*, 1991; 1992; Merrill *et al.*, 1993; Riley *et al.*, 1994). Ceramide synthesis catalyses the formation of dihydroceramide or ceramide from sphinganine and sphingosine and dihydroceramide and ceramide levels have been shown to drop in FB₁-treated cells (Harel and Futerman, 1993; Riley *et al.*, 1994).

Ultrastructural changes reported in the kidney and liver of 20-day-old broiler chicks fed OTA included the presence of abnormally shaped mitochondria in the proximal convoluted tubules, increase in size and number of mitochondrial dense granules and cytoplasmic peroxisomes, intranuclear and cytoplasmic lipid droplets and electron dense round bodies in the dilated smooth endoplasmic reticulum. In some cases accumulation of cytoplasmic glycogen and regional thickening and degeneration of the glomerular basement membrane were observed (Dwivedi *et al.*, 1984). The ultrastructural alterations in the kidney and liver

were, in general, dose dependent being more pronounced at 4 ppm OTA than at 2 ppm. Similar results were also seen in African green monkey kidney epithelial cell cultures after 24 h and 48 h exposure to ochratoxin (Engelbrecht and Purchase, 1969). Ochratoxin produced a decrease in mitosis and fragmentation of the nucleolus, as well as non-specific changes such as cytoplasmic vacuolation and pycnosis or karyorrhexis.

According to Maxwell and Burns (1987), OTA is more hepatotoxic in quail than in broilers, as well as being nephrotoxic. Pathological changes observed in the kidneys were limited to the proximal convoluted tubules (PCT) and glomeruli. In the PCT's abnormal mitochondria and excessive numbers of lipid droplets were the principle findings with glomeruli showing thickened basement membranes. Swollen mitochondria and variable glycogen deposits were the chief features present in the livers.

4.3 Effect of FB₁ and OTA on cytokine receptors on lymphocytes and neutrophils

Cytokine receptors are located on the outer membranes of the lymphocytes and neutrophils. Immunolabelling of CK, TNF and CSF receptors were performed using cell preparations from all three relevant groups. The light microscopy results (Figures 3.33 and 3.34) showed a reduction in the labelling intensity of receptors, and therefore the number of receptors, on the lymphocytes and neutrophils in all three groups after exposure to both mycotoxins. After exposure to FB₁ (25 µg/ml) for 2 h and OTA (50 µg/ml) for 23 h, the lymphocytes presented a noticeable reduction in receptor immunolabelling intensity in contrast to those exposed to the same doses of the two mycotoxins at 0 h. The same applied to the neutrophils exposed to toxin at the various time intervals. As in the viability results, the immunolabelling results confirmed that toxicity is time dependent.

Further validation of the above with the electron microscopy, and a secondary antibody tagged with an electron dense 10 nm gold colloid, clearly showed a reduction in three receptors in the cancer patient groups as compared to receptors on neutrophils and lymphocytes of non-cancerous subjects. One has to bear in mind that with the immunocytochemical experiments, our objective was to localize the antigen, so structural preservation was compromised during the fixation stage in order to preserve antigenicity. Also, the blood cells were exposed to toxin for long periods before fixation, and hence the structure of the labeled cells was severely affected. The immunolabelling results of exposure of the lymphocytes and neutrophils to FB₁ (25 µg/ml) for 2 h and OTA (50 µg/ml) for 23 h indicated a definite reduction in receptor numbers as compared to those at 0 h exposure.

The scenario again - similar results for all three groups! A possible reason could be that since cancer developed over a long period of toxin exposure, the receptors have also been exposed over this similarly long period and the cell kinetics (alteration in the number of receptors) had time to adjust to these conditions. Incubation with the mycotoxins reduced the number of receptors on the lymphocytes and neutrophils in a similar manner in our three experimental groups. As stated earlier, the experiment could only serve to indicate whether or not the receptors were affected by incubation with 25 µg/ml FB₁ and 50 µg/ml OTA.

4.4 Effect of FB₁ and OTA on cytokines

There was no significant difference in basal levels of cytokines (IL-1, IL-6, IL-8, IL-10 and TNF-α) in the plasma samples obtained from the three population groups, *i.e.* healthy volunteers, oesophageal cancer patients and breast cancer patients. When lymphocytes and

neutrophils isolated from the three groups of individuals were exposed 25 µg/ml FB₁ for 2 h or 50 µg/ml OTA for 23 h, concentrations of IL-1, IL-6, IL-8 and TNF-α increased in the supernatant. However, IL-10 levels were found to be increased in the supernatant of healthy volunteers and decreased in the supernatant of oesophageal cancer patients and breast cancer patients after exposure to both the toxins.

OCHRATOXIN A

FUMONISIN B1

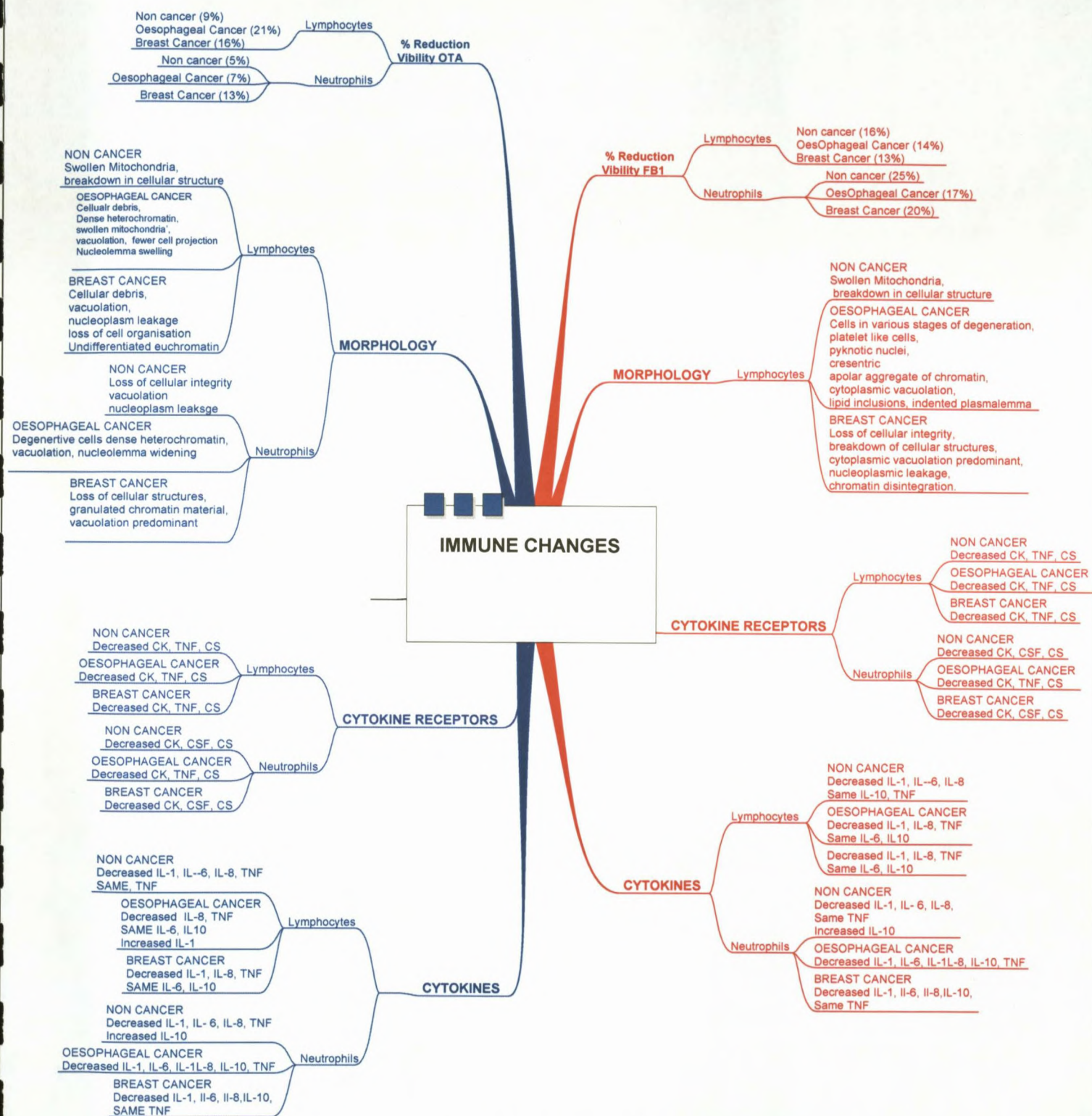


Fig. 3.51 Summary of Results

CHAPTER FIVE: CONCLUSION AND FUTURE WORK

The results of this study showed that the mycotoxins, FB₁ and OTA, produced immunomodulation of lymphocytes and neutrophils in a dose and time dependent mode. The immunotoxic effects of FB₁ and OTA on lymphocytes and neutrophils are:

- decreased viability
- increased morphological damage
- decreased CSF, TNF, and CK receptors
- decreased IL-1, IL-6, IL-8 and TNF- α concentrations

They, therefore, affect the functioning of the immune system *i.e.* they suppress the immune response. Similar results were observed in weaners fed varying concentrations of OTA for varying times (Muller *et al.*, 1999). The lymphocytes and neutrophils are involved in antibody production and killing of these cells decrease their number and could possibly indicate how the toxins escape immune surveillance and cause disease (Kowalczyk, 2002). There is evidence of this suppression being exacerbated in patients who have cancer. This could be a mechanism that causes further proliferation of the cancer cells since the homeostasis of the immune system in cancer patients is compromised. This supports a model that shows that an interaction between the molecular and cell-signalling mechanisms are involved in FB₁ and OTA toxicity.

Therefore, contamination of food with these toxins can lead to impaired immune functions, disorders of metabolism, decreased performance, and increased susceptibility to adverse environmental influences.

The mycotoxins FB₁, and OTA should be considered for further immune toxicity studies, in view of their toxicological significance and the gaps in our knowledge.

Recognizing the immune system's remarkable ability to defend the body against disease, medical scientists have long dreamed of developing a new form of treatment for cancer – immunotherapy (Dorsey *et al.*, 2002; Senderowicz, 2001). The aim of cancer immunotherapy is to bolster the immune system so that it is better able to combat cancer cells. Currently, several forms of immunotherapy are being explored in research laboratories and clinical trials. The majority of these approaches use natural biological substances to activate the immune system (Muller *et al.*, 2002). Researchers are able to reproduce these natural substances outside the body through genetic engineering and hybridoma techniques (Stripecke *et al.*, 2002). The various forms of immunotherapy fall into three main categories: immune response modifiers, monoclonal antibodies and vaccines.

Immune response modifiers are substances, either extrinsic or intrinsic to the body, that affect the immune response. One group of extrinsic modifiers is referred to as *immune potentiators*. These include BCG, *C. parvum* and endotoxin, which are all microbes or microbial products that have been shown to modify the immune response and, under certain conditions, to cause tumours to regress or grow more slowly than usual.

The intrinsic group, known as *biological response modifiers*, includes IL-1 and IL-2, interferon (alpha, beta, and gamma), TNF, B-cell growth factors and haematopoietic

growth factors (such as CSF). These agents exert their influence at different stages of the immune response (Jorkov *et al.*, 2003).

The *interleukins* activate the body's own lymphocytes to do their work. Interleukin-2 has been found to be effective in some patients with melanoma or with renal cancer when it is administered alone or with a patient's own lymphocytes that have been treated with IL-2 outside the body (Stritze *et al.*, 2003; Verheyen *et al.*, 2000). The *interferons* act on the immune system by stimulating both T cells and macrophages. They also prevent cells from multiplying. Scientists believe that these two properties together enable interferon to fight some tumours effectively. Alpha interferon was the first FDA-approved biological response modifier for the treatment of cancer (it is effective against a rare form of leukemia).

Tumour necrosis factor directly attacks and kills tumour cells. Currently, it is being tested alone and in conjunction with gamma interferon to determine its potential efficacy in the treatment of human cancers. *B-cell growth factors* stimulate the multiplication of antibody-producing cells. The *haematopoietic growth factors* step up the production of both red and white blood cells in the bone marrow, thereby giving the body additional ammunition to fight disease and protect itself against the suppressive effects on the bone marrow of radiation and chemotherapy.

Because immunotherapy uses biological substances to strengthen the body's own immune system, it promises to be a more natural and better-targeted means of treating cancer than radiation and chemotherapy. Moreover, immunological approaches, using the exquisite

recognition capacity of the immune system, should allow physicians to detect cancer earlier, which is an important advantage in controlling the disease.

Immunotherapies are expected to become a treatment option for cancers in various stages of development (Nelson and Ballow, 2003). When a cancer is localized, the usual treatment is either surgery or radiation or a combination of both. Once a cancer has spread, the primary lesion is generally removed surgically, and radiation and chemotherapy are used to kill the remaining cancer cells. Immunotherapy may one day eliminate the need for traditional treatment in the incipient stages of cancer by preventing cells from forming a solid tumour. As the cancer progresses, immunotherapy may take the place of radiation or chemotherapy by stimulating the immune system to destroy cancer cells left behind after surgery (Ostanin *et al.*, 1997). In cases where radiation or chemotherapy is used, immunotherapies have the potential to increase the efficacy of these two therapies and to lessen their toxic effects (radiation and chemotherapy can damage the immune system itself, the blood-forming system and other parts of the body).

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CHAPTER SIX: APPENDICES

APPENDIX A

CONSENT FORM FOR INCLUSION IN A RESEARCH PROJECT

I, _____ (name and surname),
agree to participate in the research that will be undertaken by _____.

I acknowledge that I have been fully informed by the researcher concerning the whole procedure that is to be conducted on myself and that I can withdraw at any stage without prejudice. The blood collected will be used to isolate blood cells for research purpose.

.....
Signature of participant Date

.....
Signature of researcher Date

(Zulu)

*Mina, _____ (igama nesibongo),
ngiyavuma ukuzibandakanya nocwaningo oluzokwenziwa aluzokwenziwe kimina ngu
_____.*

*Ngiyavuma ukuthi ngaziswe yonke iminingwane ephathelene nocwaningo
oluzokwenziwe kimina ngumcwaningi.*

.....
Sayina (isiguli) Usuku

.....
Sayina (umcwaningi) Usuku

APPENDIX B

PREPARATION OF BUFFER FOR DIFFERENTIAL CENTRIFUGATION

Phosphate buffered saline (PBS) 1% (pH 7.2-7.4)

Solution A

Disodium phosphate	1.4 g
Distilled water	100.0 ml

Solution B

Sodium dihydrogen phosphate	1.4 g
Distilled water	100.0 ml

Note: Add 84.1 ml of solution A to Solution B. Add 8.5 g of Sodium chloride and solubilize to 1000 ml (1 litre) in distilled water.

APPENDIX C

PREPARATION OF REAGENTS FOR LIGHT MICROSCOPY

(a) May - Grunwald's stain

0,3 g of the powdered dye was weighed out and transferred to a conical flask of 200-250 ml capacity. 100 ml of methanol was then added and the mixture was warmed to 50°C. The flask was then cooled to RT and was shaken several times during the day.

After standing for 24 h, the solution was filtered, and stored in a dark bottle until required.

(b) Giemsa's stain:

1 g of the powdered stain was weighed out and transferred to a conical flask. 55 ml of glycerol was then added and the mixture was heated at 56°C for 60-120 min. 66 ml of methanol was then added and after thorough mixing the solution was allowed to stand for 7 days at RT before being filtered. It was then stored in a dark bottle until required.

(c) Buffered water:

Solution A

di-Sodium hydrogen orthophosphate 9,1 g

Potassium di-hydrogen orthophosphate

Solubilize in distilled water 1000 ml (1 litre)

Solution B

di-Sodium hydrogen orthophosphate di-hydrate 11,9 g

(Sorenson's salt)

Solubilize in distilled water 1000 ml (1 litre)

APPENDIX D

PREPARATION OF REAGENTS FOR TRANSMISSION ELECTRON MICROSCOPY (TEM)

1% glutaraldehyde solution- was prepared from a 25% glutaraldehyde solution.

120 μ l of 25% glutaraldehyde was dissolved in 2 880 μ l of PBS to make up a 3 ml solution.

0.2 M sodium cacodylate buffer (pH 7.2)

1. 0.4 M solution of sodium cacodylate was prepared with:

Sodium cacodylate	21.4 g
-------------------	--------

Distilled water	250 ml
-----------------	--------

2. 0.2 M cacodylate buffer was prepared with:

0.4 M sodium cacodylate	50 ml
-------------------------	-------

0.2 M HCl	8 ml (approx. for pH 7.2) distilled water
added to make up 100 ml.	

The pH of the buffer was adjusted to 7.2 with (0.1 M) HCL.

1% Osmium tetroxide

Osmium tetroxide 1 g

0.2 M cacodylate buffer 100 ml

To make up 100 ml of 1% osmium tetroxide, 1 g of osmium tetroxide was dissolved in 100 ml of 0.2 M cacodylate buffer.

Araldite epoxy resin

This was obtained via the image-capturing department at the Nelson R. Mandela School of Medicine. Stored in a refrigerator.

50:50 (Resin/absolute alcohol) solution

>0 ml of araldite epoxy resin was dissolved in 50 ml of absolute alcohol.

Preparation of saturated ethanolic uranyl acetate

Sufficient uranyl acetate to form saturated solutions was dissolved in 50% ethanol.

Preparation of Reynold's lead citrate

The staining solution (pH 12) was prepared as follows:

Lead nitrate 1.33 g

Sodium citrate 1.76 g

Distilled water 30 ml

The mixture was shaken vigorously at intervals for about 30 min in a 50 ml volumetric flask. The completion in conversion was indicated by a uniform milky suspension. To this suspension was added 8 ml of 1 N NaOH and was mixed by inversion until the suspension cleared completely.

APPENDIX E

VIABILITY RESPONSES OF LYMPHOCYTES AND NEUTROPHILS FROM HEALTHY VOLUNTEERS, OESOPHAGEAL CANCER PATIENTS AND BREAST CANCER PATIENTS TO FB₁ AND OTA

Table 3.3 Lymphocytes and neutrophils of healthy individuals exposed to
25 µg/ml FB₁

FUMONISIN B1 NEUTROPHILS														
No.	CONTROL							TEST						
	0 TIME			2 HOUR INCUBATION				0 TIME			2 HOUR INCUBATION			
	C1	C2	Av%V	C1	C2	Av%V	%RED	T1	T2	Av%V	T1	T2	Av%V	%RED
1	58V0L	62V0L	100	46V0L	49V0L	100	0	40V10L	36V0L	85	34V8L	36V6L	76	9
2	66V4L	53V0L	100	34V4L	43V3L	91	9	43V0L	42V0L	100	26V12L	28V9L	72	28
3	66V4L	8V0L	100	8V0L	4V0L	100	0	4V1L	3V1L	78	4V4L	3V2L	59	19
4	38V0L	13V1L	93	5V2L	4V2L	69	24	20V0L	10V10L	75	11V9L	10V7L	57	18
5	55V0L	32V0L	100	17V0L	14V0L	100	0	17V3L	21V4L	81	10V8L	13V9L	52	29
6	77V2L	81V3L	97	70V3L	80V6L	93	4	71V4L	78V5L	95	30V12L	40V16L	71	24
7	12V0L	12V0L	100	3V0L	7V1L	94	6	11V1L	11V1L	92	4V3L	8V4L	62	30
8	68V0L	66V0L	100	52V4L	54V3L	94	6	70V1L	70V2L	98	54V16L	51V14L	77	21
9	38V0L	34V0L	100	30V1L	23V1L	97	3	21V1L	23V1L	96	31V20L	27V16L	63	33
10	52V0L	48V1L	99	46V3L	46V4L	93	6	49V4L	50V5L	92	28V12L	30V17L	67	25
11	26V0L	26V0L	100	15V1L	18V2L	92	8	20V2L	14V0L	94	14V9L	10V7L	60	34
12	43V0L	45V0L	100	27V1L	24V2L	94	6	18V3L	21V4L	81	15V9L	9V7L	60	21
13	15V0L	29V1L	99	15V0L	18V1L	97	2	20V0L	18V0L	100	10V6L	12V7L	63	37
14	32V0L	21V0L	100	18V0L	15V2L	94	6	20V0L	17V0L	100	11V3L	17V8L	69	31
15	46V1L	40V1L	98	30V2L	33V1L	96	2	25V0L	32V1L	97	22V7L	13V6L	71	26

5.466667

23.666667

HEALTHY INDIVIDUALS FUMONISIN B1 LYMPHOCYTES														
No.	CONTROL							TEST						
	0 TIME			2 HOUR INCUBATION				0 TIME			2 HOUR INCUBATION			
	C1	C2	Av%V	C1	C2	Av%V	%RED	T1	T2	Av%V	T1	T2	Av%V	%RED
1	59V0L	56V0L	100	32V5L	43V4L	88	12	40V9L	36V11L	80	20V12L	22V14L	67	18
2	59V2L	66V4L	96	30V10L	28V12L	73	23	42V9L	44V8L	83	23V12L	21V10L	67	16
3	59V2L	66V4L	96	30V10L	18V4L	86	10	42V9L	44V8L	83	28V12L	29V10L	72	11
4	41V0L	38V0L	100	20V0L	18V0L	100	0	26V2L	24V4L	89	19V19L	10V10L	50	39
5	59V0L	55V0L	100	32V5L	34V4L	89	11	40V9L	36V11L	79	22V14L	20V12L	63	16
6	62V1L	65V1L	98	54V4L	64V4L	94	4	74V3L	67V4L	95	42V18L	39V14L	72	23
7	140V0L	155V4L	98	148V3L	140V3L	98	0	142V4L	145V3L	97	80V30L	88V26L	75	22
8	127V0L	118V0L	100	69V15L	60V14L	82	18	62V2L	67V0L	99	34V18L	48V26L	65	34
9	41V0L	55V0L	100	40V1L	38V0L	99	1	40V1L	44V2L	97	31V17L	22V19L	60	37
10	34V0L	36V1L	99	23V5L	20V5L	81	18	21V8L	34V7L	78	26V20L	28V22L	56	22
11	69V0L	71V0L	100	46V6L	43V4L	90	10	57V3L	54V4L	94	38V20L	33V18L	66	28
12	70V3L	65V0L	98	56V4L	55V2L	95	3	59V3L	55V2L	97	48V20L	32V14L	70	27
13	66V2L	64V2L	96	50V3L	52V3L	94	2	60V2L	61V4L	92	40V18L	46V20L	69	23
14	54V0L	50V1L	99	50V2L	44V2L	96	3	36V4L	30V2L	92	28V14L	35V16L	68	24
15	48V2L	44V2L	96	35V4L	30V5L	88	8	40V9L	36V7L	83	28V11L	24V14L	68	15

8.2

23.666667

C1= first control, C2= second control, T1= first test, T2= second test
Av V (%) = average viability (expressed in %), RED = reduction in viability

Table 3.4 Lymphocytes and neutrophils of healthy individuals exposed to
50 µg/ml OTA

HEALTHY INDIVIDUALS OCHRATOXIN A LYMPHOCYTES														
No.	CONTROL						TEST							
	0 TIME			2 HOUR INCUBATION			0 TIME				2 HOUR INCUBATION			
	C1	C2	Av%V	C1	C2	Av%V	%RED	T1	T2	Av%V	T1	T2	Av%V	%RED
1	55V0L	56V0L	100	29V18L	24V10L	60	40	44V0L	43V0L	100	31V17L	25V10L	65	35
2	59V2L	56V4L	95	30V0L	42V10L	91	91	45V5L	45V5L	75	13V7L	15V10L	63	12
3	55V0L	45V5L	94	14V4L	12V5L	74	74	35V6L	24V10L	60	12V12L	15V9L	56	4
4	41V0L	38V2L	100	15V7L	14V5L	67	33	18V11L	19V9L	65	9V8L	8V8L	54	11
5	59V0L	55V0L	100	21V0L	23V0L	100	0	34V4L	35V3L	91	15V10L	16V8L	65	26
6	87V2L	96V3L	98	60V15L	50V14L	79	19	85V4L	98V5L	95	25V15L	22V13L	63	32
7	127V0L	118V0L	100	75V10L	69V9L	88	12	81V0L	91V0L	100	44V20L	46V24L	68	32
8	41V0L	45V0L	100	28V10L	26V10L	73	27	45V1L	40V2L	99	15V7L	18V10L	66	33
9	34V0L	36V1L	99	13V5L	15V4L	76	23	52V0L	23V0L	100	7V3L	5V2L	71	29
10	69V1L	71V1L	99	22V10L	35V8L	75	24	61V2L	59V0L	99	18V10L	25V8L	70	29
11	88V0L	84V0L	100	40V4L	45V8L	88	12	72V0L	71V2L	99	22V8L	17V11L	77	22
12	44V0L	49V0L	100	26V2L	20V3L	90	10	45V0L	40V0L	100	14V8L	12V6L	66	34
13	54V1L	38V1L	98	28V4L	31V4L	88	10	56V2L	48V2L	97	18V10L	10V4L	68	29
14	62V2L	55V0L	99	38V4L	35V7L	87	12	54V4L	50V3L	94	20V9L	15V8L	67	27
15	48V2L	58V3L	96	20V3L	15V2L	88	8	48V3L	44V0L	97	14V8L	10V6L	64	33

16.933333

25.866667

OCHRATOXIN A NEUTROPHILS														
No.	CONTROL						TEST							
	0 TIME			23 HOUR INCUBATION			0 TIME				23 HOUR INCUBATION			
	C1	C2	Av%V	C1	C2	Av%V	%RED	T1	T2	Av%V	T1	T2	Av%V	%RED
1	42V0L	40V0L	100	38V0L	36V0L	100	0	30V0L	26V8L	90	28V14L	24V9L	76	14
2	34V4L	43V3L	91	14V6L	10V4L	70	21	18V2L	14V2L	80	8V4L	8V4L	67	13
3	6V0L	8V0L	100	6V2L	5V2L	73	27	4V1L	2V0L	90	4V2L	4V3L	54	36
4	6V2L	4V2L	89	2V2L	5V2L	80	9	2V1L	2V0L	83	2V2L	2V1L	59	24
5	38V0L	32V0L	100	10V0L	9V0L	100	0	21V7L	23V4L	80	8V3L	6V4L	57	23
6	66V0L	66V0L	100	30V2L	35V0L	93	7	40V0L	44V3L	97	21V13L	18V7L	67	30
7	47V2L	48V1L	97	18V2L	12V0L	95	22	40V2L	42V4L	93	18V8L	8V5L	63	30
8	36V0L	39V0L	100	15V2L	10V3L	83	17	25V3L	26V4L	88	8V3L	10V6L	68	20
9	59V0L	65V0L	100	19V2L	24V0L	90	10	50V1L	45V2L	97	30V15L	18V7L	70	27
10	75V1L	69V1L	99	24V4L	20V3L	87	12	64V2L	60V1L	98	14V7L	12V7L	65	33
11	42V2L	38V1L	97	19V2L	17V1L	92	5	35V0L	32V1L	99	10V3L	11V4L	75	24
12	52V0L	45V0L	100	20V5L	16V9L	72	28	45V1L	40V1L	98	18V4L	15V7L	75	23
13	32V0L	24V0L	100	14V4L	14V5L	76	24	26V2L	20V3L	90	13V8L	8V4L	64	26
14	23V0L	29V1L	99	15V4L	8V2L	80	19	20V2L	15V0L	96	4V1L	5V2L	76	20
15	49V0L	54V1L	99	8V0L	10V1L	96	13	44V0L	38V1L	99	6V4L	5V3L	62	37

12.266667

16.666667

C1= first control, C2= second control, T1= first test, T2= second test
Av V (%) = average viability (expressed in %), RED = reduction in viability

Table 3.5 Lymphocytes and neutrophils of oesophageal cancer patients exposed to 25 µg/ml FB₁

OESOPHAGEAL CANCER PATIENTS FUMONISIN B1 LYMPHOCYTES														
No.	CONTROL						TEST							
	0 TIME			2 HOUR INCUBATION			0 TIME				2 HOUR INCUBATION			
	C1	C2	Av%V	C1	C2	Av%V	%RED	T1	T2	Av%V	T1	T2	Av%V	%RED
1	47V/1L	47V/1L	98	42V/2L	41V/1L	98	0	28V/2L	44V/0L	97	26V/4L	32V/2L	88	9
2	25V/1L	25V/1L	98	24V/1L	23V/0L	98	0	48V/2L	54V/1L	97	25V/12L	25V/8L	72	25
3	65V/0L	65V/0L	100	44V/0L	42V/0L	100	0	56V/3L	54V/3L	96	35V/18L	34V/12L	72	24
4	47V/0L	47V/0L	100	31V/0L	43V/0L	100	0	36V/0L	35V/0L	100	25V/6L	25V/6L	84	16
5	27V/0L	27V/0L	100	25V/2L	25V/1L	98	2	23V/0L	24V/0L	100	12V/8L	15V/4L	80	20
6	139V/0L	120V/1L	100	130V/3L	127V/5L	97	3	137V/3L	131V/4L	98	70V/14L	74V/12L	85	13
7	77V/2L	74V/1L	98	45V/1L	49V/4L	95	5	58V/2L	44V/1L	98	24V/14L	46V/22L	66	32
8	126V/1L	123V/1L	99	89V/1L	84V/2L	99	0	77V/5L	85V/8L	93	40V/24L	34V/8L	72	21
9	41V/1L	42V/1L	98	47V/1L	36V/1L	98	0	28V/2L	44V/0L	97	26V/4L	32V/3L	89	8
10	29V/1L	52V/1L	98	24V/1L	23V/0L	98	0	48V/2L	54V/1L	97	28V/7L	25V/8L	77	20
11	48V/0L	44V/2L	98	60V/1L	40V/1L	98	0	46V/0L	40V/2L	98	24V/24L	18V/9L	59	39
12	20V/0L	21V/0L	100	15V/0L	12V/0L	100	0	23V/1L	10V/0L	98	21V/5L	24V/3L	84	14
13	42V/0L	48V/0L	100	28V/0L	26V/0L	100	0	43V/0L	48V/0L	100	40V/8L	37V/2L	89	11
14	41V/0L	40V/0L	100	21V/0L	24V/0L	100	0	41V/5L	49V/0L	100	24V/5L	20V/4L	83	17
15	47V/0L	46V/0L	100	31V/0L	43V/0L	100	0	28V/0L	34V/0L	100	20V/6L	21V/4L	81	19

0.5333333

19.2

OESOPHAGEAL CANCER PATIENTS FUMONISIN B1 NEUTROPHILS														
No.	CONTROL						TEST							
	0 TIME			2 HOUR INCUBATION			0 TIME				2 HOUR INCUBATION			
	C1	C2	Av%V	C1	C2	Av%V	%RED	T1	T2	Av%V	T1	T2	Av%V	%RED
1	21V/0L	17V/0L	100	21V/0L	18V/1L	97	3	20V/0L	25V/0L	100	24V/2L	26V/2L	93	7
2	49V/0L	44V/0L	100	19V/0L	18V/0L	100	0	19V/0L	19V/0L	100	12V/0L	15V/2L	94	6
3	35V/0L	34V/0L	100	23V/0L	20V/0L	100	0	31V/2L	34V/2L	94	4V/1L	5V/1L	82	12
4	23V/0L	20V/0L	100	12V/2L	14V/1L	90	10	20V/0L	20V/0L	100	10V/6L	12V/4L	69	31
5	35V/0L	32V/3L	96	18V/2L	22V/1L	93	3	22V/2L	23V/2L	83	15V/7L	11V/4L	71	12
6	144V/1L	140V/1L	99	100V/2L	102V/3L	98	1	98V/2L	90V/2L	98	55V/12L	37V/16L	77	21
7	26V/1L	24V/1L	99	18V/3L	21V/3L	83	16	36V/2L	31V/4L	91	10V/5L	9V/3L	71	20
8	14V/0L	19V/0L	100	16V/1L	16V/1L	94	6	16V/0L	15V/2L	94	16V/3L	10V/4L	77	17
9	21V/0L	17V/0L	100	21V/0L	16V/1L	97	3	20V/1L	18V/1L	95	15V/4L	12V/6L	73	22
10	49V/0L	44V/0L	100	24V/1L	20V/2L	94	6	44V/0L	40V/1L	99	18V/8L	14V/7L	68	31
11	43V/0L	40V/0L	100	35V/2L	30V/2L	95	5	38V/1L	36V/1L	97	14V/8L	8V/5L	63	34
12	22V/0L	20V/1L	98	18V/1L	15V/1L	95	3	20V/2L	15V/1L	93	18V/9L	12V/6L	67	26
13	32V/1L	25V/0L	99	15V/1L	16V/1L	94	5	26V/2L	24V/1L	95	15V/7L	12V/5L	70	25
14	26V/0L	30V/2L	97	24V/0L	23V/2L	96	1	26V/0L	24V/2L	96	13V/8L	8V/4L	65	31
15	48V/0L	44V/2L	98	35V/1L	30V/2L	96	2	48V/2L	46V/1L	97	24V/12L	20V/4L	75	22

4.2666667

21.133333

C1= first control, C2= second control, T1= first test, T2= second test
Av V (%) = average viability (expressed in %), RED = reduction in viability

Table 3.6 Lymphocytes and neutrophils of oesophageal cancer patients exposed to 50 µg/ml OTA.

OESOPHAGEAL CANCER PATIENTS OCHRATOXIN A NEUTROPHILS														
No.	CONTROL						TEST							
	0 TIME			23 HOUR INCUBATION			0 TIME			23 HOUR INCUBATION				
	C1	C2	Av%V	C1	C2	Av%V	%RED	T1	T2	Av%V	T1	T2	Av%V	%RED
1	21V0L	17V0L	100	20V8L	31V0L	85	15	35V0L	31V0L	100	8V2L	11V6L	63	37
2	40V0L	44V0L	100	32V1L	31V0L	99	1	22V0L	19V0L	100	8V1L	5V3L	76	24
3	35V0L	34V0L	100	16V0L	14V0L	100	0	33V1L	35V0L	98	16V9L	15V4L	72	26
4	22V0L	20V0L	100	11V4L	10V0L	80	20	22V0L	18V0L	100	8V6L	12V4L	66	34
5	35V0L	32V1L	99	16V1L	14V2L	91	8	23V2L	23V2L	93	4V1L	8V2L	81	12
6	16V0L	20V0L	100	22V2L	13V1L	93	7	46V1L	45V1L	99	9V3L	15V4L	77	22
7	32V0L	28V0L	100	20V3L	27V4L	87	13	30V2L	30V1L	96	15V8L	10V8L	61	35
8	20V0L	21V0L	100	10V1L	8V2L	86	14	20V2L	13V2L	89	12V8L	11V6L	73	16
9	49V0L	44V0L	100	32V4L	21V0L	95	5	22V0L	19V0L	100	8V1L	5V3L	76	24
10	48V0L	51V0L	100	15V2L	10V1L	90	10	40V0L	20V0L	100	15V10L	4V2L	64	36
11	27V0L	29V1L	98	17V2L	13V1L	91	7	29V1L	24V1L	97	18V9L	9V4L	66	31
12	23V1L	21V0L	98	7V1L	5V0L	94	4	20V1L	18V0L	98	3V1L	5V1L	79	19
13	25V1L	32V0L	98	11V2L	15V1L	90	8	24V1L	29V2L	95	9V2L	6V4L	71	24
14	28V0L	33V1L	98	14V2L	11V1L	90	8	24V2L	26V1L	94	5V4L	7V1L	71	23
15	36V0L	32V0L	100	18V2L	14V1L	89	11	39V1L	29V2L	96	7V4L	5V2L	67	28

8 733333

26 133333

8.733333

26.133333

OESOPHAGEAL CANCER PATIENTS OCHRATOXIN A LYMPHOCYTES														
No.	CONTROL						TEST							
	0 TIME			23 HOUR INCUBATION			0 TIME			23 HOUR INCUBATION				
	C1	C2	Av%V	C1	C2	Av%V	%RED	T1	T2	Av%V	T1	T2	Av%V	%RED
1	20V0L	21V0L	100	10V1L	5V4L	58	42	24V4L	20V4L	87	33V16L	30V10L	71	16
2	25V0L	25V1L	98	24V0L	22V2L	98	0	22V0L	20V5L	75	17V11L	15V11L	66	9
3	55V0L	52V0L	100	45V5L	40V4L	96	4	58V0L	62V0L	100	35V18L	35V18L	60	40
4	47V0L	48V0L	100	38V2L	34V1L	96	4	40V0L	38V0L	100	21V6L	18V5L	78	22
5	27V0L	29V0L	100	17V0L	27V3L	94	6	21V0L	24V0L	100	11V1L	9V0L	96	4
6	126V1L	123V1L	99	40V1L	32V1L	97	2	80V1L	77V2L	98	18V10L	16V7L	67	31
7	26V2L	23V1L	95	28V2L	26V2L	93	2	48V0L	38V1L	98	21V10L	34V21L	65	33
8	49V1L	52V1L	98	17V4L	19V2L	91	7	46V1L	48V1L	98	16V7L	18V7L	70	28
9	55V0L	52V0L	100	15V0L	18V1L	97	3	48V0L	44V1L	98	8V5L	10V5L	65	32
10	52V0L	60V0L	100	20V1L	14V1L	94	6	50V0L	52V2L	98	10V6L	5V3L	63	35
11	21V0L	17V0L	100	24V4L	20V4L	84	16	35V0L	31V0L	100	8V7L	5V4L	55	45
12	42V0L	48V1L	99	31V4L	23V2L	91	8	48V0L	46V0L	100	11V7L	15V11L	60	40
13	50V0L	48V0L	100	29V0L	30V0L	100	0	37V0L	44V0L	100	19V4L	15V3L	83	17
14	44V0L	41V0L	100	13V0L	20V0L	100	0	45V0L	46V0L	100	3V3L	3V1L	63	37
15	35V1L	33V0L	99	10V0L	7V1L	94	5	36V0L	30V1L	99	10V5L	6V4L	64	35

7

28.333333

C1= first control, C2= second control, T1= first test, T2= second test
Av V (%) = average viability (expressed in %), RED = reduction in viability

Table 3.7 Lymphocytes and neutrophils of breast patients exposed to 25 µg/ml FB₁

OTHER CANCER PATIENTS														
FUMONISIN B1 LYMPHOCYTES														
No.	CONTROL					TEST								
	0 TIME			2 HOUR INCUBATION			0 TIME			2 HOUR INCUBATION				
	C1	C2	Av%V	C1	C2	Av%V	%RED	T1	T2	Av%V	T1	T2	Av%V	%RED
1	41V0L	45W0L	100	40V0L	38V0L	100	10	41V0L	44V0L	100	31V0L	22V2L	96	4
2	127V0L	118V0L	100	69V20L	60V20L	76	24	62V0L	67V0L	100	34V10L	40V12L	77	23
3	69V0L	71V0L	100	46V6L	43V4L	90	10	57V3L	54V1L	89	38V10L	33V18L	72	17
4	52V0L	48V0L	100	40V4L	38V2L	93	7	49V11L	50V10L	83	31V17L	22V19L	59	24
5	34V0L	36V1L	93	23V5L	20V2L	85	12	21V8L	34V7L	77	28V26L	28V28L	49	18
6	40V1L	55V2L	97	42V2L	45V0L	91	8	42V3L	44V1L	96	31V4L	27V7L	83	23
7	62V1L	25V1L	97	54V4L	64V4L	94	3	70V3L	67V1L	98	40V18L	39V18L	69	29
8	27V0L	18V0L	100	15V3L	10V5L	75	25	26V3L	23V1L	93	10V7L	8V5L	61	32
9	41V1L	59V1L	98	45V1L	55V2L	93	7	44V2L	52V1L	96	20V6L	22V10L	73	23
10	34V1L	36V0L	99	25V2L	23V3L	91	8	32V3L	30V3L	91	18V10L	16V8L	66	25
11	69V2L	71V1L	98	38V5L	43V4L	90	8	60V4L	61V5L	93	28V10L	22V15L	69	24
12	54V0L	60V1L	99	31V4L	35V7L	86	13	45V2L	49V4L	94	44V10L	26V13L	74	20
13	74V2L	75V2L	97	35V5L	39V4L	90	7	70V5L	63V7L	92	31V14L	24V12L	68	24
14	53V2L	49V1L	97	44V4L	28V7L	87	10	48V7L	40V5L	88	27V9L	18V10L	70	18
15	49V0L	38V0L	100	28V0L	29V3L	95	5	36V5L	31V2L	91	23V10L	20V9L	70	21
9														
21 656667														

OTHER CANCER FUMONISIN B1 NEUTROPHILS															
No.	CONTROL							TEST							
	0 TIME			2 HOUR INCUBATION				0 TIME			2 HOUR INCUBATION				
	C1	C2	Av%V	C1	C2	Av%V	%RED	T1	T2	Av%V	T1	T2	Av%V	%RED	
1	38V0L	34V0L	100	30V0L	21V0L	100	0	21V0L	23V0L	100	31V3L	27V4L	89	11	
2	68V0L	66V0L	100	54V3L	56V4L	93	7	64V0L	54V0L	100	45V16L	40V14L	74	26	
3	50V0L	55V0L	100	40V0L	48V0L	100	0	42V6L	46V0L	94	26V8L	27V0L	72	22	
4	34V0L	36V1L	98	30V0L	21V3L	94	4	21V8L	34V7L	96	21V4L	20V9L	80	16	
5	52V0L	48V4L	92	6V2L	6V3L	71	21	49V11L	50V10L	82	2V2L	2V1L	42	40	
6	77V2L	81V3L	97	70V5L	80V6L	93	4	71V4L	78V5L	95	30V12L	40V16L	71	24	
7	12V1L	12V1L	92	3V2L	7V1L	74	18	11V1L	11V1L	92	4V3L	8V5L	60	32	
8	68V0L	66V0L	100	54V4L	56V2L	94	6	70V0L	74V2L	99	34V16L	31V14L	69	30	
9	38V0L	34V0L	100	30V1L	21V1L	96	4	21V0L	23V0L	100	27V9L	18V12L	68	32	
10	52V0L	48V2L	98	36V2L	33V4L	92	6	50V2L	45V5L	93	25V15L	29V17L	63	30	
11	69V1L	54V1L	99	35V4L	40V3L	93	7	40V3L	50V4L	94	24V12L	20V12L	65	29	
12	36V0L	34V0L	100	19V4L	25V4L	85	15	35V2L	35V3L	94	17V9L	13V10L	61	33	
13	83V2L	75V1L	99	45V4L	49V6L	91	8	71V4L	65V3L	93	31V4L	36V18L	68	27	
14	33V0L	54V1L	100	21V4L	32V3L	88	12	33V0L	37V2L	98	24V15L	18V12L	61	37	
15	28V0L	30V0L	100	33V2L	23V5L	88	12	26V2L	24V1L	95	8V3L	7V4L	69	26	

C1= first control, C2= second control, T1= first test, T2= second test
Av V (%) = average viability (expressed in %), RED = reduction in viability

Table 3.8 Lymphocytes and neutrophils of breast cancer patients exposed to
50 µg/ml OTA

OTHER CANCER PATIENTS OCHRATOXIN A LYMPHOCYTES													
No.	CONTROL						TEST						
	0 TIME			23 HOUR INCUBATION			0 TIME			23 HOUR INCUBATION			
	C1	C2	Av%V	C1	C2	Av%V	%RED	T1	T2	Av%V	T1	T2	%RED
1	41V0L	45V0L	100	28V0L	6V6L	91	9	45V0L	40V0L	100	21V5L	24V3L	85
2	100V0L	98V0L	100	75V10L	69V0L	88	12	81V0L	90V0L	100	44V20L	46V24L	67
3	69V0L	71V0L	100	40V6L	42V2L	91	9	50V2L	48V2L	98	22V20L	18V10L	58
4	52V0L	48V4L	96	36V2L	34V0L	96	0	50V2L	48V6L	92	26V2L	28V4L	90
5	34V0L	36V1L	95	10V1L	9V1L	90	5	32V2L	30V4L	91	6V2L	8V1L	82
6	87V2L	96V3L	98	60V15L	50V10L	81	17	85V4L	98V5L	95	25V15L	22V13L	63
7	27V0L	18V0L	100	20V0L	16V6L	87	13	29V0L	18V0L	100	10V2L	8V3L	78
8	41V0L	45V0L	100	45V1L	40V2L	97	3	36V0L	40V1L	99	15V5L	21V8L	74
9	34V1L	36V2L	96	27V1L	22V3L	92	4	35V1L	30V1L	97	16V13L	12V10L	55
10	69V0L	71V0L	100	54V5L	56V5L	92	8	55V2L	56V1L	97	22V22L	21V18L	52
11	48V1L	53V1L	99	29V3L	26V2L	92	7	44V0L	46V0L	100	18V10L	20V9L	67
12	39V0L	48V0L	100	21V2L	20V4L	87	13	36V2L	40V4L	93	20V9L	18V3L	78
13	53V2L	55V0L	98	26V3L	30V2L	92	6	50V3L	45V2L	95	19V2L	24V6L	85
14	63V1L	61V0L	99	30V1L	28V3L	94	5	59V1L	60V4L	96	20V10L	19V4L	75
15	44V0L	38V0L	100	18V3L	24V4L	86	14	44V0L	36V0L	100	21V4L	24V3L	87
							8.333333						

OTHER CANCER PATIENTS OCHRATOXIN A NEUTROPHILS													
No.	CONTROL						TEST						
	0 TIME			23 HOUR INCUBATION			0 TIME			23 HOUR INCUBATION			
	C1	C2	Av%V	C1	C2	Av%V	%RED	T1	T2	Av%V	T1	T2	%RED
1	38V0L	34V0L	100	14V0L	12V0L	100	0	31V0L	38V0L	100	13V2L	10V2L	85
2	68V0L	66V0L	100	61V9L	50V6L	88	12	36V2L	60V8L	92	48V25L	40V31L	61
3	50V0L	34V0L	100	28V4L	30V2L	90	10	40V0L	38V2L	90	30V8L	32V12L	76
4	34V0L	36V1L	98	28V2L	28V6L	88	10	20V6L	28V3L	84	18V6L	20V4L	79
5	52V0L	48V4L	92	5V0L	8V2L	90	2	34V6L	31V6L	82	4V2L	4V2L	50
6	66V0L	66V0L	100	62V8L	55V3L	92	8	64V9L	60V4L	91	34V20L	32V18L	64
7	38V0L	34V0L	100	20V0L	19V1L	98	2	34V1L	32V0L	99	28V14L	23V12L	66
8	52V0L	48V4L	96	44V2L	33V2L	95	1	44V0L	42V1L	99	30V10L	31V14L	72
9	44V0L	24V0L	100	39V2L	26V1L	96	4	33V6L	31V5L	86	15V7L	18V10L	66
10	36V0L	33V0L	100	22V2L	29V5L	89	11	32V2L	30V0L	97	20V8L	16V4L	76
11	50V1L	54V0L	99	20V7L	22V4L	80	19	48V1L	44V2L	97	23V5L	19V4L	83
12	54V0L	34V1L	99	25V3L	24V6L	85	14	50V2L	31V3L	93	33V16L	30V11L	70
13	47V0L	43V0L	100	29V5L	26V4L	86	14	38V2L	40V3L	94	24V7L	17V5L	77
14	35V1L	31V0L	99	16V2L	18V1L	92	7	29V0L	29V1L	99	15V4L	10V9L	66
15	29V0L	39V0L	100	17V4L	15V5L	78	22	25V2L	23V0L	97	18V4L	20V5L	81
							9.066667						

C1= first control, C2= second control, T1= first test, T2= second test
Av V (%) = average viability (expressed in %), RED = reduction in viability

APPENDIX F

STANDARD CURVES FOR CYTOKINES, IL-1, IL-6, IL-8, IL-10 AND TNF- α

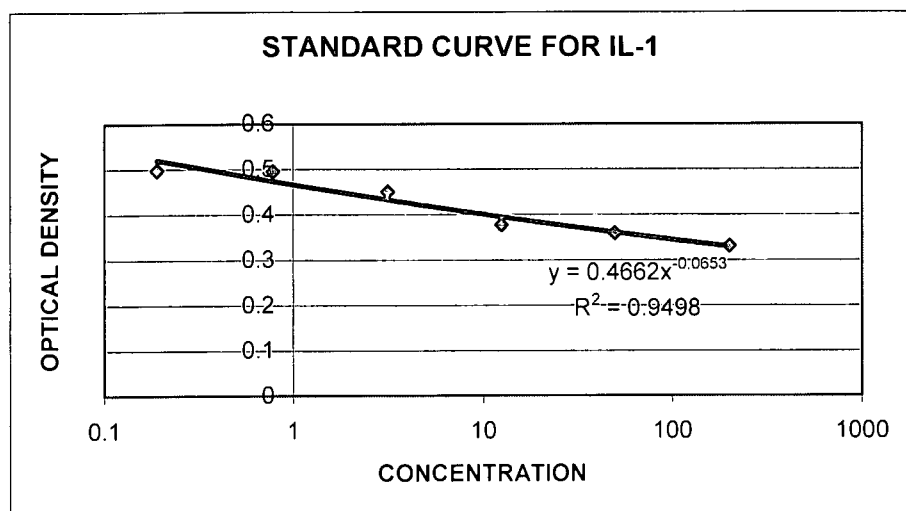


Fig 3.41 Standard curve of optical density vs concentration for IL-1, showing as concentration of IL-1 increases, the optical density decreases.

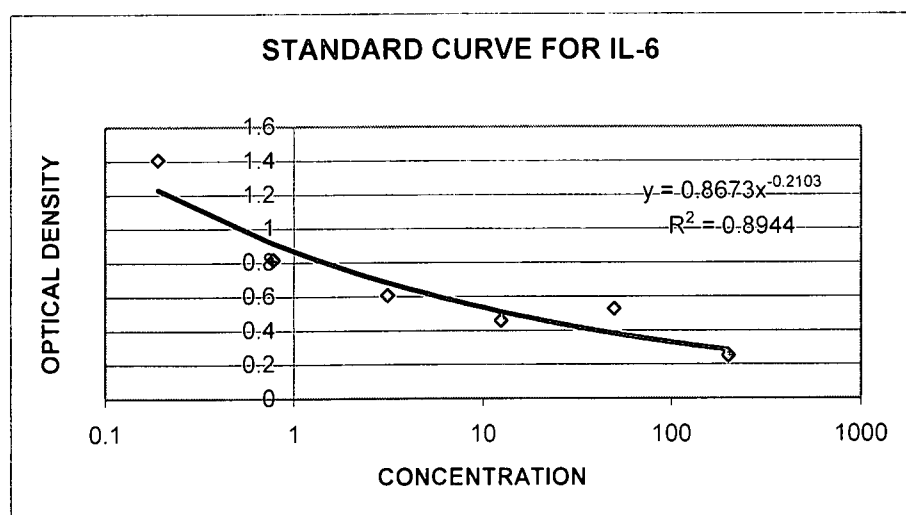


Fig 3.42 Standard curve of optical density vs concentration for IL-6, showing as concentration of IL-6 increases, the optical density decreases.

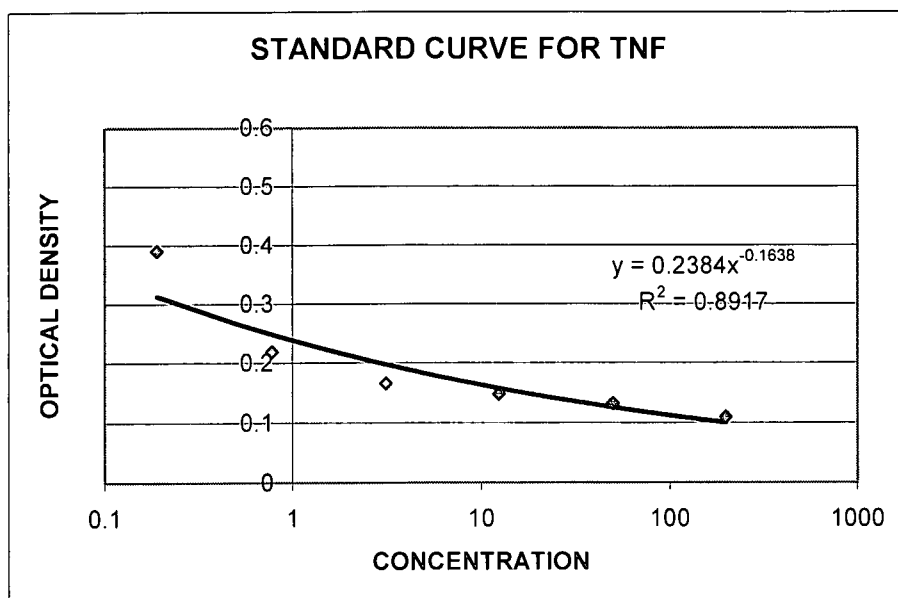


Fig 3.43 Standard curve of optical density vs concentration for TNF-α, showing as concentration of TNF-α increases, the optical density decreases.

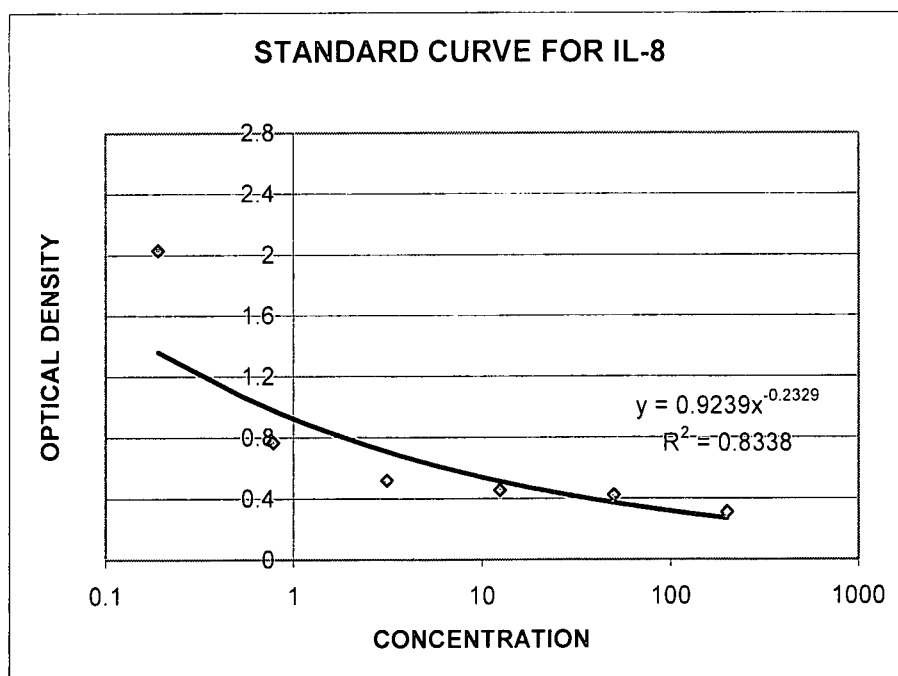


Fig 3.44 Standard curve of optical density vs concentration for IL-8, showing as concentration of IL-8 increases, the optical density decreases.

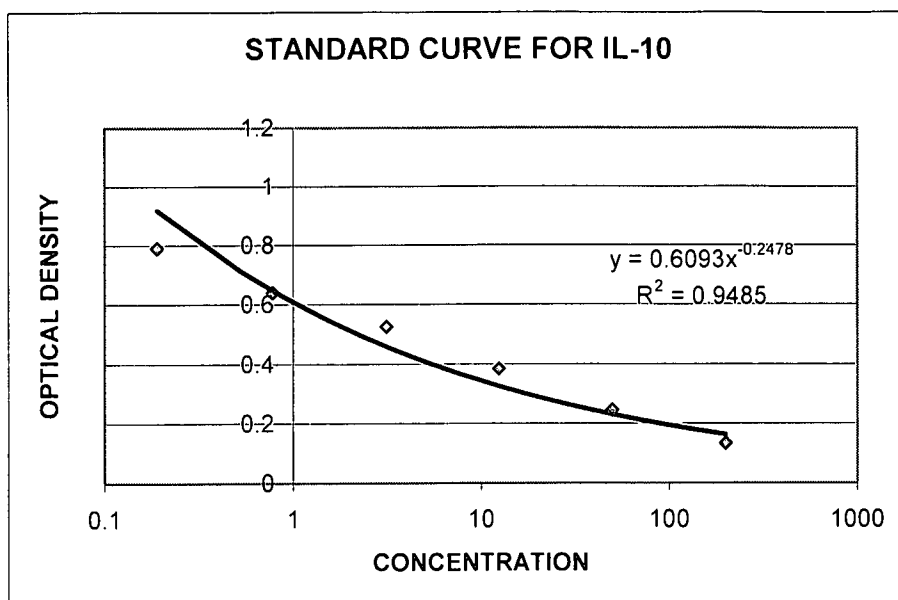


Fig 3.45 Standard curve of optical density vs concentration for IL-10, showing as concentration of IL-10 increases, the optical density decreases.