THE CYTOTOXIC EFFECTS OF AFLATOXIN B_1 AND FUMONISIN B_1 ON CULTURED HUMAN CELLS

By

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ABSTRACT

Aflatoxin B_1 (AFB_1) and Fumonisin B_1 (FB_1), potentially cytotoxic and carcinogenic mycotoxins are common contaminants of agricultural commodities in South Africa and thus could be detrimental to the human immune system. Many of the cytotoxic effects of AFB_1 require its bioactivation to an epoxide, which will bind covalently to macromolecules to form protein and DNA adducts. Fumonisin B_1 is a competitive inhibitor of sphingosine and sphinganine *N* aceyltransferase, which are key components in the pathways for sphingolipid biosynthesis. Accumulation of free sphingoid bases, which are both cytotoxic and mitogenic, could provide a plausible explanation for the toxicity and carcinogenicity of FB_1. The cytotoxic effects of AFB_1 and FB_1 on normal human lymphocytes, individually and in combination were assessed using the methylthiazol tetrazolium (MTT) bioassay. Two different methods of treatment were used, the treatment of isolated normal human lymphocytes for 12, 24, 48, 72 and 96 hours and whole blood treated for 12 hours. Flow cytometry and fluorescent microscopy were used to determine whether AFB_1 and FB_1 (5μM and 50μM), individually or in combination, were capable of inducing apoptosis, necrosis or nuclear fragmentation in isolated lymphocytes and whole blood treated for 12 hours. DNA damage was evaluated using the comet assay.

The results showed that AFB_1 routinely induced higher levels of cytotoxicity in isolated lymphocytes than FB_1. In the combination treatment, the mitogenic properties of FB_1 appeared to partially counteract the cytotoxic effect exerted by AFB_1. When whole blood was treated with the same concentration and ratio of toxin, FB_1 was shown to be more cytotoxic than AFB_1. The combination treatment of whole blood was shown to be cytotoxic in a dose dependant manner. The toxins appeared to exert a greater cytotoxic effect, when treated in combination than individually at higher concentrations. Aflatoxin B_1 induced increased levels of apoptosis and necrosis in isolated lymphocytes while treatment with the FB_1 resulted in increased levels of apoptosis at both concentrations. Treatment with the combination also resulted in increased levels of apoptosis. The levels of apoptosis were reduced in whole blood lymphocytes when compared to isolated lymphocytes. However, treatment with AFB_1 and FB_1 resulted in increased levels of apoptosis. Both AFB_1 and FB_1 are capable of inducing nuclear fragmentation. Treatment with FB_1 (5μM and 50μM) resulted in greater degree of fragmentation than AFB_1. The most nuclear fragmentation was induced by the 5μM combination treatment. The 50μM combination treatment of isolated lymphocytes induced the most DNA damage. As both toxins are common contaminants and have been known to coexist, this could be a potential area of concern for public health.

AUTHOR'S DECLARATION

The experimental work presented in this thesis represents the original work by the author and has not been submitted in any form to any other University. Where use was made of work of others, it was duly acknowledged in the text.

The research described in this study was carried out under the supervision of Ms. R. Myburg in the Department of Physiology, Nelson R. Mandela School of Medicine, Durban, during the period of March 2002 to December 2003.

M.E van der Stok

Miss ME van der Stok

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AFB_1:	Aflatoxin B_1
ALS:	Alkali liable sites
APAF:	Apoptotic protease activating factor
BAX:	Bcl-2 associated protein
BCL-2:	B cell lymphoma/leukemia gene
CCM:	Complete culture medium
CD:	Cell domain
CO_2:	Carbon dioxide
CTL:	Cytotoxic T lymphocyte
DFF:	DNA fragmention factor
DISC:	Death initiating signalling complex
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DSB:	Double stranded DNA breaks
EDTA:	Ethidium diamine tetra acetate
ELEM:	Equine leukoencephalomalacia
FADD:	Fas associated death domain
FB_1:	Fumonisin B_1
FCS:	Foetal calf serum
FLICE:	Fas activated protein like ICE
GST:	Glutiathione-S-transferase
HBSS:	Hank's balanced salt solution
HCC:	Hepatocellular carcinoma
HET-1A:	Human oesphageal epithelial cells
HL cells:	Helen Langer cells
IARC:	International agency for research on cancer
ICE:	Interleukin-1β converting enzyme
IL:	Interleukin
LMPA:	Low melting point agarose
MDCK:	Modin Darby canine kidney cells
MTT salt:	Methylthiazol tetrazolium salt
Na_2EDTA:	Disodium ethidium diamine tetra acetate

NaCL:	Sodium chloride
NaOH:	Sodium hydroxide
PBS:	Phosphate buffered saline
PI:	Propidium iodide
PS:	Phosphatidyl serine
RPMI:	Rosewell Park Memorial Institute
SCGE:	Single cell gel electrophoresis
SK:	Swine kidney cells
SSB:	Single stranded breaks
TCR:	T cell receptor
TNF:	Tumour necrosis factor
UDS:	Unscheduled DNA synthesis
USA:	United States of America

CHAPTER 1

Introduction

Mycotoxins have been defined as low molecular weight secondary metabolites of certain filamentous fungi, which when ingested, inhaled, or absorbed through the skin cause lowered performance, sickness or death in man and animals (Pitt, 1996). Currently there are over 300 mycotoxins that have been reported. There is also ample evidence that the inhabitants of sub-Saharan Africa are experiencing heavy dietary exposure to food-borne mycotoxins, particularly aflatoxins and fumonisins (Bankole and Adebanjo, 2003).

Parts of South Africa have a tropical climate with all year round high ambient temperature and relative humidity that provide optimal condition for the growth of toxigenic fungi. The sub-region also has poorly developed infrastructures such as processing facilities, storage, transportation and skilled human resources. According to the World Development Report (1993), diseases caused by mycotoxins lead to reduced life expectancy in developing countries. In several parts of Africa, the need to eat outweighs other considerations such as food safety and as such, this has made food-borne intoxications a serious problem in many parts (Miller, 1996).

According to Miller (1996), 40% of the productivity lost to diseases in developing countries is due to diseases exacerbated by aflatoxins. Regrettably, many of the people in the affected regions are not even aware of the effect of consuming fungal contaminated products. Due to the poor education levels and other socio-economic factors, even if steps are taken to make food products safe, the consumers will be unwilling to pay the extra costs and will still prefer to buy the cheaper commodities (Bankole and Adebanjo, 2003).

Mycotoxins have attracted worldwide attention due to the significant losses associated with their impact on human and animal health and consequent economic implications. Losses from rejected shipments and lower prices for inferior quality can devastate developing country export markets. Costs to farmers include reduced income from outright food or feed losses and lower selling prices for contaminated commodities. The economic impact on livestock production includes mortality as well as reductions in productivity, gain, feed efficiency, fertility and ability to resist disease (Ramesh and Vasanthi, 2003).

The toll of the effects on human health includes the cost of mortality and the cost of morbidity. There is the cost of productive capacity lost when people die prematurely. There are losses resulting from hospitalisation and the cost of health care services, both public and private. Finally, there is the intangible cost of pain, suffering, anxiety and reduction of the quality of life (Ramesh and Vasanthi, 2003).

Thus the united efforts of researchers from a broad research field, with culminated ideas from disciplines like molecular biology, biochemistry, physiology, microbiology, botany and basic science, are crucial for a more coherent picture of these mycotoxins and their mode of actions in human and animal systems.

1.1 Objectives

Two mycotoxins were selected for this study, Aflatoxin B_1 (AFB_1) and Fumonisin B_1 (FB_1); due to their wide spread contamination of agricultural commodities in South Africa and subsequently could prove to be detrimental to the human immune system. The objectives of this study were to determine:

- The cytotoxicity of AFB_1 and FB_1, individually and in combination, on normal human lymphocytes
- The genotoxicity of AFB_1 and FB_1
- The apoptotic inducing ability of AFB_1 and FB_1, individually or in combination.

CHAPTER 2

Literature review

Man's knowledge of the more unpleasant microbial activities in his environment has often stemmed from the occurrence of such natural disasters as epidemics of bacterial, fungal, protozoal or viral diseases (Austwick, 1975).

2.1 Introduction

Throughout history it has been shown conclusively that several species of bacteria, protozoa and viruses are extremely hazardous to man. In recent years, fungi have attracted considerable interest from scientists as they produce toxic secondary metabolites, known as mycotoxins, during growth on substrates destined as food for man or animals. When ingested, inhaled or absorbed through the skin, mycotoxins cause lowered performance, sickness or death in man and animals, including birds (Pitt, 1996).

In 1989, it was estimated that 25% of the world's annual crops were affected by mycotoxins. This equates to a direct cost of billions of rands due to loss of crops and animals. In addition, the hidden indirect costs of monitoring the level of mycotoxins in crops as well as the decreased performance of farm animals that ingest contaminated food containing mycotoxins must be taken into account (Trail, 1995).

Many species of fungi from a wide range of taxa are known to be toxigenic. Current research, has been focussed on the genera *Aspergillus*, *Penicillium* and *Fusarium* because each contains several toxigenic species of importance in many parts of the world (Moss and Smith, 1985).

2.2 Aflatoxins

2.2.1 Production of Aflatoxins

The most significant species of the genus *Aspergillus* are *Aspergillus flavus* and *Aspergillus parasiticus*, which are primarily responsible for the production of aflatoxins. Aflatoxins were first identified as aetiological agents for animals in the 1960s, when ducklings, calves and approximately 100 000 turkeys died from ingesting contaminated peanut meal. Microscopical examination of the deadly meal revealed indisputable evidence that prior to or during harvesting and processing it had been contaminated by a fungus. The fungus was then isolated and identified as a highly toxic strain of *A. flavus,* along with its alcohol-extractable toxins that became known as aflatoxins (Asplin and Carnaghan, 1961).

The fungi belonging to the genus *Aspergilli,* are ubiquitous and grow on a variety of agricultural products. When growing under conditions of temperature (27°C) and a high relative humidity (85%), which normally prevail in tropical climates, almost any food commodity can be contaminated with aflatoxins. Aflatoxins mainly contaminate moist grain and nut products, but have also been found routinely in maize and cottonseed meal. They have also been found in cocoa, fishmeal, milk, cheese, rice, coconut, cassava, peas, cottonseed, corn, potatoes, peanuts, pistachios, wheat, cured hams, sausages and soybeans (Bennett and Christensen, 1983).

Aspergillus species are known as storage fungi because they invade stored food products, but the fungus can also invade pre-harvest crops. Conditions for toxin production are much narrower than those for growth. In general, production of aflatoxins is affected by a variety of parameters including dissemination of the fungus, predisposing stresses on the host plant, cultivation techniques, insect damage, plant variety, weather (moisture/drought) and the type and duration of the storage conditions of the fruit or vegetable (Sinz and Shier, 1991).

Many substances can affect both fungal growth and aflatoxin production when present in the growth medium. Most of these compounds inhibit toxin production by inhibiting fungal growth, but a limited number of substances do exert their effects directly on aflatoxin production. For example organophosphates inhibit aflatoxin production by blocking certain steps in the biosynthetic pathway. The nitrogen source can also be an important factor in toxin production (Ellis *et al.*, 1991).

2.2.2 Structure of Aflatoxins

Aflatoxins are derived from acetate-malonate condensation and are therefore known as acetogenins or polyketide derivatives. The four major naturally occurring aflatoxins are known as aflatoxin B_1, B_2, G_1 and G_2 (Figure 2.1). The blue and green fluorescent colours produced by these compounds under ultraviolet light illumination are referred to as 'B' and 'G' respectively, while the subscript numbers 1 and 2 are used to indicate the relative position of the compounds on the thin layer chromatography plates (Sinz and Shier, 1991).

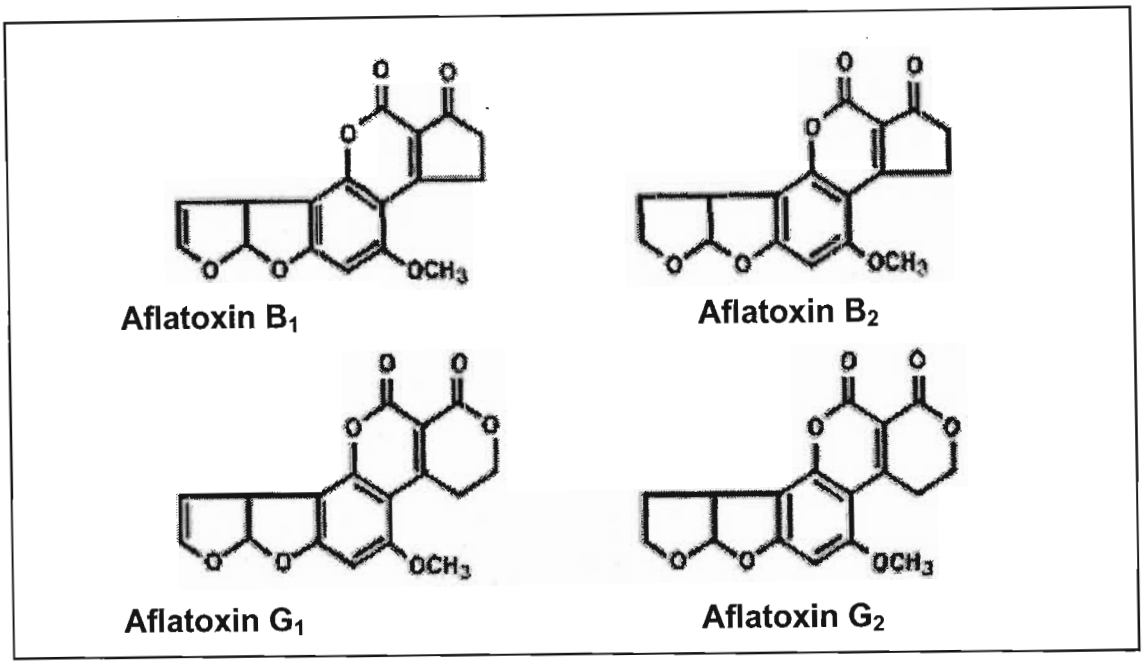

Figure 2.1: Molecular structure of Aflatoxins B_1, B_2, G_1, G_2 (Prescott *et al.*, 1996).

Aflatoxins contain a coumarin nucleus fused to a bisdihydrofurano moiety present in a cis fashion and to either a five membered lactone for aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2) or a six membered lactone for aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2). Aflatoxin B_2 and AFG_2 are the 15, 16-dihydro derivatives of AFB_1 and AFG_1 respectively. Other derivatives of AFB_1 and AFB_2 include aflatoxin M_1 and aflatoxin M_2, which are found in milk products from several species including cattle, as well as mouldy peanuts and corn. The relative proportions of the four major aflatoxins produced by *Aspergillus* cultures vary with both the genetic constitution of the fungi and with the environmental parameters associated with fungal growth (Sinz and Shier, 1991). Aflatoxin B_1 is the major metabolite produced by aflatoxigenic strains. *Aspergillus flavus* isolates usually produce only B aflatoxins and less than 50% of isolates are toxigenic, while *A. parasiticus* isolates produce both G and B aflatoxins and are invariably toxigenic (Bennett and Christensen, 1983).

2.2.3 Bioactivation of Aflatoxin B_1

Aflatoxin B_1 is the most prevalent, potently cytotoxic and carcinogenic of the aflatoxins. Of the toxic actions associated with AFB_1 exposure, the most serious are mutagenicity and carcinogenicity, which have been linked to metabolic activation of the molecule. Garner *et al.* (1972) were the first to show that metabolic activation of the AFB_1 is necessary for mutagenic activity. The mutagenicity and carcinogenicity of AFB_1 are believed to result from the activation of AFB_1 to the AFB_1-8, 9-epoxide, the ultimate mutagen/carcinogen (Figure 2.2) (Essigmann *et al.*, 1982). The 8-9 epoxide has not been isolated from biological systems due to its reactivity.

It has been demonstrated categorically that AFB_1 itself is not mutagenic, nor does it bind covalently to macromolecules such as DNA in the absence of a bioactivation system (Hecht and Trushin, 1988). Since bioactivation of AFB_1 is required for carcinogenicity, much attention has focussed on the biotransformation of this naturally occurring mycotoxin. However the major DNA adduct formed *in vitro* and *in vivo* in the presence of an activation system is 8, 9-dihydro-8-(N^7-gaunyl)-9-hydroxy AFB_1 (AFB_1 gaunine) (Essigmann *et al.*, 1982; Harrison and Garner, 1991; Ball *et al.*, 1990). Thus, independent of the species or tissue under investigation, activation of AFB_1 to the 8,9 epoxide is an absolute requirement for AFB_1 to manifest its mutagenic, carcinogenic and DNA-binding actions.

The initial metabolism of AFB_1 can involve four types of reactions: O-dealkylation, hydroxylation, epoxidation or ketoreduction (Figure 2.2). While the reduction of AFB_1 to aflatoxicol is believed to be catalysed by a cytosolic NADPH-dependent reductase, the other reactions are primarily carried out by a cytochrome P450 (P450) dependent polysubstrate monooxygenase enzyme superfamily. However other activation systems can play a role (Massey *et al.*, 1995).

Cytochrome P450 is the principal enzyme system involved in the oxidative biotransformation of AFB_1. This conclusion is based on the following observations:

1. Molecular oxygen and NADPH are required for metabolism *in vitro*.
2. The extent of microsomal activation is altered following treatment with P450 inducers (e.g. phenobarbital).
3. Inhibitors of P450 e.g. cobalt chloride, carbon monoxide inhibit microsomal activation.
4. Susceptibility of animals to AFB_1-induced hepatocarcinogenesis is affected by treatment with P450 inducers and inhibitors.

5. AFB_1 is activated in systems containing purified P450, NADPH cytochrome P450 reductase, NADPH and phospholipids.
6. Antibodies against P450 isoform inhibit activation catalysed by microsomes or purified P450.
7. Systems which express the cDNA of P450 isoforms are capable of AFB_1 activation

(Hecht and Trushin, 1988; Busby and Wogan, 1984; Essigmann *et al.*, 1982; Shimada and Guengerich, 1989; Aoyama *et al.*, 1990; Crespi *et al.*, 1991).

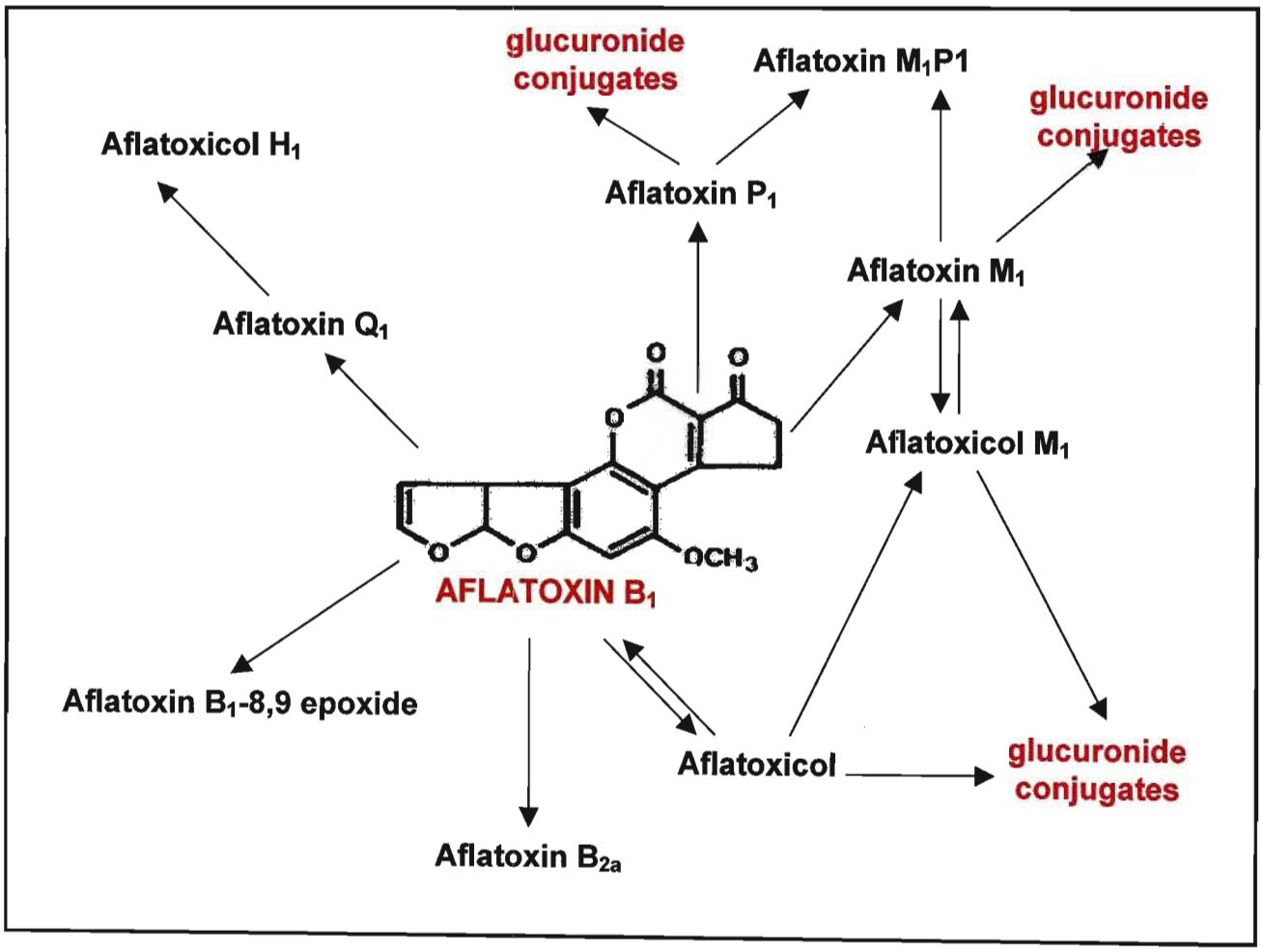

Figure 2.2: Biotransformation pathways for Aflatoxin B_1 (Eaton and Groopman, 1994).

In addition to the recognised pathways of aflatoxin bioactivation, metabolic detoxification of aflatoxins and their relative metabolites have also been demonstrated. An important detoxification mechanism involves the conjugation of the reactive epoxides with glutathione (GSH) (Figure 2.3). The conjugation of GSH is an important reaction in determining the susceptibility of different species to the toxic effects of AFB_1 (Hayes *et al.*, 1991).

As shown schematically in Figure 2.3, the fate of AFB_1 is dependent on the relative activity of several biotransformation pathways, in addition to other factors such as DNA repair rates. The amount of the mycotoxin that is going to exert carcinogenic or toxic effects will depend on the amount converted to various metabolites, as well as on the biological activity of those metabolites (Eaton and Groopman, 1994).

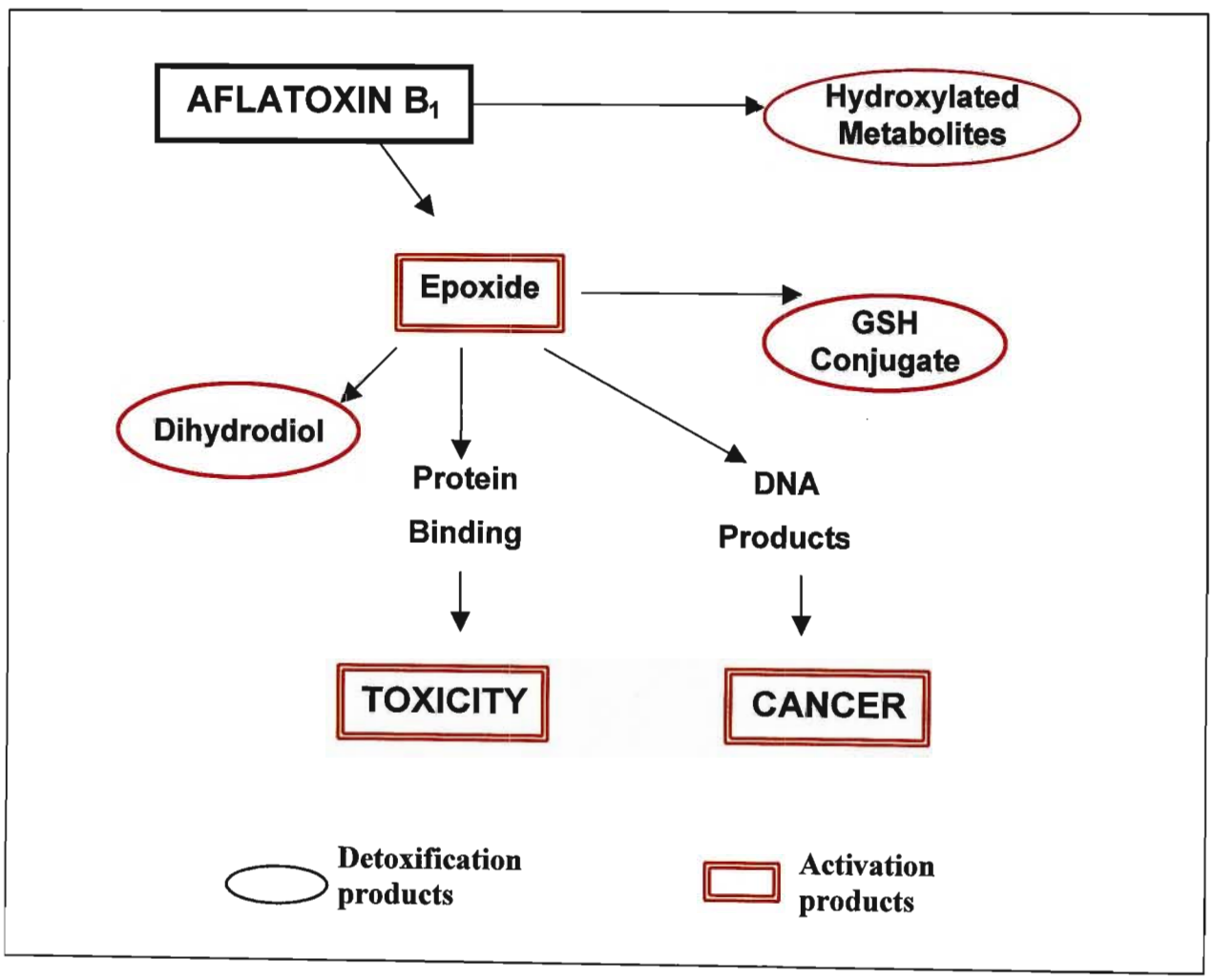

Figure 2.3: Schematic representation of the role of various biotransformation pathways in the disposition, toxicity and carcinogenecity of Aflatoxin B_1 (Eaton and Groopman, 1994).

2.2.4 Toxic effects of Aflatoxin B_1

The role of aflatoxins as human carcinogens has been under investigation ever since the realisation that liver cancer incidence is high in regions with high endemic aflatoxin concentrations and the extreme potency of these mycotoxins as carcinogens was established in laboratories. It is estimated that 250 000 deaths occur annually in certain parts of China and sub-Saharan Africa due to hepatocellular carcinoma (HCC). The major factors that contribute to this high rate of HCC are aflatoxin ingestion and hepatitis B virus infection (Massey *et al.*, 1995). Aflatoxins are carcinogenic, as expressed by the International Agency for Research on Cancer (IARC), which concluded in 1987 that there is sufficient evidence for the carcinogenicity of aflatoxins to humans (International Agency for Research on Cancer (IARC), 1987).

The liver is the primary target in many different animal species (hepato-carcinogenic) (Ellis *et al.*, 1991). Of the aflatoxins AFB_1 is the most carcinogenic, the most frequently occurring and the most potent hepatocarcinogen to rats and mice (Massey *et al.*, 1995). Mixtures of aflatoxins and AFB_1 have been tested extensively for carcinogenicity by various routes of administration in several species. Species tested include various strains of mice (Dix, 1984), rats (Hertzog *et al.*, 1980), hamsters (Moore *et al.*, 1982), several strains of fish (Curtis *et al.*, 1995), ducks (Cova *et al.*, 1990), tree shrews (Li *et al.*, 1999) and monkeys (Adamson *et al.*, 1976).

Following their oral administration, mixtures of aflatoxins and AFB_1 caused hepatocellular and/or cholangiocellular liver tumours, including carcinomas, in all species tested except mice (Ellis *et al.*, 1991). In rats, renal-cell tumours and a low incidence of tumours at other sites, including the colon, were also found. In monkeys, liver angiosarcomas, osteogenic sarcomas and adenocarcinomas of the gall bladder and pancreas developed, in addition to hepatocellular and cholangiocellular carcinomas (Adamson *et al.*, 1976). In adult mice, AFB_1 administered intraperitoneally increased the incidence of lung adenomas. Intraperitoneal administration of AFB_1 to infant mice, adult rats and toads produced high incidences of liver-cell tumours in all of these species (Jennings *et al.*, 1994). Subcutaneous injection of AFB_1 resulted in local sarcomas in rats. Intraperitoneal administration of AFB_1 to rats during pregnancy and lactation induced benign and malignant tumours in mothers and their progeny in the liver and in various other organs, including those of the digestive tract, the urogenital system and the central and peripheral nervous systems (Raisudden *et al.*, 1990). Exposure of fish embryos to AFB_1 induced a high incidence of hepatocellular adenomas and carcinomas (Curtis *et al.*, 1995).

In several species, AFB_1 administered by different routes induced foci of altered hepatocytes, the number and size of which was correlated with later development of hepatocellular adenomas and carcinomas. Aflatoxins in general and AFB_1 in particular are known to cause cancer in different animal species as shown in Table 2.1 (Ellis *et al.*, 1991).

Table 2.1: Carcinogenicity of Aflatoxin B_1, depicting the dosage, period of oral exposure and tumour frequency (Ellis *et al.*, 1991).

	Dose per kg body weight	Period of exposure	Tumour frequency (%)
Duck	30µg	14 months	8/11 (72%)
Trout	8µg	1 year	27/65 (40%)
Monkey	100 – 800mg	2 years	3/42 (7%)
Rat	100µg	54 – 88 weeks	28/28 (100%)
Mice	150µg	80 weeks	0/60 (0%)

In addition to being present in foodstuffs, AFB_1 can contaminate respirable grain dust and thus the respiratory system is a potential target for carcinogenesis (Daniels and Massey, 1992). To study the potential effects of AFB_1 in the upper airways, the metabolism of AFB_1 was investigated in tracheal cultures and purified tracheal cultures and purified tracheal microsomes from rabbit, hamster and rat. Ball and Coulombe (1991) concluded that the rabbit upper airway epithelium contains metabolic activity primarily involved in AFB_1 bioactivation, whereas AFB_1 detoxification pathways predominate in the hamster.

The mutagenic properties of different aflatoxins were assessed using a modification of the Ames test with *Salmonella typhimurium*. It was found that AFM_1 and AFB_1 had specific mutagenic activites of 13 and 121 revertants/ng respectively (Bujons *et al.*, 1995). The cytotoxic properties of compounds can be assessed using the MTT-cell culture assay. Aflatoxin B_1 was used in the MTT test using different cell types. In swine kidney (SK) cells, Madin-Darby canine kidney (MDCK) cells and Henrietta Lacks (HeLa) cells, AFB_1 was found to be cytotoxic at the following levels: 50µg/ml to SK cells, >200µg/ml to MDCK cells and 25µg/ml to Hela cells (Hanelt *et al.*, 1994).

2.2.5 Aflatoxin and the Immune Response

Aflatoxins, besides adversely affecting several vital processes, possess an immunosuppressive property. Prolonged immunosuppression renders animals and humans extremely susceptible to infection and even to cancer (Ray *et al.*, 1987a; Ray *et al.*, 1987b; Raisudden *et al.*, 1990). Aflatoxins have previously demonstrated their immunotoxic effects in birds (Giambrone *et al.*, 1981); pigs (Panagala *et al.*, 1986); horses (Angsubhakaran *et al,*, 1981); mice (Galikeev *et al.*, 1968) and rats (Ellis *et al.*, 1991). Some of the immunological responses evoked by the aflatoxins are summarised in Table 2.2. It has been observed that birds and swine are the most sensitive species for aflatoxin immunotoxicity (Giambrone *et al.*, 1981; Panagala *et al.*, 1986). Both cellular and humoral immune responses are affected by aflatoxin treatment. However it is believed that cellular immunity is more severely hampered than the humoral one (Giambrone *et al.*, 1981). It has also been shown that aflatoxin exposure results in decreased resistance against pathogenic organisms such as bacteria, fungi and viruses (Hamilton *et al.*, 1971; Pier *et al.*, 1970; Pier *et al.*, 1972).

Table 2.2: Effects of Aflatoxin B_1 on Immune functions.

Parameter	Species	Dose	Effect	
Cell mediated immunity	Chicken Pig	2.5 ppm 300 500	Impaired Impaired	Giambrone *et al.*, 1981 Panagala *et al.*, 1986
Humoral immunity	Chicken Pig	2.5 ppm 300 500	Impaired Impaired	Giambrone *et al.*, 1981 Panagala *et al.*, 1986
Complement activity	Chicken Pig	0.625-10ppm 300 500ppm	Impaired Impaired	Hamilton *et al.*, 1971 Panagala *et al.*, 1986
Resistance against pathogens	Chicken Pig	0.625-10ppm 300 500ppm	Reduced Reduced	Hamilton *et al.*, 1971 Panagala *et al.*, 1986
Lymphocyte population	Pig Horse	300 500ppm not detected	Depleted Depleted	Panagala *et al.*, 1986 Angsubhakaran *et al,*, 1981
Antibody production	Chicken Mouse	2.5ppm 30-700ppm	Reduced Reduced IgG, IgM	Giambrone *et al.*, 1981 Reddy *et al.*, 1983
Lymphocyte transformation	Mouse Cow Human	30-700ppm 0.25 µg/ml 0.5-2.0 µg/ml	Reduced Reduced Reduced	Reddy *et al.*, 1983 Bodine *et al.*, 1984 Aleksandrowicz *et al.*, 1974
Phagocytosis	Chicken Rat	0.625-10 ppm 70-700µg/ kg bw	Reduced Reduced	Chang *et al.*, 1979 Raisudden *et al.*, 1990
Tumour challenge	Mouse	70 -700µg/ kg bw	Enhanced mortality	Raisudden *et al.*, 1990
Vaccination	Turkey	0.25-0.5 ppm	Enhanced mortality	Pier *et al.*, 1970; Pier *et al.*,1972

Further detailed studies on the immunological responses of AFB_1 at cellular and subcellular levels have been undertaken. One of the ways that the immunotoxic effects of a compound can be determined is by investigating the effect of the compound on the production of Interleukin (IL-)-2 and IL-5. The influence of AFB_1 was measured by incubating the thymona cell line with various concentrations. It was shown that AFB_1 did not affect IL-2 or IL-5 production at concentrations up to 10μg/ml (Marin *et al.*, 1996). The respiratory tract cilia represent one of the most important biological barriers between human organs and the environment. It has been shown by Jesenska and Bernat (1994) that AFB_1 inhibits movement after 6 days at a concentration of 30μg/l. Aflatoxin B_2 and AFM_1 were almost as effective as AFB_1, while much less ciliostatically effective were AFG_1 and AFG_2.

In humans AFB_1, AFB_2, AFG_1, AFG_2 have proven to have significant mitogenic effects on human T_4 lymphocytes, while no adverse reactions were seen upon administration of doses of 10, 50, 100μg on a modest clinical basis to volunteers (Griffiths *et al.*, 1996). Aflatoxin B_1 induced inhibition of phagocytosis of Kuppfer cells at concentrations as low as 0.01 pg/ml. The metabolites AFQ_1 and AFM_1 have similar effects on phagocytosis, but were less potent than AFB_1 (Cusumano *et al.*, 1995). When rat bone marrow cells were exposed to AFB_1 at a concentration of 1mg/kg body weight, approximately 1/5 of the LD_{50} for young male rats, granulopoietic toxicity was observed. There were also changes in the production of humoral regulatory factors involved in the granulopoietic development pathway. The granulocyte-monocyte colony forming units were transiently suppressed by AFB_1, the peak in granulopoietic activity was preceded by an increased colony stimulating activity and interleukin formation and elevated interleukin synthesis and increased T-cell activation paralleled the peak in granulopoietic activity (Cukrova *et al.*, 1992).

2.3 Fumonisins

2.3.1 Production of Fumonisins

Fumonisins are a well-known group of mycotoxins produced by *Fusarium verticillioides* and to a lesser extent by *F. proliferatum*, *F. anthophilum* and *F. subglutinans*. Several other *Fusarium* species also produce fumonisins (Gelderblom *et al.*, 1988). *Fusarium moniliforme* (now known as *F. verticillioides*) is a non-obligate pathogen which is not host specific. Thus, the fungus has been sometimes found in sorghum, wheat, cotton, beans, tomatoes, peanuts, bananas, soybean, green peppers and several forages. However, almost 90% of contamination by the fungus is found in maize (MacKenzie *et al.*, 1998). The life cycle of the fungus, which is found in maize, is composed of a saprophytic stage and a parasitic stage, as shown in Figure 2.4.

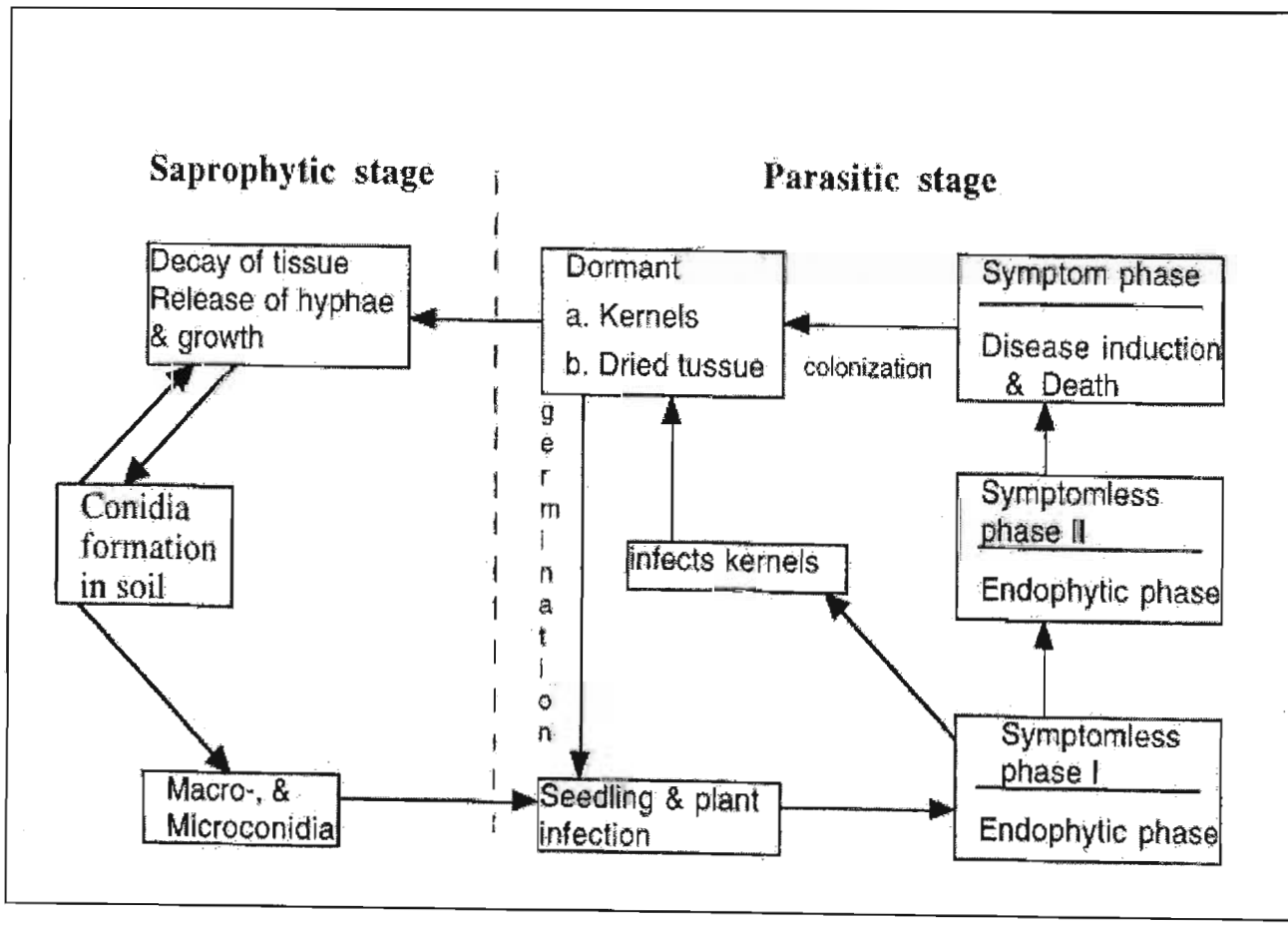

Figure 2.4: Life cycle of *Fusarium verticillioides* on corn (Fumonisin Home page).

During the saprophytic stage, *F. verticilliodes* can obtain nutrients from non-living tissues such as dried tissues, thereby producing infective structures for disease establishment. On the other hand, during the parasitic stage, the fungus mostly gets nutrients from living host cells after intracellular colonisation. Usually, most non-obligate pathogens kill their host cells in advance of infection and then obtain nutrients from the non-living tissues. Disease symptoms and death of maize plants, which may not be common causes of the parasitic stage, are responsible for most of the economic losses on corn. Several major diseases causing the economic losses are attributed to *F. verticilliodes* inducing seedling blight, stalk rot, ear rot (Figure 2.5), root rot and seed rot. The highest amounts of fumonisins are occurring in disease tissues as evidenced by the high concentrations occurring in maize screenings, which often include the shattered maize by the diseases (Moss and Smith, 1985).

Figure 2.5: Photograph of Ear Rot (Fusarium Ear Rot).

2.3.2. Occurrence of Fumonisin B_1

Fumonisins, unlike other mycotoxins are not restricted to certain countries. They have been found in all continents of the world, thus indicating that their prevalence is not climate dependent (Table 2.3). Egypt and the European countries showed high levels of the toxin in healthy and plain maize (Sydenham *et al.*, 1991; Doko *et al.*, 1995). Blue and yellow maize meal showed highest levels of fumonisins in the USA. Puffed maize had a very high level of the toxin in Italy (Doko and Visconti, 1994). The Far East countries had high levels of fumonisin in maize kernels (Ueno *et al.*, 1993). Studies of maize from the Transkei (Sydenham *et al.*, 1990) where Equine leukoencephalomalacia was found to occur showed the highest levels of the toxin in plain maize. In Sardinia ear rot maize showed an extremely high level of the toxin (Bottalico *et al.*, 1995).

Table 2.3: Occurrence of Fumonisin B_1 in foods and feeds.

Commodity	Country	Average Conc. µg/kg (ppb)	Reference
Maize			
Healthy	Egypt	2 380	Sydenham *et al.*, 1991
	Transkei	1 530	Rheeder *et al.*, 1992;
		1 600	Sydenham *et al.*, 1990
	USA	1 048	Sydenham *et al.*, 1991
Flour	China	100	Ueno *et al.*, 1993
Plain	European country	3 500	Doko *et al.*, 1995
	Zambia	1 710	Doko *et al.*, 1995
	Italy	2 250	Logrieco *et al.*, 1995
	Transkei	1 990	Rheeder *et al.*, 1992
	South Africa	+ tve	Dutton *et al.*, 1994
Meal (blue)	USA	8 484	Pestka *et al.*, 1994
Meal (yellow)	USA	5 824	Pestka *et al.*, 1994
	USA	1 050	Sydenham *et al*, 1990
	Peru	660	Sydenham *et al.*, 1991
	South Africa	138	Sydenham *et al.*, 1991
Rice	Japan	500	Ueno *et al.*, 1993
Tortilla/popcorn	USA	1410	Hopmans *et al.*, 1993
Puffed maize	Italy	61 000	Doko and Visconti, 1994
Kernels	Mozambique		
	Botswana	20	Doko *et al.*, 1995
	Malawi		
	China	6 800	Ueno *et al.*, 1993
	USA		
	South Africa	1600	Ueno *et al.*, 1993
	Argentina		
Pre-harvest	Sardinia	250 000	Bottalico *et al.*, 1995
Seeds	France	5 000	Logrieco *et al.*, 1995

2.3.3 Structure of Fumonisin B_1

Fumonisins are group of toxins characterised by an aminopolyol eciosane backbone and two tricarballylic acid esters (Musser *et al.*, 1996). To date six fumonisins have been identified and purified, namely Fumonisin A_1, A_2 and Fumonisin B_1, B_2, B_3, B_4. Of these the most important fumonisin with regard to health risks is FB_1 (Cawood *et al.*, 1991). In 1988, Gelderblom *et al* first isolated FB_1 and FB_2 from cultures of *F. moniliforme* MRC 826 from South Africa. Buizenhout *et al.*, (1988) later elucidated the structures of FB_1 and FB_2. Fumonisin B_1 is the diester of propane-1,2,3-tricarboxylic acid and 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyicosane, in which the C_{14} and C_{15} hydroxyl groups are esterified with one of the terminal carboxyl groups of the tricarboxylic acid (Figure 2.6). The presence of a free amino group and four free carboxyl groups means the fumonisins are strongly polar and water soluble. They are also soluble in methanol and acetonitrile/water but are not soluble in non-polar solvents (Scott and Lawrence, 1992).

The fumonisins can be heat stable but temperatures greater than 150°C for 1 hr may reduce fumonisin levels. Fumonisin B_1 is stable during most types of processing. Dry milling of maize results in the distribution of FB_1 into the bran, germ and flour. Fumonisin B_1 is stable in polenta (maize porridge). However, the concentration of FB_1 is reduced during the manufacture of cornstarch by wet milling, since FB_1 is water-soluble. A number of factors make it difficult to extract FB_1 from processed food (Bullerman and Tsai, 1994; Scott and Lawrence, 1995; Norred *et al.*, 1998 ;). Nixtamalization [calcium hydroxide processing], is a traditional processing step used to manufacture tortilla flour. This process followed by ammoniation lead to hydrolysed FB_1 and aminopentol respectively. These treatments reduce the fumonisin content, while increasing the concentration of hydrolysed fumonisins, which are almost as toxic as unmodified fumonisins (Norred *et al.*, 1992; Kuiper Goodman *et al.*, 1995). During ethanol fermentations, fumonisins tend to be concentrated in the distilled dried grain, which is used for animal feed, but are not found in the ethanol. The formation of Schiff's bases between primary amines of FBs and reducing sugars (glucose or fructose) allows the fumonisins to transform into non-harmful non-enzymatic browning products. The similarity of FB_1 to sphingosine (Figure 2.6) has led to the hypothesis that the toxicity of fumonisins is mediated by impaired sphingosine metabolism (Wang *et al.*, 1991).

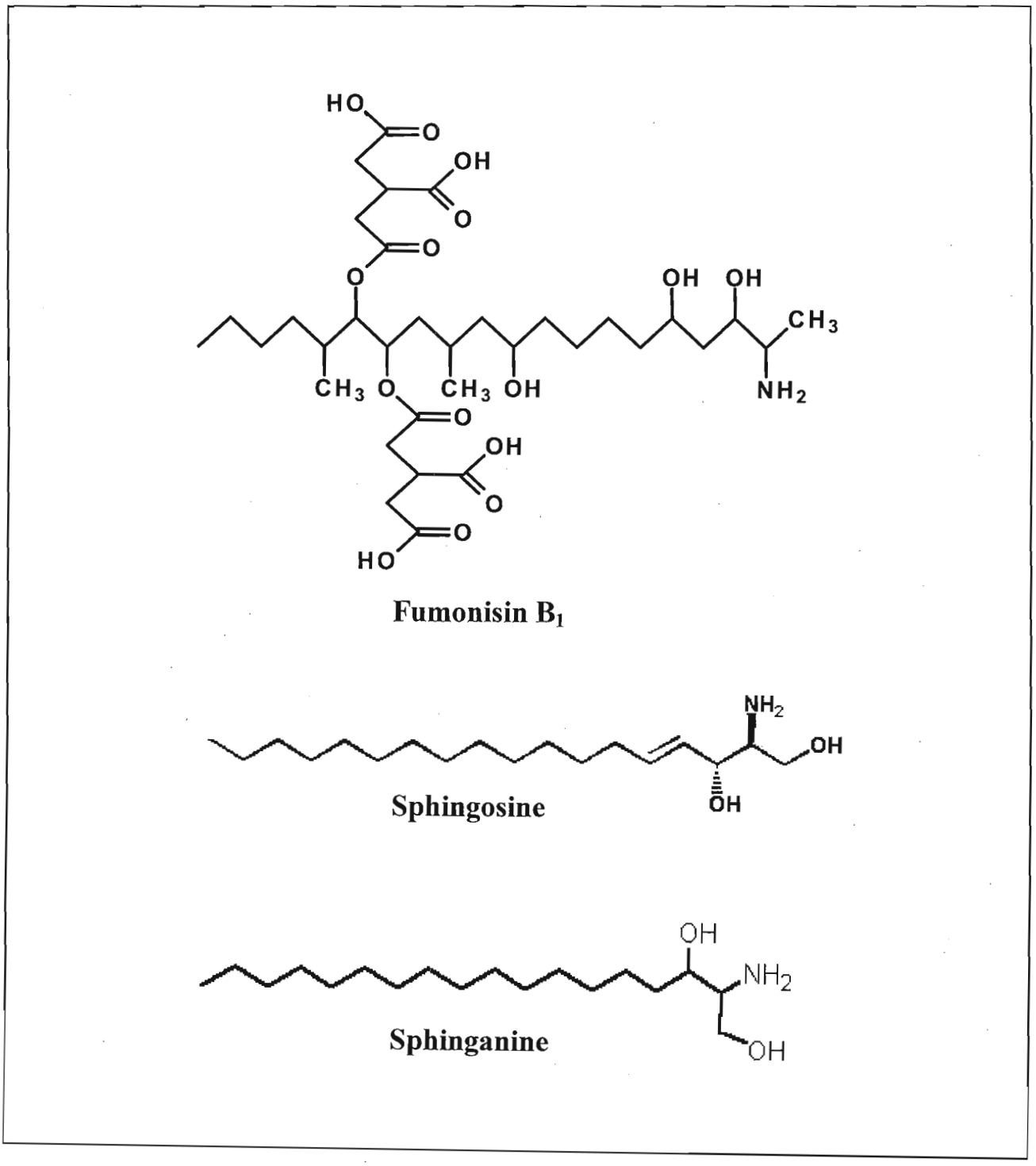

Figure 2.6: Structure of Fumonisin B_1, Sphingosine and Sphinganine (Wang *et al.*, 1999).

2.3.4 Mechanism of Action of Fumonisin B_1

Fumonisins structurally resemble some sphingoid bases such as sphinganine, an intermediate in the biosynthesis of complex sphingolipids. As a result fumonisins act as inhibitors of ceramide synthetase (N-Acyltransferase), a key enzyme involved in *de novo* sphingolipid biosynthesis and in the reacylation of free sphingoid bases derived from sphingolipid turnover (Di Bartolomo *et al.*, 2000). . The site of inhibition by FB_1 is shown in Figure 2.7

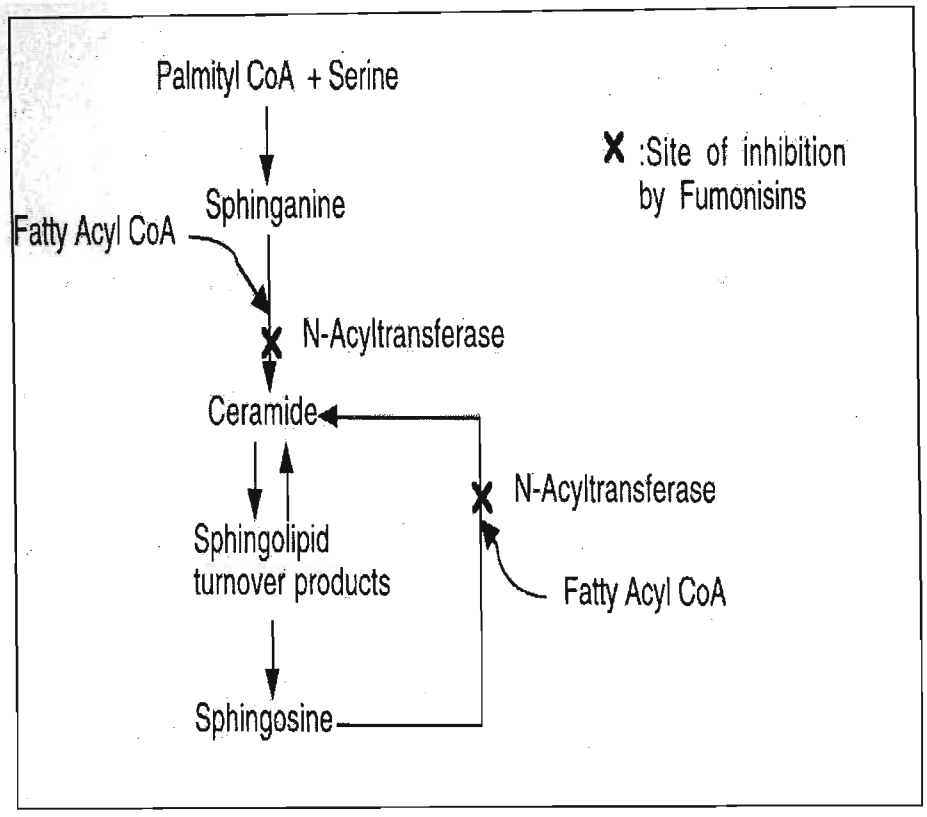

Figure 2.7: Sphingolipid pathway (Wang *et al.*, 1999).

The inhibitory effect exerted by FB_1 leads to accumulation of free sphinganine and sphingosine in the cells. These highly bioactive molecules play key roles in protein kinase activity, cell growth and differentiation, carcinogenicity and programmed cell death. This results in disturbances in cellular processes such as cell growth, cell differentiation, endothelial cell permeability and apoptosis. Inhibition of biosynthesis of sphingolipids is seen at different levels of the process and is reflected in changes of the ratio sphinganine/ sphingosine (Wang *et al.*, 1999). In addition, several studies have suggested the involvement of lipid peroxidation or an increase in oxygen transport in FB_1 cytotoxicity, as well as the activation of some well known apoptotic pathways (Sakakura *et al.*, 1996; Sweeney *et al.*, 1996; Schmelz *et al.*, 1998).

2.3.5 Toxic effects of Fumonisin B_1

Fumonisin B_1 is poorly absorbed but rapidly distributed and eliminated in many animal species including laying hens, swine, cows, rats, mice and non-human primates (Prelusky *et al.*, 1994). However a small but persistent pool of FB_1 or its metabolites appears to be retained in the liver and kidney. It has been shown that FB_1 is not transferred through the placenta, or into the milk in several animal species (Collins *et al.*, 1998b).

Fumonisin B_1 has a low acute oral toxicity in several animal species (Eriksen and Alexander, 1998). In addition to the studies with rodents, pigs and horses, there have been several subacute toxicity studies performed with animal species such as poultry, rabbits, hamsters, non-human primates, lambs and cattle. Many of the studies used contaminated feed rather than pure FB_1 (Kuiper Goodman 1995). The major target organs are the liver and kidney in most animals but particularly in the rat and mouse. For the rat, whether the kidney or liver is the most sensitive organ, depends on the strain or gender (Gelderblom *et al.*, 1996).

Although most animals' species show at certain dose levels nephrotoxic and hepatotoxic effects, the most relevant effect of FB_1 in pigs is the porcine pulmonary oedema syndrome, which is characterised by dyspnoea, weakness, cyanosis and death (Gumprecht *et al.*, 1998). In horses and ponies, FB_1 has been implicated in a fatal neurotoxic syndrome known as equine leukoencephalomalacia (ELEM) (Kellerman *et al.*, 1990). Equine leukoencephalomalacia is characterised by the presence of liquefactive necrotic lesions in the white matter of the cerebrum. The first symptoms of ELEM are lethargy and head pressing, followed by the convulsions, ataxia and death after several days (Kellerman *et al.*, 1990).

Epidemiological studies performed in South Africa and China revealed that there might be a correlation between the intake of fumonisin B_1 and increased oesophageal cancer in humans (Mackenzie *et al.*, 1997). However similar studies performed in Italy did not establish any correlation between the intake of FB_1 and the oesophageal cancer incidence (Pascale *et al.*, 1995). As a result in 1993, the IARC evaluated FB_1 and classified it in Group 2B: "possible carcinogenic to humans." It was concluded that there was inadequate evidence in humans for carcinogenicity. The available studies are inconclusive and no quantitative data enabling a risk assessment on human data is possible (IARC, 1993).

On the basis of negative genotoxicity data from experiments covering several endpoints including the Ames test and *in vitro* and *in vivo* UDS assays (Norred *et al.*, 1992) and positive results in a non-validated type of bacteria test (Sun and Stahr, 1993). A very limited *in vitro* study was undertaken, in which chromosomal aberration and micronucleus tests were tested (Knasmüller *et al.*, 1997), the overall conclusion is that there is no adequate evidence that FB_1 is genotoxic.

2.3.6 Fumonisin B_1 and the Immune System

A study has shown reduced thymus weight, thymus necrosis and elevated immunoglobin M (IgM) in rats after intraperitoneal administration of 7.5mg/ FB_1/kg body weight for 4 days (Eriksen and Alexander, 1998).

Tryphonas *et al.* (1997) studied the effects of FB_1 in the immune system of Sprague-Dawly rats. Groups of rats (15/sex/group) were gavaged daily for 14 days with doses of 0, 5, 15 and 25 mg/ FB_1/kg bw and the primarily IgM response to sheep expressed as plaque- forming cell number/ 10^6 spleen monuclear leukocytes, PFC/10^6 splenocytes and PFC/ spleen was determined. There was a significant dose related linear trend toward decreased PFC/ 10^6 spenocytes and pFC/ spleen in the male rats. Body weights were significantly reduced in male rats administered 15 and 25mg FB_1/kg bw.

There was a significant dose-related increase in *Listeria monocytogenes* number in spleen at 24 hours post infection also indicating decreased immune function. There was a weakly significant dose-related increase in immunoglobulin class G_1 (IgG_1). There was no effect seen on organ weights, haematology, mitogen-induced leukocyte transformation, calcium mobilisation, killer cell activity and phagocytosis.

Martinova *et al.* (1998) showed that both sphingomyelin cycle products and FB_1 affect the T lymphocyte surface antigen expression, disrupt the balance between different subpopulations of lymphocytes, inhibit DNA synthesis in normal lymphocytes and suppress an immune response to T-dependent antigens *in vivo*.

2.4 The Immune Response

All vertebrates, including humans are continuously exposed to products such as microorganisms, their own metabolic products, or other foreign macromolecules that can cause disease. Immunity refers to the particular ability of the host to resist a particular disease. The immune response that results is a specific and complex series of defensive actions widely distributed throughout the animal's body (Figure 2.8). Each particular immune response is a unique local sequence of events, shaped by the nature of the challenge (Prescott *et al.*, 1996).

Non-specific resistance refers to those general mechanisms inherited as part of the innate structure and function of each animal. These act in concert as the first line of defence against infectious microorganisms, their products or foreign macromolecules before they cause disease. The various non-specific defence mechanisms include biological barriers (inflammation, phagocytosis), chemical barriers (enzymatic action, interferons) and general barriers, such as fever and physical barriers (skin, mucous membranes) (Reeves and Todd, 1996).

If non-specific defences are breached, specific immunity (the immune response) is called upon to protect the host. This system consists of several immunological mechanisms in which lymphocytes recognise the presence of particular foreign agents termed antigens and act to eliminate them. Elimination can occur by direct lymphocyte destruction of the antigens, or by formation of specialised proteins called antibodies that either disrupt antigen function or target the antigen for destruction by other cells (Reeves and Todd, 1996).

Acquired immunity refers to the type of specific immunity a host develops after exposure to a suitable antigen, or after transfer of antibodies or lymphocytes from an immune donor. Acquired immunity can be obtained actively or passively by natural or artificial means. Naturally acquired active immunity occurs when an individual's immune system contacts an antigenic stimulus such as an infection during normal activities. The immune system responds by producing antibodies and sensitised lymphocytes that inactivate or destroy the antigen. The immunity produced can be either lifelong, as with measles or last for only a few years, such as tetanus. Naturally acquired passive immunity involves the transfer of antibodies form one host to the other. For example, some of a pregnant woman's antibodies pass across the placenta to her foetus. Unfortunately naturally acquired immunity only lasts a short time (Reeves and Todd, 1996).

Artificially acquired active immunity results when an animal is immunised with a vaccine. A vaccine consists of a preparation of killed microorganism, living, weak (attenuated), microorganisms or inactivated bacterial toxins that are administered to an animal to induce immunity artificially. Artificially acquired passive immunity results when antibodies that have been produced either in an animal or by specific methods *in vitro* are introduced into a host. Although the type of immunity is immediate, it is short lived. An example of this type of immunity includes botulism antitoxin produced in a horse and given to a human suffering from botulism food poisoning (Reeves and Todd, 1996).

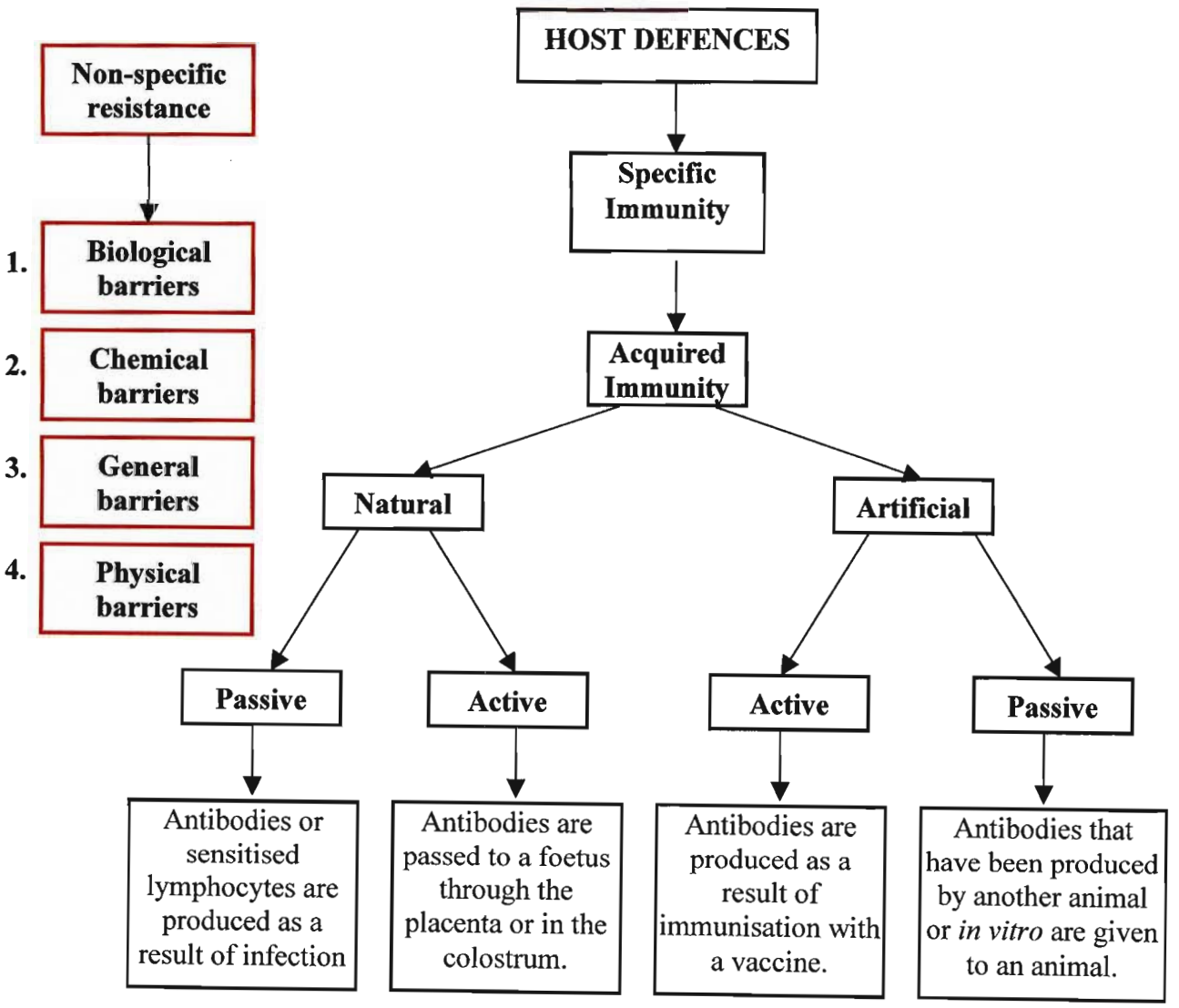

Figure 2.8: Schematic diagram of the different Host defences (Prescott *et al.*, 1996).

2.5 Lymphocytes

2.5.1 Origin of Lymphocytes

Lymphocytes are small, non-phagocytic, mononuclear leukocytes that are immunologically competent, or are precursors of such cells. They lack stainable cytoplasmic granules and are formed in lymphatic tissue. Undifferentiated lymphocytes are derived from bone marrow stem cells. They are produced in the bone marrow at a very high rate, approximately 10^9 cells per day. Some lymphocytes migrate through the circulatory or lymphatic systems to the secondary lymphoid tissue (thymus, spleen, aggregated lymph nodules in the intestines and lymph nodes) where they produce lymphocyte colonies (Boyd *et al.*, 1993).

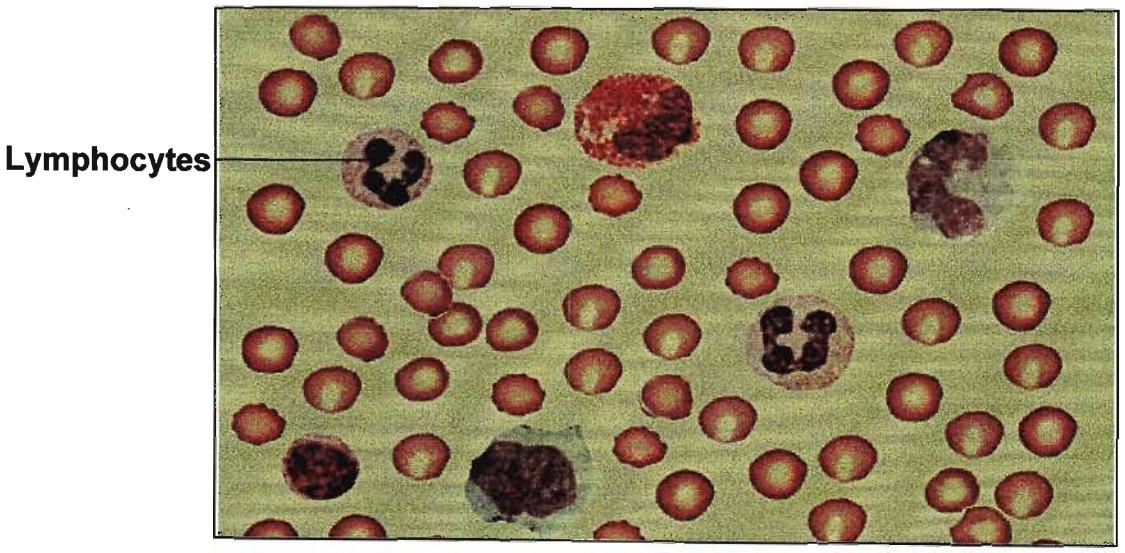

Figure 2.9: Picture of whole blood (University of Leicester, 1996).

The undifferentiated lymphocytes that migrate to the thymus undergo special processing and become T cells or T lymphocytes, the letter T representing the thymus. Some T cells are transported away from the thymus and enter the bloodstream where they comprise 70% to 80% of the circulating lymphocytes. This thymus dependent differentiation of T cells occurs during early childhood and by adolescence the secondary lymphoid organs of the body generally contain a full complement of T cells (Boyd *et al.*, 1993).

B cells or B-lymphocytes differentiate in the foetal liver and adult bone marrow. B cells are distributed by the blood and make up 20 to 30% of the circulating lymphocytes. They also settle in the various lymphoid organs along with the T cells. Table 2.4 summarises the various classes of lymphocytes (Boyd *et al.*, 1993).

There is a population of lymphoid cells that do not have characteristics of either T or B cells. These are called null cells because they lack the specific surface markers of B cells and T cells and can be distinguished from them by the presence of cytoplasmic granules. It is believed that this population of cells contains the most natural killer cells antibody-dependent cytotoxic T cells. Although these cells are probably of bone marrow origin, their exact lineage is uncertain (Boyd *et al.*, 1993).

Table 2.4: Classes of lymphocytes (Boyd *et al.*, 1993).

Lymphocyte	Role
T Cells	
T_H (helper) cells, also called CD4 cells	Provide "assistance", or potential expression of immune function by other lymphocytes
T_S (suppressor) cells, also called CD8 cells	Suppress or impair expression of immune function by other lymphocytes
T_C (cytotoxic) cells, also called CD8 cells	Brings about cytolysis and cell death of "targets"
B cells	
B lymphocytes	Proliferate and mature into antibody-producing cells
Plasma cells	Are mature active antibody-producing cells
Null cells	
Natural killer cells	Bring about cytolysis and death of target cells

2.5.2 Function of Lymphocytes

Plasma cells are fully differentiated antibody-synthesising cells that are derived into from B-lymphocytes. They respond to antigens by secreting antibodies into the blood and lymph tissue. Antibodies are glycoproteins produced by plasma cells after the B cells in their lineage have been exposed to antigens. Antibodies are specifically directed against the antigen that caused their formation. Since antibodies are soluble in blood and lymph fluid they provide humoral (Latin *humor*, a liquid) immunity or antibody-mediated immunity (Roitt *et al.*, 1993). The humoral immune response defends mostly against bacteria, bacterial toxins and viruses that enter the body's various fluid systems (Figure 2.10).

T cells do not secrete antibodies. Instead they attack host cells that have been parasitised by viruses or micro-organisms, tissue cells that has been transplanted from one host to another and cancer cells. They also produce cytokines, chemical mediators that play specific augmenting and regulatory roles in the immune system. Since T cells must physically contact foreign cells or infected cells in order to destroy them, they are said to provide cell-mediated immunity. Null cells destroy tumour cells and virus- and other parasite infected cells. They also help regulate the immune response. Null cells often exhibit antibody dependent cellular cytotoxicity (Roitt *et al.*, 1993).

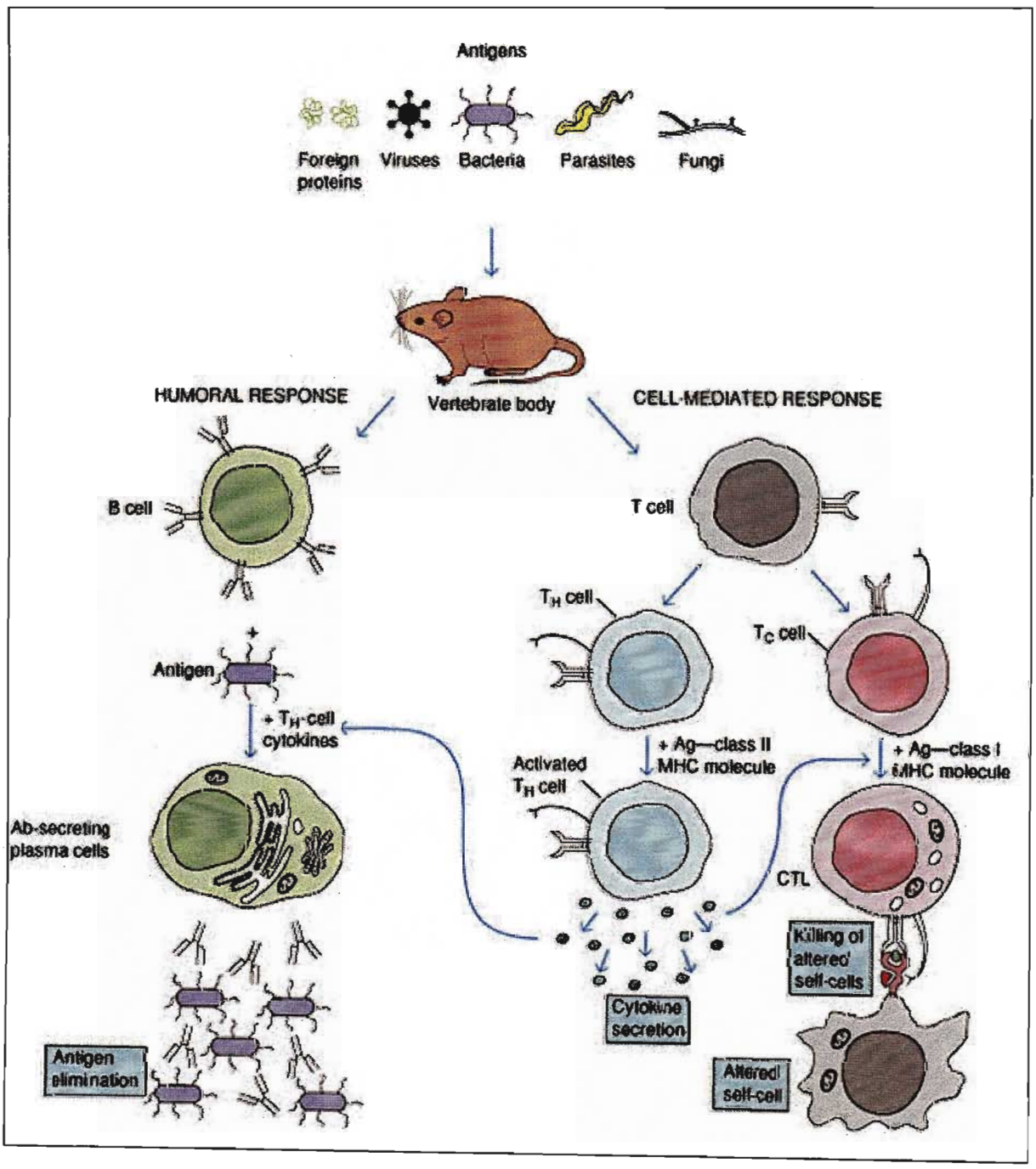

Figure 2.10: Picture of the immune response (Sears, 1997).

2.5.3 Cytokines

The two major classes of lymphocytes B and T cells specifically recognise antigens and form immune proteins called immunoglobulins or antibodies and sensitised lymphocytes, respectively. These are the immune products that interact with the antigens. Chemical mediators, such as cytokines, that transmit messages between immune system cells, aid this defence. The term cytokine is a generic term for the proteins released by one cell population that acts as an intracellular mediator. When these proteins are released from mononuclear phagocytes, they are called monokines and when released from T lymphocytes they are known as lymphokines. However when produced by a leukocyte and the action is on another leukocyte, these proteins are known as interleukins. If their effect is to stimulate the growth and differentiation of the immature leukocytes in the bone marrow, they are called colony stimulating factors (Prescott *et al.*, 1996).

Cytokines can affect the same cell responsible for their production (autocrine function), nearby cells (paracrine cells), or can be distributed by the circulatory system to their target cells (endocrine function). Their production is induced by non-specific stimuli such as viral, bacterial, or parasitic infection, cancer, inflammation, or the interaction between a T cell and antigen. Some cytokines also can induce the production of other cytokines. Cytokines exert their effect primarily by binding to specific plasma membrane receptors called CD's (cell associated differentiation antigens on target cells. Cytokines function in four different ways, as mediators of natural/non-specific immunity, activators of effectors cells, mediators of mature cell activation, differentiation, behaviour and growth. Finally cytokines also act as mediators of immature cell growth and differentiation. These functions, along with examples of relevant cytokines are shown in Table 2.5 (Prescott *et al.*, 1996).

Table 2.5: Functions and Examples of selected cytokines (Prescott *et al.*, 1996).

Cytokine	Producer	Mechanism of action
Mediators of Non-specific Immunity		
Interferon-α	Macrophages/ monocytes	Protects neighbouring cells by inhibiting viral DNA or RNA and protein synthesis Activates lytic activity of NK cells
Interferon-β	Fibroblasts/ other cells	Induces antiviral state, differentiation of fibroblasts Modulates antibody function, cytotoxic T-cell Stimulates the lymphocytes/ monocytes to express MHC molecules Enhances secretion of Interleukin-1α and tumour necrosis factor - α
Tumour necrosis factor- α	Phagocytes	Mediates expression of genes for growth factors and cytokines, transcription, receptors inflammatory mediators Mediator of the inflammatory response, Cytotoxic for tumour cells
Interleukin 1-α **Interleukin 1-β**	Monocytes, macrophages, endothelial cells, fibroblasts	Variety of effects on the differentiation and function of cells involved in inflammatory, immune responses
Interleukin 6	T cells, monocytes, macrophages, fibroblasts, hepatocytes, endothelial cells, neuronal cells	Activates hematopoietic cells; induces growth of T cells, B cells, hepatocytes, keratinocytes and nerve cells, stimulates the production of acute phase proteins
Chemokines	Monocytes, macrophages, endothelial cells, fibroblasts, T cells, platelets	Leukocyte chemotaxis
Activators of Effector cells		
Interferon-γ	T cells, NK cells	Activation of T cells, macrophages, neutrophils and NK cells Increases class I and II MHC molecules
Lymphotoxin	T Cells	Activation of neutrophils and endothelial cells
Interleukin 5	T Cells	Growth and activation of B cells and eosinophils; activation of eosinophil function, chemotactic
Migration inhibition factor	T Cells	Conversion from otile to immotile state (macrophages/ monocytes)
Mediators of Mature Cell Activation, Growth and Differentiation		
Interleukin -2	T cells	Stimulates T cell proliferation and differentiation, enhances cytotoxic activity of NK cells, promotes proliferation and immunoglobulin secretion of activated B cells
Interleukin -4	T cells, macrophages, mast cells, basophils, B cells	Induces differentiation of naïve $CD4^4$ into T helper like cells; induces proliferation and differentiation of B cells; exhibits diverse effects on T cells, monocytes, granulocytes, fibroblasts, endothelial cells
Transforming growth factor-β	Bone cells, platelets, fibroblasts, monocytes	Inhibits growth of many cells, inhibits NK cell activity and T cell proliferation, with Iinterleukin4 stimulates IgA secretion
Mediators of Immature Cell Growth and Differentiation		
Interleukin-3	T cells, mast cells	Production and differentiation of macrophages
Interleukin 7	Bone marrow stromal cells, fetal liver cells	Supports the growth of pre B and pro B cells; supports the proliferation of T cells; stimulates the cytotoxic activity, proliferation and generation of cytotoxic T cells
Granulocyte / macrophage colony stimulating factor	T cells, neutrophils, macrophages, fibroblasts, endothelial cells	Stimulates various functions of monocytes and macrophages, promote the growth and development of macrophage colonies from undifferentiated precursors

2.6 Cellular death

For every cell there is appointed a time to live and a time to die. The existence of two different pathways for cellular death has long been postulated: the passive and traumatic process leading to necrosis and the active program characterised by organelle integrity known as apoptosis (Cossarizza *et al.*, 1995). Briefly, necrosis ("accidental" cell death) may be defined as the pathological process, which occurs when cells are exposed to a serious physical or chemical insult. Apoptosis ("normal" or "programmed" cell death) is the physiological process by which unwanted or useless cells are eliminated during development and other normal biological processes. Other schools of thought maintain that necrosis does not indicate a form of cell death but refers to the changes "secondary" to cell death by any mechanism including apoptosis (Majno and Joris, 1995).

For disease therapy to be effective, knowledge of the difference between apoptosis and necrosis is essential. Whether the cell death in pathological diseases is apoptotic or necrotic, the prevention is of medical importance. This requires an elucidation of the mechanisms of the particular cell death pathway associated with various disorders (Uchiyama, 1995).

2.6.1 Apoptosis

The Greek name apoptosis is appropriate as it suggests the discrete image of leaves dropping off here and there (apó, meaning from, ptósis, meaning a fall) as opposed to massive cell death (Majno and Joris, 1995). There are key structural features associated with apoptosis. The dying cell membrane separates from its neighbours, usually with loss of specialised membrane structures such as microvilli and desmosomes. The membrane undergoes a period of blebbing and contortion. The blebs are membrane-invested extensions of cytosol that are usually devoid of organelles and are reversibly extruded and resorbed (Wyllie, 1997).

This is followed by rapid, irreversible condensation of cytoplasm, accompanied by an increase in cell density, compaction of cytoplasmic organelles and condensation of the nuclear chromatin to form dense granular caps or torodal structures underlying the nuclear membrane. Nuclear pores disappear from the membrane subjacent to these chromatin condensations whilst, within the nucleus, the proteinaceous centre of the nucleolus separates from its surrounding shell of osmiophilic transcription complexes (Wyllie, 1997). Around this time, the cell splits into a cluster of membrane bounded bodies, each containing a variety of organelles.

Apoptotic cells within the tissues or even *in vitro*, are the objects of phagocytosis by their viable neighbours. The dying cells disappear rapidly from the tissue without generating an inflammatory response (Wyllie, 1997).

2.6.2 Necrosis

There are distinct morphological differences between apoptosis and necrosis (Figure 2.11). During necrosis, the dying cells swells, both cytosolic and nuclear structures alter, but the general disposition of hetero- and euchromatin is maintained, as are the nuclear pores. The plasma membrane ruptures and internal materials reach the extracellular space, where some of them (notably some mitochondrial proteins) induce an inflammatory reaction.

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

Some of the features of necrosis can become superimposed on those of apoptosis if the dead cell for some reason fails to be recognised and engulfed by a phagocyte. Thus apoptotic cells *in vitro*, after the initial brisk rise in buoyant density show a progressive fall in density to subnormal levels over the few hours. Their membranes become permeable to dyes that that had previously been excluded e.g. propidium iodide (PI) and their ultrastructure is a series of distended cytosolic membrane profiles, although the conspicuous blocks of condensed chromatin remain in the nucleus. Similar structural features can be seen *in vivo* in apoptotic cells. This has been called secondary necrosis (Wyllie, 1997).

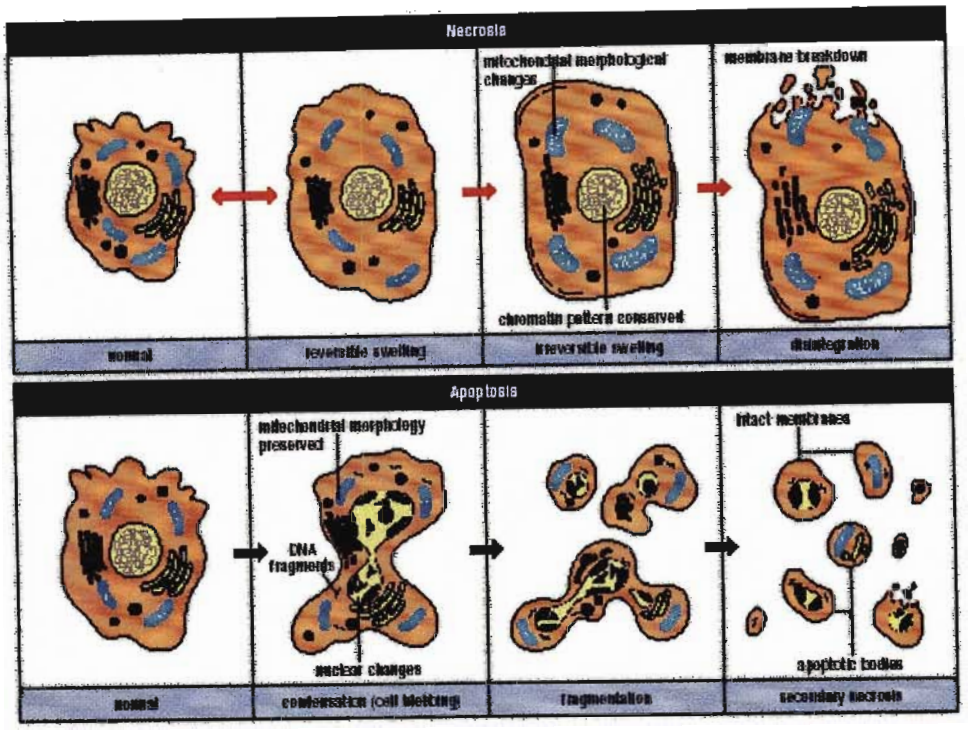

Figure 2.11: Structural differences between necrosis and apoptosis (Roche, 2002).

2.6.3 Mechanisms of Apoptosis

The coordinated structural changes that make up the process of apoptosis are driven by a set of molecular interactions, sometimes known as terminal effector events. Most living cells contain the molecules that participate in these events, but in a form that requires activation. The process of death is the result of an interaction between initiating stimuli, which can be physiological or the result of injury in various parts of the cell and factors that determine the susceptibility of the cell to activation of the terminal effector events.

2.6.3.1 Caspases

Specific genes regulate apoptosis. A family of over a dozen related genes is present in the mammalian genome collectively called caspases (cysteine-containing aspartases) (Whyte, 1996). The caspases are the key effector proteins of apoptosis in mammalian cells. They are produced as inactive precursors that in order to be activated are cleaved at aspartate residues and assembled into tetramers. In all of them the active catalytic residue is a cysteine. Once activated, these caspases cleave their substrate at aspartate residues; some caspases can activate their own precursors or other caspases. Many proteins are cleaved once these caspases become active and endonucleases are released that gain access to the chromatin. The cell then displays the characteristic apoptotic morphology.

The founder member of these caspases is Caspase 1, formerly known as interleukin-1β converting enzyme (ICE), which is known to be responsible for the cleavage and activation of the inflammatory cytokine 1L-1β. There remains some doubt of over the role of Caspase 1 itself in mammalian cell death. However other caspases are clearly central to mammalian cell death; they are activated during apoptosis, death is forestalled by specific inhibitors and germ-line knock out of their genes produces abnormal development with retention of cells which are normally deleted (Whyte, 1996).

Activation of the terminal effector events leads to the formation of a signalling complex that subsequently activates a family of so-called initiator caspases, e.g. caspase 8 (Fraser and Evan, 1996). Initiator caspases are responsible for the first proteolytic events, e.g. cleavage of cytoskeletal and related proteins including vimentin (van Engeland *et al.*, 1997), actin (Kayalar *et al.*, 1996) and fodrin (a membrane-associated cytoskeletal protein) (Martin *et al.*, 1995; Greidinger *et al.*, 1996). Amongst others, these early apoptotic events are thought to be responsible for the characteristic blebbing of the cell surface (McCarthy *et al.*, 1997). Cleavage of translocase (flippase) and/or activation of scramblase (floppase) results in a subsequent flip of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. This is known to be a very early event during the initiation stages of apoptosis (Martin *et al.*, 1995). Cells use externalisation of phosphatidylserine as a signal for events such as the attraction of macrophages, induction of the coagulation cascade or induction of syncytial fusion.

Initiator caspases are positioned at the top of the caspase hierarchy (Cohen, 1997). Subsequently they cleave and thus activate a second subpopulation of caspases known as the execution caspases (Mignotte and Vayssiere, 1998). Irreversible progression of the apoptosis cascade commences with activation of the latter. The bcl-2 family, an acronym for the B cell lymphoma/leukaemia gene (Reed, 1994), of mitochondrial proteins tightly regulates execution caspase activation. The various members of this family promote, for example, bcl-2-associated X protein (bax) and bcl-2-antagonist/killer (bak), or inhibit, such as bcl-2, bcl-2 like protein (bcl-xL) cleavage of execution caspases (Mignotte and Vayssiere, 1998).

Irreversible progression of apoptosis by activated execution caspases (e.g., caspase 3) requires that these enzymes remain active for a critical minimum period of time (Marks *et al.*, 1998). This varies from minutes to hours depending on the size, type and functional state of the cell.

Once activated, the execution caspases, either directly or by means of other proteases, cleave a broad array of proteins critical for cell survival. The latter include intermediate filament proteins, such as cytokeratin 18 (Caulin *et al.*, 1997), nuclear envelope proteins, for example lamins A and B (Greidinger *et al.*, 1996), proteins involved in DNA maintenance and repair (such as poly-(ADP-ribose) polymerase (PARP); Tewari *et al.*, 1995), enzymes involved in relaxation of the DNA-helix and separation of chromosomes during mitosis (topoisomerase IIa; Nakajima *et al.*, 1996), the catalytic subunit of the DNA-dependent protein kinase (DNA-PKCS; Song *et al.*, 1996).

Execution caspases activate DNA fragmentation factor (DFF) and other endonucleases resulting in specific fragmentation of DNA (Liu *et al.*, 1997). Finally, execution caspases cause characteristic intracellular shifts and activation of proteins such as of: TIAR [T-cell-restricted intracellular antigen-related protein, possibly involved in cytoplasmic DNA cleavage (Taupin *et al.*, 1995)], which is shifted from a nuclear to a cytoplasmic localisation and transglutaminase II, which protects the endangered plasmalemma, by cross-linking proteins and forming large sub-plasmalemmal protein scaffolds (Cummings, 1996; Fesus *et al.*, 1996) that is shifted from a more diffuse cytoplasmic localisation to directly underneath the plasma membrane.

2.6.3.2 Surface Receptors

Cell domain 95 (CD95) is a member of the tumour necrosis factor (TNF) receptor family and binds a TNF-family ligand (Wallach, 1997). Ligand binding initiates trimerisation of the receptor and this permits the immediate recruitment of several proteins that form a complex around the cytoplasmic moiety of the receptor - the death initiating signalling complex (DISC). The death initiating signalling complex proteins bind to each other and CD95 through a series of homologous domains (Yaun, 1993; Muzio *et al.*, 1996).

Thus the C terminus of the CD95 contains an amino acid sequence (called the death domain), that binds a similar sequence on the C terminus of a DISC protein which contains a Fas associated death domain (FADD). The FADD contains a distinct N-terminal domain (called the death effector domain) that binds to a homologous region in the N terminal of a third protein, Fas activated protein like ICE (FLICE). Fas activated protein like ICE is so named because its C terminus has structural and functional homology to the caspases and can activate them. Thus a cytokine receptor on the cell surface is coupled to the heart of the caspase engine by a remarkable direct, non-transcriptional pathway (Muzio *et al.*, 1996).

Members of many transcriptional modifying pathways have also been implicated in the regulation of apoptosis. An example of this is the expression of the proto-oncogene *c myc*, which increases susceptibility to apoptosis. *C myc* is well known as an immediate early response gene in growth factor dependent entry to the cell cycle. Transcriptional activation of *c-myc* is thought to initiate a state of susceptibility to both apoptosis and cell proliferation. A second signal is required to determine which one is selected. In the presence of cytokine survival factors, not necessarily mitogens, apoptosis is aborted and cell proliferation occurs. In this way, the potentially dangerous process of cell proliferation is made dependent on dual controls. This theory is supported by the observation that apoptosis frequently occurs in conjunction with proliferation in tissues, with and without injury (Evan *et al.*, 1992).

2.6.3.3 Cytochrome C (Mitochondrial pathway)

Aside from the ligation of death receptors there are a number of other mechanisms through which the caspase cascade can be activated. The mitochondria are also key regulators of the caspase cascade and apoptosis. Release of cytochrome C from mitochondria can lead to the activation of caspase 9 and then of caspase 3. This effect is mediated through the formation of an apoptosome, a multi-protein complex consisting of cytochrome C, apoptotic protease activating factor 1 (Apaf-1), pro-caspase 9 and ATP (Huppertz *et al.*, 1999). The formation of the apoptosome is illustrated below in Figure 2.12 .

Cytochrome C released from the mitochondria binds to the cytosolic protein Apaf-1. This interaction results in a conformational change in Apaf-1 which, when stabilised by the binding of ATP, allows molecules of Apaf-1 to associate with each other and results in the formation of a wheel-like structure that contains 7 molecules each of Apaf-1, cytochrome C and ATP (Huppertz *et al.*, 1999).

This wheel-like structure, known as the apoptosome, permits the recruitment of 7 molecules of procaspase-9 to the complex. The exact mechanism of caspase activation is still uncertain although two possibilities have been proposed. In one case the Apaf-1, cytochrome C and procaspase-9 complex can act as a stage to activate cytosolic procaspase-9 as it is recruited to the apoptosome. In the other scenario two apoptosomes have been proposed to interact with each other and to activate the caspase-9 located on the other apoptosome (Huppertz *et al.*, 1999).

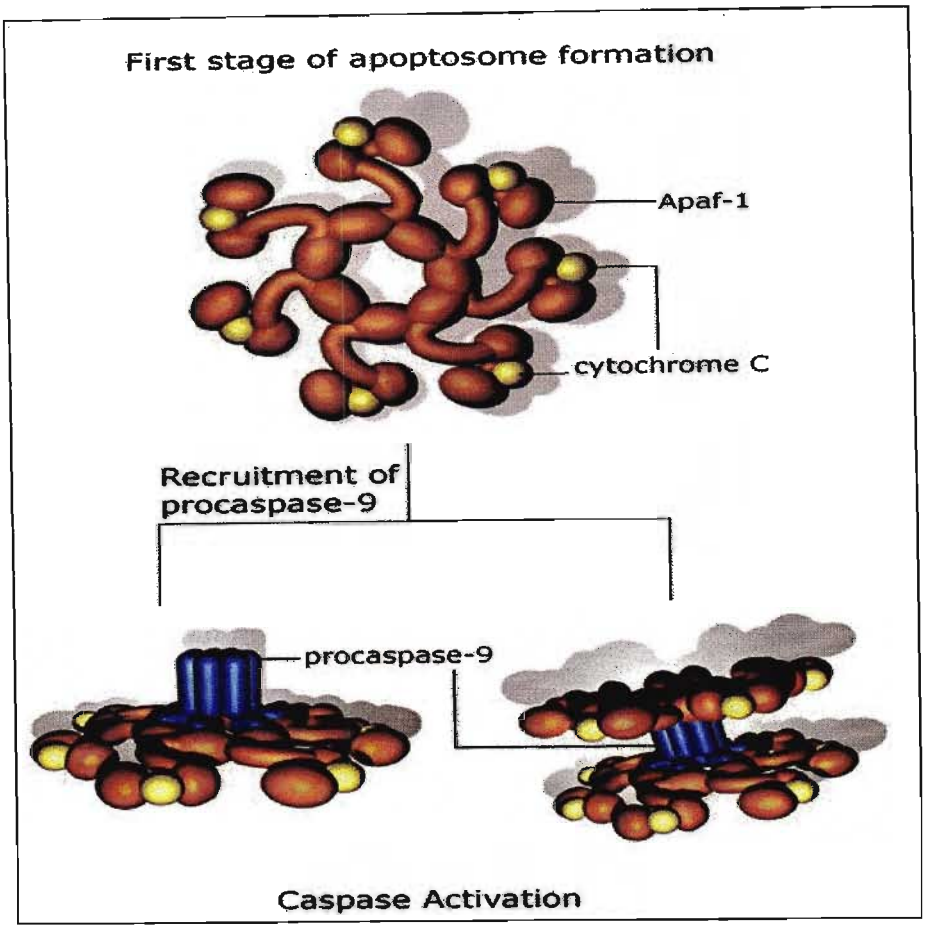

Figure 2.12: Formation of an apoptosome (Reproductive and Cardiovascular Group, 2003).

2.6.4 Apoptosis in Response to Cellular Injury

Apoptosis is a critical component of the cellular response to injury:

2.6.4.1 DNA Damage

Along with evidence at the electron microscope level, the study of DNA fragmentation patterns remains the gold standard for the visualisation of apoptotic cell death. The cleavage of the DNA occurs at internucleosomal linker regions, resulting in a "ladder" of 180-200 base pair fragments and multiples thereof and this can be determined on agarose gel electrophoresis. The cause of the ladder type DNA fragmentation has been attributed to the activity of a calcium and magnesium ion (Ca^{2+}, Mg^{2+})- activated endonuclease, DNAse I or DNAse II, all of which are capable of generating internucleosomal cleavage of DNA (Payne *et al.*, 1995).

DNA injury can initiate apoptosis by a powerful, early activated mechanism dependent on the nuclear phosphoprotein p53. The p53 protein is activated by both transcriptional and post-translational means and is a critical element in the cellular response to double stranded DNA breaks. Such breaks appear for example, following damage inflicted by ionising radiation.

The p53 protein is also critical in the response to damage by UV light, where the initial events do not involve strand breakage but the generation of nucleotide dimers. In a manner not completely understood, p53 forms part of a decision fork in which the cell is directed either towards the completion of repairs or to apoptosis. It is also clear that cells possess pathways that couple DNA injury to apoptosis in the complete absence of p53 (Clarke *et al.*, 1993).

2.6.4.2 Injury to Cell Membranes

Injury to cell membranes and in particular the plasma membrane, activates acid sphingomyelinase and so generates the second messenger ceramide from membrane lipids (Haimovitz-Friedman *et al.*, 1994; Santana *et al.*, 1996). Sphingosine-based ceramides have been strongly implicated in the process of inducing apoptosis (amongst other methods) through stimulation of the Fas (Cifone *et al.*, 1994) and TNF receptors by UV and x-ray irradiation (Wright *et al.*, 1994), or through treatment with certain chemotherapy drugs (Jaffrezou *et al.*, 1996). Furthermore, the addition of synthetic ceramides themselves has also been shown to induce apoptosis in various cell types (Martin *et al.*, 1995). Ceramide, perhaps through modification of the usage of MAP kinase versus Jun kinase signaling pathways, alters cellular susceptibility to apoptosis (Xia *et al.*, 1995).

2.6.4.3 Mitochondrial Injury

Mitochondrial physiology is disrupted in cells undergoing either apoptosis or necrosis. During apoptosis mitochondrial permeability is altered and apoptosis specific protease activators are released from mitochondria. Specifically, the discontinuity of the outer mitochondrial membrane results in the redistribution of cytochrome C to the cytosol followed by subsequent depolarisation of the inner mitochondrial membrane. The release of Cytochrome C (Apaf-2) further promotes caspase activation by binding to Apaf-1 and therefore activating Apaf-3 (caspase 9). AIF (apoptosis inducing factor), released in the cytoplasm, has proteolytic activity and is by itself sufficient to induce apoptosis (Kroemer, 1997).

2.6.4.4 Cytotoxic T Cell Killing

Cytotoxic T cell killing is effected by at least two major pathways. Following recognition of the target cell by the cytotoxic T lymphocyte (CTL), fas is activated through the binding of the Fas ligand expresses on the CTL surface (Rouvier *et al.*, 1993). The CTL also releases the contents of its granules, amongst them perforin (which effects increased permeability of the target cell membrane) and a group of proteases of which the best defined is the serine protease granzyme B, which directly activates the target cell caspases (Greenberg, 1996).

2.6.5 Apoptosis and the Immune System

The immune system is responsible for the complex task of providing defence against a vast array of potential pathogens, whilst ensuring that the same protective mechanisms are not turned against self. Mechanisms of apoptosis play key roles in the development, regulation and functioning of the immune system. Malfunctioning of the cell death process can cause autoimmune disease, immunodeficiencies and lymphoid malignancies (Ekert and Vaux, 1997).

2.6.5.1 Developmental Apoptosis in Lymphocytes

The vast majority of developing T and B-lymphocytes die during development. As these cells differentiate from progenitor cells, they rearrange the genes for their antigen receptors and express them on the plasma membrane. Rearrangement of immunoglobulin (Ig) genes in B cells occurs independently of antigen and generates diversity in Ig receptor antigen specificity. As the cells progress through the pro B to pre BI and to pre BII stages, the rearrangements of the receptor genes follow a recognisable pattern. Many rearrangements produce genes in a reading frame that cannot be translated into functional proteins (Tonegawa, 1983). Yet other rearrangements are successfully transcribed and translated but the receptor is not expressed on the cell membrane or does not transduce a signal. In all instances, the cell fails to receive survival signals, the apoptotic pathway is activated and the cell dies by neglect. When the Ig genes are correctly rearranged and Ig is expressed on the cell surface, it sends a signal to the cell that interrupts default activation of the cell death process (Tonegawa, 1983).

Thymocyte development is marked by a progression of changes in the expression of surface antigens, including the CD4 and CD8 markers (Godfrey and Zlotnik, 1993). These developing cells undertake genetic rearrangements of the T cell receptor (TCR) genes in a process similar to the rearrangement of the B cell antigen receptor genes. Their fate, either survival and clonal expansion, or deletion, is dependent on the specificity of the interactions between the TCR and MHC antigens. Those cells failing to make successful receptor gene rearrangements die by initiating apoptosis. The development and differentiation of immune cells is governed by the autocrine, paracrine and endocrine actions of various factors. The interleukins are a family of cytokines with important developmental and regulatory functions in the immune system. For example interleukin 7 promotes proliferation and hence survival of pre B cells, in its absence the cells undergo apoptosis (Raulet *et al.*, 1985).

2.6.5.2 Positive and Negative Selection

Lymphocytes that manage to express antigen receptors must undergo further selection. In the thymus, apoptosis is used to remove T cells with receptors that fail to interact with self MHC peptides (positive selection) and is used to remove T cells with receptors that have high affinity for self antigens (negative selection) (Nossal, 1994). T cells not positively selected will be removed by apoptosis. Negative selection of B cells is required to remove populations which express antibodies recognising self-antigens. The rearrangements occurring during B cell development lead to the surface expression of IgM and IgD receptors. One mechanism whereby self reactive B cells are suppressed is clonal deletion by apoptosis.

This process of deletion involves first, an arrest of maturation and then secondly activation of the intrinsic cell death pathway in the self reactive lymphocyte, which can be delayed at least in the bone marrow by the over expression of the anti-apoptotic gene bcl-2 (Nemazie and Burki, 1989).

2.6.5.3 Apoptosis of Activated Lymphocytes and Homeostasis of Lymphocyte Numbers

After development of lymphocytes in the primary lymphoid organs, lymphocytes are exported to the secondary lymphoid organs, where they come across a variety of antigens (Nossel, 1994). Once mature T and B cells have been exposed to an antigen, they become activated, enter the cell cycle, proliferate and differentiate. The lymphocytes must be removed on completion of their respective functions, such as producing antibodies (B cells) or secreting cytokines or killing target cells (helper and cytotoxic T cells). In most cases apoptosis appears to be mediated by the induction on cells of both CD95 (Fas/APO-1) and its ligand, leading to the induction of suicide or fractricide by the activated cells (Alderson *et al.*, 1995). Other TNF receptor members, such as TNFR2, have also been implicated in apoptosis.

2.6.5.4 Apoptosis Activation Pathways, Effector Mechanisms and Regulation in the Immune System

As with immature lymphocytes, the survival of mature T and B cells can be regulated by signals from soluble cytokines and molecules expressed on their own surface or on the surface of other cells, for example, the CD 95 (Fas/Apo) molecule (Itoh *et al.*, 1991).

Bcl-2 is a member of a family of proteins, which regulate apoptosis. Ueno *et al.*, (1995) suggested that apoptosis could be caused as a result of the suppression of bcl-2 proteins. The B-cell lymphoma/leukaemia-2 gene was first discovered due to its involvement with B cell malignancies. However it is more commonly associated with inhibition of programmed cell death (Reed, 1994). The bcl-2 proteins appear to act by preventing the presence of damaged DNA from being translated into a signal for activation of the genes involved in apoptosis, or by blocking the action of the products of those genes once induced. The ability of bcl-2 proteins to protect cells form a wide variety of pathological, as well as physiological, stimuli indicates that many triggers can serve to activate the same suicide pathway, even some stimuli thought to cause necrosis and not physiological cell death (Vaux, 1993).

Apoptosis plays a vital role in the normal development of the immune system and in the regulation of the immune response. Apoptosis contributes to the pathology of many human diseases, including disorders of immune functions, either as a secondary phenomenon activated by the underlying abnormality or as a primary phenomenon where the abnormality is a dysregulation of apoptosis.

2.7 Mechanisms of cell death induced by Aflatoxin B_1 and Fumonisin B_1

Aflatoxin B_1 and FB_1 have been shown to have many cytotoxic effects on different cell lines. However a link needs to be established between the cytotoxic effects of AFB_1 and FB_1 and the mechanisms of cell death in normal human lymphocytes. Irreversible damage to lymphocytes results in immuno-compromised organisms. Thus the toxic effect of AFB_1 and FB_1 are both direct and indirect.

Aflatoxin B_1 and FB_1 have also been shown to exhibit nephrotoxic, immunosuppressive teratogenic and carcinogenic properties in animals (Corrier, 1991). Although the molecular basis for many of the specific immunosuppressive effects of many mycotoxins is presently unclear, inhibition of DNA, RNA and protein synthesis via a variety of different mechanisms appears to be directly or indirectly responsible for the immunosuppressive action of many mycotoxins. The immunotoxic effect of AFB_1 and FB_1 has been demonstrated on animals, however there is not enough information in the case of humans (Berek *et al.*, 2001). This lack of information and the reported severe cases of human toxicoses (Ehling *et al.*, 1997) has prompted this study to investigate how AFB_1 and FB_1 affect the human immune system, in particular lymphocytes.

CHAPTER 3

Cytotoxicity of Aflatoxin B_1 and Fumonisin B_1 on cultured normal human lymphocytes

3.1 Cell Culture

Mammalian cells are often used as alternatives to animals in toxicology studies and the culture of human and animal cells is common practice in modern research laboratories. *In vitro* cultures allow for a more controlled and manipulated environment, thus also allowing the biochemical mechanisms of toxicity to be analysed more specifically. This type of experimentation involves a reduction of animal studies that are often in the public protest limelight and also reduces the financial costs incurred by animal studies (Benford and Hubbard, 1987).

Presumably the best environment for growing cells is one providing as closely as possible the conditions they experience *in vivo*. The essential requirements for an optimum cell culture environment are temperature, pH, gas phases and media containing the necessary nutrients (Freshney, 1983). However the culture medium is by far the most important single factor in culturing cells. Its main function is to provide the physical conditions of pH and osmotic pressure required for survival and the complicated chemical substances required by the cells, which they cannot synthesise for themselves (Freshney, 1983).

In 1966, Moore developed a specific medium for the growth of human leukaemia cells in monolayer or suspension cultures using a serum supplement (Moore *et al.*, 1969). This cell culture medium was developed at the Roswell Park Memorial Institute, so became known as the acronym RPMI-1640 (Appendix 1.1). RPMI-1640 is now a commercially available growth medium that contains essential amino acids, vitamins and salts. However additional proteins are required to enhance cell growth. Various types of animal sera, which contain important proteins such as albumin and globulins, are routinely used for supplementation. The serum used is normally from a non-human source, such as foetal calf serum (FCS), to exclude human antibodies, which could be inhibitory to the subsequent isolation of human viruses. A complete culture medium (CCM) will usually contain FCS, RPMI-1640 and antibiotics and therefore sustain continuous cell growth (Freshney, 1983).

In addition to CCM, Hanks balanced salt solution (HBSS) (Appendix 1.2) containing inorganic salts, glucose and phenol red is routinely used to wash cultured cells in order to remove cellular debris and dead cells.

Histopaque-1077, a solution of polysucrose and sodium diazoate adjusted to a density of 1077μg/ml, is commonly used for the isolation of mononuclear cells from whole blood. The rapid recovery of viable mononuclear cells from small quantities of blood is facilitated by Histopaque-1077 using density gradient centrifugation.

3.1.1 Cytotoxicity Testing

As the number of potential toxic agents has increased in current years so the need for a rapid and efficient *in vitro* assay for assessing cytotoxicity has developed. Numerous bioassays using cell culture techniques have been described for the toxicological characterisation of mycotoxins (Robb and Norval, 1983; Thompson and Wannemacher, 1986). The main criteria for these tests are based on cell density and morphology viz simple macroscopic examination of cell damage. However the disadvantage of such morphological evaluation is the subjective judgement of cytotoxicity (Robb and Norval, 1983). Other assays involve functional criteria, such as measurement of protein and DNA synthesis using radiolabelling techniques (Porsher *et al*, 1987). These techniques however are limited by general health hazards caused by radioactive material, high costs of laboratory equipment and recycling of radioactive refuse.

3.1.1.1 The Methylhiazol Tetrazolium (MTT) Bioassay

Slater *et al.* (1963) described the staining reactions of four tetrazolium salts caused by the intact respiratory chains of rat liver mitochondria. The main point of interest was the applicability of these observations for measuring the mitochondrial activity of living cells. Mossman (1983) modified this study and described a colorimetric cell culture assay using the tetrazolium salt 3-{4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), to measure cell proliferation and survival. The principle of this reaction was based on the reduction of the yellow coloured MTT salt to purple formazan crystals by the succinate tetrazolium reductase system which belongs to the respiratory chain of mitochondria in viable cells (Altmann, 1976).

Another theory suggests that the bioreduction of the MTT is associated with the enzymes of the endoplasmic reticulum and involves the reduced nicotinamide nucleotides NADH. Succinate is a weak electron donor for mitochondrial MTT reduction (Berridge *et al.*, 1996). Therefore the cellular reduction of MTT is more related to the glycolytic rate and thus to NADH production rather than cellular respiration. Berridge *et al.*, (1996) showed that MTT is reduced by microsomal enzymes that require nicotinamide nucleotides but agree that succinate can also act as an electron donor in MTT reduction through mitochondrial succinate dehydrogenase, however this reduction is slow and contributes little to the total cellular MTT reduction.

An increase in the number of viable cells results in an increase of the overall enzyme activity in the sample. This augmentation leads to a greater quantity of formazan dye being produced which correlates with the number of metabolically active cells in the culture. Based on these principles, MTT assays have been used for numerous medical, microbiological and toxicological tests (Hanelt *et al.*, 1994).

The MTT assay uses microtitre plates and scanning plate readers that measure absorbance values in respective wells, allowing replicate testing of xenobiotics, chemotherapeutic agents, potential toxicological agents or drugs, at multiple concentrations on many different cell lines. The object of this investigation was to assess the cytotoxicity of AFB_1 and FB_1 on both isolated normal human lymphocytes and whole blood, using the MTT dye reduction bioassay.

3.2 Materials and Methods

3.2.1 Ethical Approval

Institutional ethical approval was obtained for this study (Reference H027/03).

3.2.2 Materials

Mycotoxins standards (AFB_1 and FB_1), Histopaque 1077, 3-{4,4-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide were purchased from Sigma, SA. Lithium heparin tubes, alcohol swabs, needles, gloves, disposable 3ml Pasteur pipettes and vacutainers were obtained from Shalom, SA. Hank's balanced salt solution, RPMI-1640, FCS, Penstrep-Fungizone, 96 well microtitre plates and sterilin tubes were purchased pre-sterilized from Adcock Ingram. Ethanol, methanol and DMSO were acquired from Merck, SA.

3.2.3 Methodology

3.2.3.1 Preparation of Cell Culture Media

Foetal calf serum was added (5% -10%) to RPMI-1640 (500ml) as the additional nutrient source. To counteract the problem of microbial contamination, the media was supplemented with Penstrep-Fungizone (1ml), a commercially available cocktail of the antibiotics Penicillin (against gram negative) and Fungizone (against fungal contaminants).

Dimethylsulphoxide (DMSO) was added at a concentration of 10% to CCM as the freezing medium for the cells stored at -70°C. Dimethylsuphoxide was used in freezing because it does not crystallise at -70°C and therefore surrounds the cell preventing water from entering and crystallising within the cells. Additional FCS (20%) was used to provide extra supplementation.

3.2.3.2 Environment

All cell culture procedures were carried out in a laminar flow cabinet containing a UV light. The unit was swabbed/ sterilized with a mixture of ethanol and methanol (70:30) and G-cide 2% (G-cide international) before use. Prior to each session hands were washed with habitant. This serves to sterilize and moisten the hands and thereby reduce the risk of dry skin blowing onto the cultures as well as reduce loosely adherent micro-orgamisms, which are the greatest risk to the culture (Freshney, 1983). Gloves were thereafter used and frequently swabbed with 70% ethanol.

The optimal temperature for human cell cultures is dependent on human body temperature, which is between 36.5°C and 37°C. Cultured cells tolerate considerable drops in temperature, can survive several days at 4°C and can be cooled and frozen to -196°C, but they are unable to tolerate more than 2°C above normal (39°C) for more than a few hours and die rapidly at 40°C and over (Freshney, 1983).

3.2.3.3 Cell Counting and Viability

Trypan Blue was used in a dye exclusion procedure for viable cell counting (Sigma, 1996). The stain was used at a concentration of 0.4% (w/v) in sterile water. Cell numbers were determined using a Neubauer haemocytometer counting chamber that consists of two chambers; each divided into 1mm squares (Figure 3.1). A cover glass was supported 0.1mm over these squares so that the total volume over each square was $0.1mm^3$. Since $1cm^3$ is equivalent to 1ml, the cell concentration per millilitre was the average count per square $\times 10^4$. The trypan blue solution (0.1ml) was transferred to an eppendorf and a 100µl aliquot of the cell suspension added (dilution factor =5) and mixed thoroughly. The mixture was allowed to stand for 5 - 15minutes. The method is based on the principle that viable cell are unable to take up certain dyes while non-viable cells do. It was important to note that if the cells were exposed to trypan blue for a long period of time, viable cells took up the dye. With coverslip in place, a sterile Pasteur pipette was used to transfer a small amount of the trypan blue mixture to both chambers of the haemocytometer.

Starting with chamber 1 of the haemocytometer, all the cells in the 1mm square and 4mm square were counted (Figure 3.1). Cells were counted on top and left touching middle line of the perimeter of each square. Cells that touched the middle line at the bottom and right sides were not counted. Non-viable cells were stained blue and a separate count of viable and non-viable cells was kept.

The procedure was repeated for chamber 2. If greater than 10% of the cells appeared clustered, the entire procedure was repeated. If less than 200 or greater than 500 cells were observed in the 10 squares, the procedure was repeated adjusting to an appropriate dilution factor. A second sample was then withdrawn and the count repeated for accuracy.

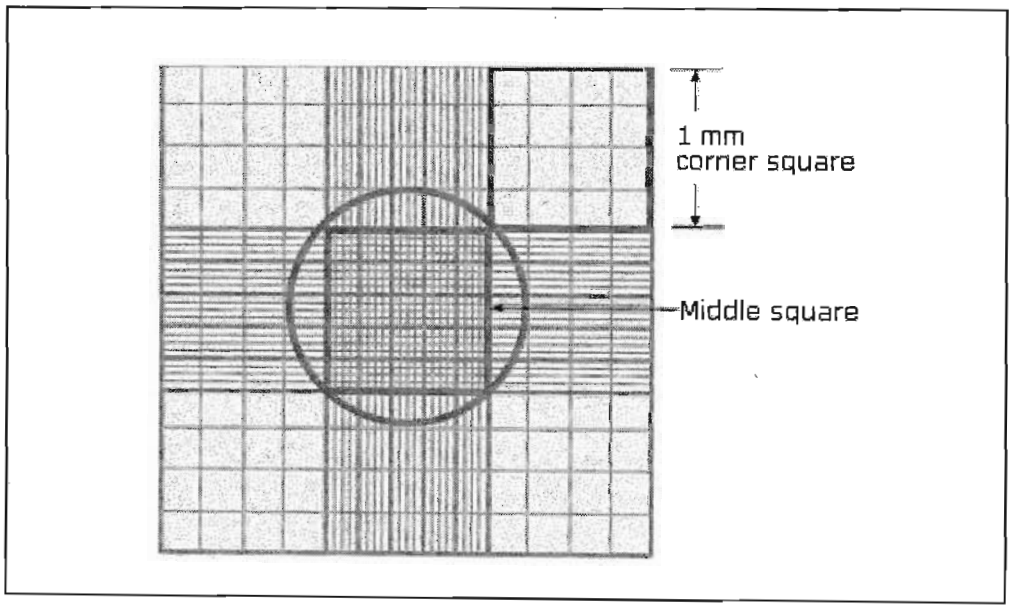

Figure 3.1: Diagram of a haemocytometer (Sigma 1996).

Cell viability was calculated as follows:

Cell viability (%)	= Total viable cells [unstained]/ Total cells [stained + unstained] × 100
Cells per ml	= Average count per square × dilution factor × 10^4 (count 10 squares)
Total cells	= Cells per ml × Original volume of fluid form which cell sample was removed

3.2.3.4 Collection of Blood

Peripheral venous blood was used in all experiments. The source of the blood was a healthy 23 year old female donor. Throughout this study the same donor was used. Whole venous blood (60ml) was collected in 5ml sterile lithium heparin tubes. This blood was used for the isolation of lymphocytes.

3.2.3.5 Isolation of Lymphocytes

All the reagents were stored at 4°C and prior to use warmed to 37°C. Whole blood (5ml) was carefully layered onto an equal volume of Histopaque in a sterile sterilin tube (15ml). Care was taken not to disturb the tubes, as mixing could delay the sedimentation of the different cell types. The tubes were then centrifuged using a bench top centrifuge at 400g for 30 minutes at room temperature (RT). After centrifugation, four distinct layers were obtained as shown in Figure 3.2.

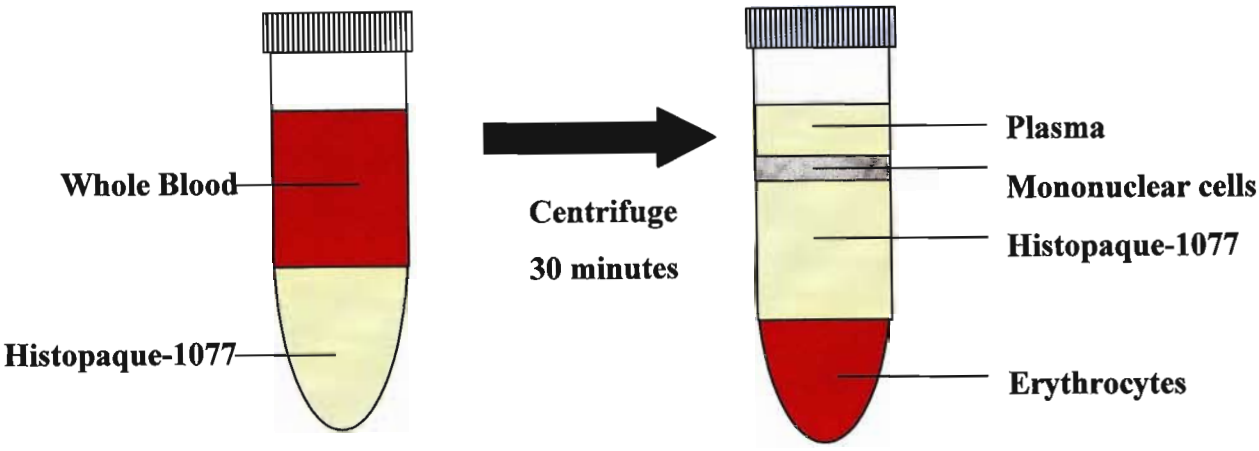

Figure 3.2: The isolation of mononuclear cells from normal human blood using density gradient centrifugation.

The upper plasma layer was aspirated and discarded. The mononuclear cell layer, which contained the lymphocytes, was carefully removed using a 3ml sterile pasteur pipette and transferred to a sterile sterilin tube. These cells were washed in HBSS (5ml; 37°C) by centrifugation (400xg; 20 minutes; RT), which removed extraneous platelets. The HBSS washing step was repeated twice or until erythrocyte contamination was negligible. All supernatants were discarded. Finally the pelleted cells were resuspended in CCM.

The viability and the number of lymphocytes were determined using the trypan blue haemocytometer method (Section 3.2.3.3) and the resulting suspension stored at 37°C for further tests.

3.2.3.6 Preparation of the Mycotoxin Stock Solutions and MTT salt

Mycotoxin standards, AFB_1 (1mg) and FB_1 (1mg) were partially dissolved in ethanol (100µl) and made up to 10mls using CCM for a final concentration of 0.1mg/ml. Serial dilutions of both mycotoxins were prepared with a range in concentration from 50µM to 5µM. The control solution consisted of 100µl ethanol, made up to 10ml using CCM.

The MTT salt solution was prepared by dissolving 5mg MTT salt in 1ml HBSS. The mixture was vortexed until all the particles were dissolved.

3.2.3.7 The MTT Bioassay on Cultured Human Lymphocytes

The suspension of cultured human lymphocytes was adjusted to give a cell number of 1×10^4 cells/ml. Aliquots of the cell suspension (200µl) were dispensed into each of the 96 wells of the microtitre plate to give a final cell count of 2000 cells/well. After vortexing, a 100µl aliquot of the 5µM, 10µM, 25µM and 50µM serial dilutions of AFB_1 were transferred to individual wells of the microtitre plate using a micropipette. This procedure was repeated with the FB_1 serial dilutions. A 50µl aliquot of each concentration of AFB_1 and 50µl of the same concentration of FB_1 were added to the same well to evaluate the cytotoxic effects of the combination of mycotoxins.

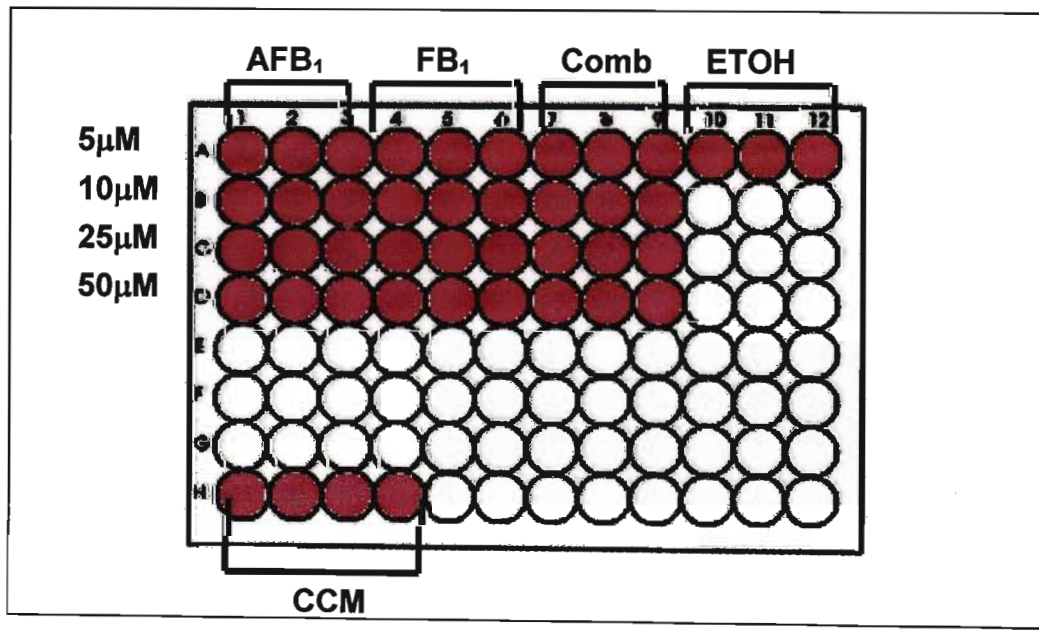

Figure 3.3: Schematic diagram of the treatment of human lymphocytes with mycotoxins for the MTT assay in a 96 well microtitre plate.

There were six replicates for each test concentration for the control, AFB_1, FB_1 and combination. After each incubation period, 12 hours, 24 hours, 48 hours, 72 hours and 96 hours, two 96 well plates were centrifuged at 1 600g for 20 minutes at RT using a Eppendorf Centrifuge 5702 R (Beckmann Instruments). The supernatant was discarded and the cells resuspended in 100µl fresh CCM. A 10µl aliquot of the MTT solution was added to each well. After 4 hours incubation (37°C) the plates were centrifuged at 1 600g for 20 minutes at 25°C. After the supernatant was discarded, a 100µl aliquot of DMSO added to each well and the plates incubated at 37°C for one hour. The optical densities of the treated and untreated cells were measured spectrophotometrically, at a dual wavelength of 595nm and 655nm, on a Biorad Microplate Reader.

3.2.3.8 The MTT Bioassay on Whole Blood

Whole venous blood (60ml), from the same donor, was collected in 5ml sterile lithium heparin tubes. A 2ml aliquot of the blood was treated with a 1ml aliquot of each different concentration of toxin in sterile tubes. These tubes were then incubated for 12H at 37°C in a controlled environmental incubator shaker at 150 rpm. The lymphocytes were then extracted as outlined in 3.2.3.5. The isolated pellets from the individual and combined treatments were then resuspended in 1ml CCM and a 100µl aliquot of each suspension dispensed into 6 replicate wells of a 96 well microtitre plate. The MTT assay was then preformed as outlined in 3.2.3.7. This experiment was done in duplicate to ensure reproducibility.

A dose respondent graph was drawn using the mean values of the absorbance readings obtained in relation to increasing toxin concentration.

3.2.3.9 Statistical Analysis

All data were presented as means ± standard deviations (SD). The results of the cytotoxicity assay were analysed using the Student's t test. A probability (p) value less than 0.05 was considered statistically significant. Mean absorbencies were expressed as percentage cleavage activity in comparison to the control cells (100%).

$$\text{Cleavage activity (\%)} = \frac{\text{mean absorbance of toxin treated cells}}{\text{mean absorbance of control cells}} \times 100$$

$$\text{Cell mortality} = 100\% - \%\ \text{Cleavage activity}$$

3.3 Results and Discussion

The MTT assay offers a quantitative, convenient method for evaluating a cell population's response to external factors, whether it is an increase in cell growth, no effect, or a decrease in growth due to necrosis or apoptosis. Among the applications for this method are drug sensitivity, cytotoxicity, response to growth factors and cell activation. This study looked at both the individual and combined cytotoxic effects of AFB_1 and FB_1 on isolated normal human lymphocytes using the MTT assay. Different time periods (12, 24, 48, 72 and 96 hours) were used to assess whether the cytotoxic effects of the mycotoxin were sub-acute, acute or chronic.

After 12 hours incubation, AFB_1 was shown to be significantly cytotoxic to normal human lymphocytes at the lower concentrations of 5μM and 10μM with a percentage cell mortality of 40% and 41% respectively (Figure 3.4). This correlates with a study by Corrier *et al.* (1991) which showed that when immune cells were directly treated with AFB_1, cell proliferation and antibody formation were decreased. Aflatoxin B_1 is both lipid and water soluble, which enables it to pass easily through cell membranes and into cellular organelles (Massey *et al.*, 1995).

Many of the cytotoxic effects of AFB_1 require its bioactivation to the 8,9 epoxide metabolite by a cytochrome P450 (P450) dependent polysubstrate monooxygenase enzyme. It has been shown that cytochrome P450 is expressed in human peripheral blood lymphocytes (Song *et al.*, 1990; Haufroid *et al.*, 2003). Once activated, some of the epoxide will bind covalently to macromolecules to form protein and DNA adducts (Eaton and Gallagher, 1994). These results indicate that human lymphocytes have the ability to rapidly biotransform AFB_1 to its epoxide at lower concentrations, thereby causing cytotoxicity and other functional alterations.

Fumonisin B_1 has been characterised as a tumour initiator and a tumour promoter (Rumora *et al.*, 2002). It has been also been shown that FB_1 can exhibit both proliferation enhancing (Tolleson *et al.*, 1996; Lim *et al.*, 1996); and growth-inhibiting effects (Dombrink –Kurtzman *et al.*, 1994; Tolleson *et al.*, 1996) in a panel of cultured cell lines. Therefore it can probably be assumed that a final toxic effect of FB_1 will depend on the balance between the relative intensity of these two counteracting processes. After normal human lymphocytes were treated with FB_1 for 12 hours, all the concentrations had a greater than 100% cell viability, possibly due to the mitogenic properties of FB_1.

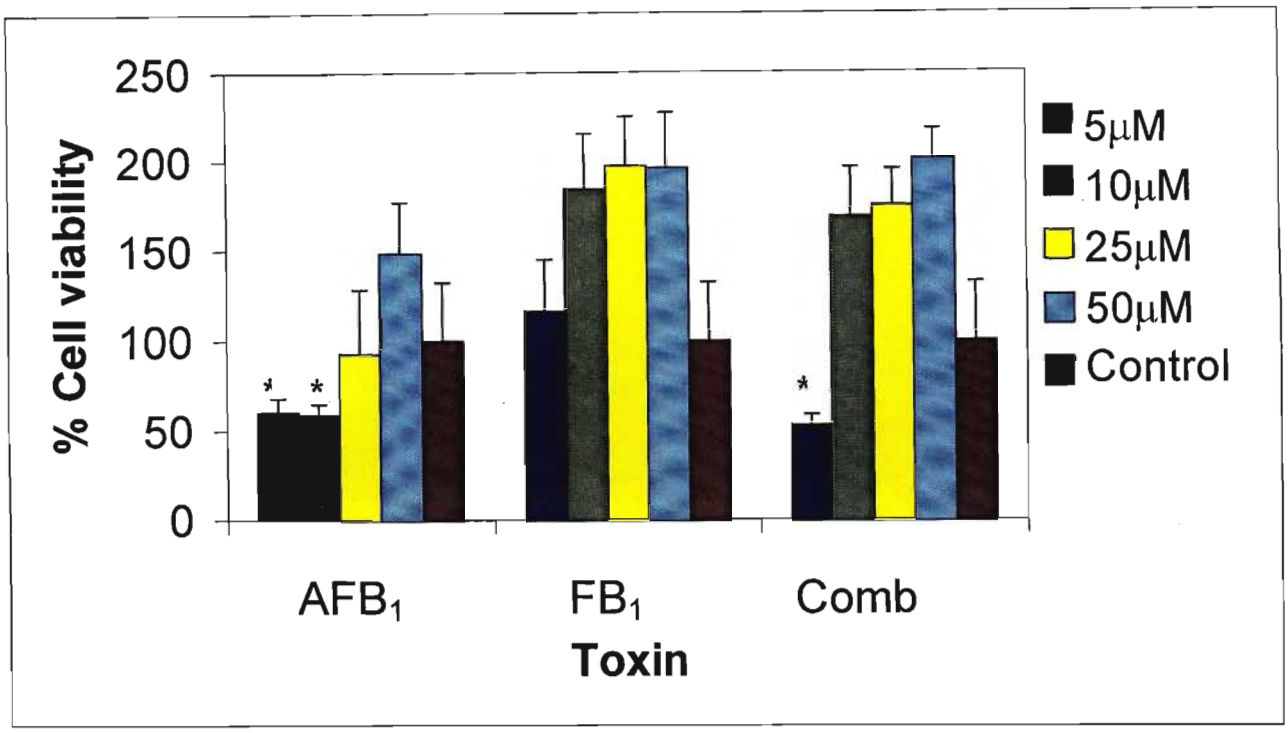

Figure 3.4: Bar graph showing the individual and combined effect of Aflatoxin B_1 and Fumonisin B_1 on normal human lymphocytes after an incubation period of 12 hours.

* = significant difference from the control ($p < 0.05$) using Student's t test.

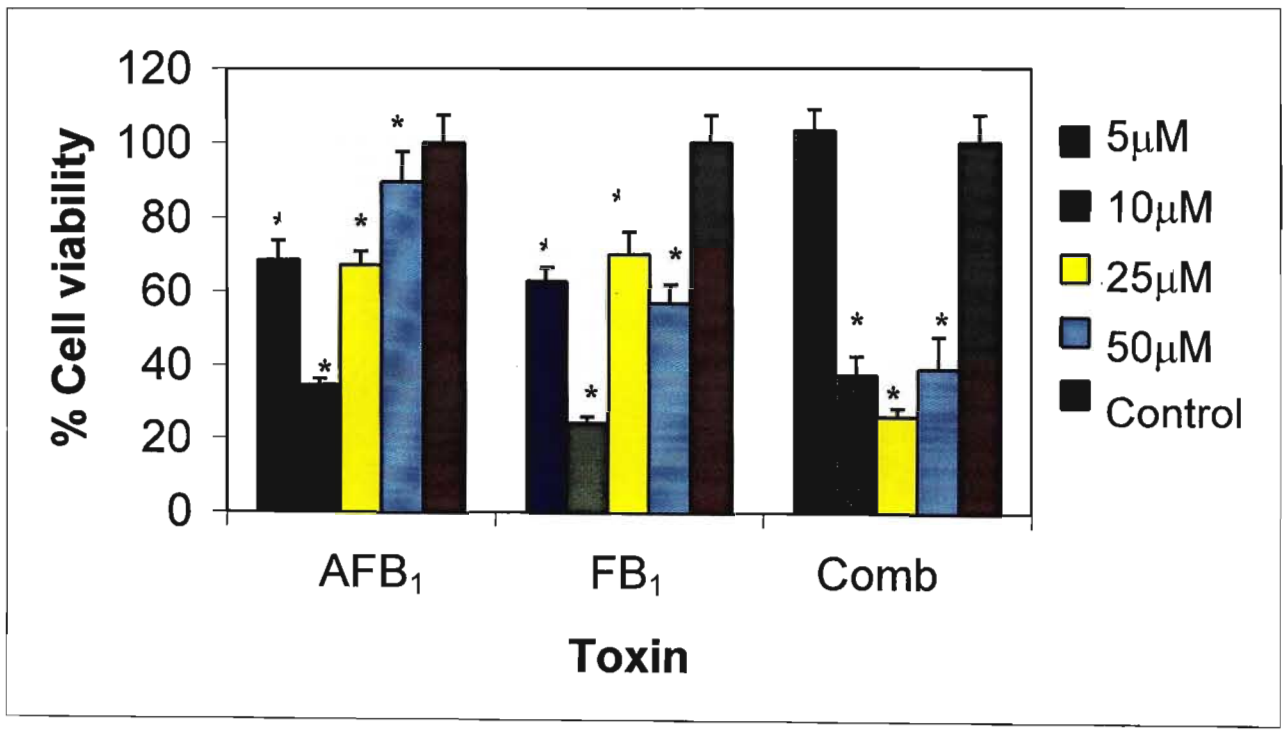

Figure 3.5: Bar graph showing the individual and combined effect of Aflatoxin B_1 and Fumonisin B_1 on normal human lymphocytes isolated after treatment of whole blood with toxin for an incubation period of 12 hours.

* = significant difference from the control ($p < 0.05$) using Student's t test.

The presence of an amino group and the location of the hydroxyl on the C_{14}/C_{15} of FB_1 may play an important role in both the toxic and cancer initiating activities of FB_1. It has been shown that the presence of the amino group facilitates the conjugation of FB_1 via gluteraldehyde to protein carriers (Azcona-Oliviera *et al.*, 1992). This could provide a plausible mechanism by which FB_1 exerts its mitogenic effect.

A single compound may effect different reactions within one biological system while it may also exhibit additive, antagonistic, or synergistic interactions with other compounds (Carpenter *et al.*, 1998). Several studies have reported on the co occurrence of AFB_1 and FB_1 (Gao and Yoshizawa, 1997; Ueno *et al.*, 1997; Zhang *et al*, 1997). The co-occurrence of mycotoxins together with various endogenous and exogenous risk factors such as nutrition, viral infections, alcohol and tobacco that may modulate cell proliferation and drug metabolism, are known to act synergistically with respect to the development of cancer in animals and possibly in humans (Yu *et al.*, 1996). The multiplicity and complexity of these interactions needs to be elucidated in order to clarify the adverse health and environmental effects of a mixture of mycotoxins and their subsequent regulation.

The combination of AFB_1 and FB_1 was shown to be cytotoxic to isolated lymphocytes only at the 5μM concentration. When comparing this data to the individual cytotoxicity data for the toxins, it suggests that at the lowest combined concentration, the AFB_1 was cytotoxic to the normal human lymphocytes, with the FB_1 having minimal effects. From 10μM concentration, it appears that the proliferation enhancing effects of FB_1 negated the cytotoxic properties of AFB_1. This could indicate that at lower concentrations FB_1 is more effective at inducing cytotoxicity. However as can be seen from the lowest concentration, this does not necessarily mean that the combination of AFB_1 and FB_1 is not potentially harmful to normal human lymphocytes.

The treatment of normal human lymphocytes with mycotoxins and determining their cytotoxic effects using the MTT assay can provide useful information about the cytotoxic potential of theses toxins. However as this is an *in vitro* situation, it is not necessarily an accurate representation of what actually occurs when mycotoxins are absorbed into the human blood stream. Human blood not only consists of lymphocytes, but also contains many different types of cells such as red blood cells, neutrophils and macrophages. As a result it is extremely difficult to recreate the complex mechanisms and interactions between the different cells present in an *in vivo* situation which may contribute to the cytotoxicity of the toxins to normal human lymphocytes.

Whole blood was treated with AFB_1 and FB_1 and incubated for 12 hours. The cell numbers, toxin concentrations and time period were kept consistent with the treatment of the isolated normal human lymphocytes. Following incubation, the lymphocytes were extracted and their cell viability determined (Figure 3.5). When whole blood was treated with AFB_1 and FB_1 and the lymphocytes extracted, all the concentrations except for the 5μM combination treatment, were shown to be cytototoxic to the lymphocytes. This effect was more pronounced at the 10μM concentration.

In contrast to treatment of isolated normal human lymphocytes, treatment of whole blood and then isolation of lymphocytes showed FB_1 to be very cytotoxic to the lymphocytes. Different studies have shown that FB_1 is only slightly cytotoxic after 48 hours. Liu *et al.* (2002) found no statistically significant decrease in primary swine alveolar macrophages after treatment for 48 hours. Cawood *et al.* (1994) noted a low cytotoxic response in primary hepatocytes. However FB_1 has also been shown to be cytotoxic to certain mammalian cell lines (Abbas *et al.*, 1993; Shier *et al.*, 1991), suggesting that some tissue may accumulate over time or may be more susceptible to the cancer-promoting activity of FB_1. Quereshi and Hagler (1992) reported that the exposure of chicken peritoneal macrophages to 10μg/ml of FB_1 for only 2 hours could reduce the cell viability to 80% of the controls.

Fumonisin B_1 is known to be a competitive inhibitor of sphingosine and sphinganine *N* aceyltransferase, which are key components in the pathways for *de novo* biosynthesis of sphingolipids (Wang *et al.*, 1991). The effects of sphingolipids on cell growth are complex. Free sphingoid bases both stimulate and inhibit cell growth and therefore the accumulation of sphinganine in FB_1 treated cells is likely to play a role in the alteration of cell growth and viability by fumonisins (Merrill *et al.*, 1996). Studies with a renal epithelial cell line, LLC-PK_1, have shown a close association between the concentrations of FB_1 that inhibit sphingolipid biosynthesis and cause growth inhibition and toxicity. This inhibition is supposed to be an early event in the cytotoxicity of FB_1 (Yoo *et al.*, 1992).

The greater cytotoxic effect exerted by FB_1 on lymphocytes isolated from treated blood could be attributed to the presence of other cells. In an *in vivo* situation, FB_1, which is poorly absorbed would be rapidly excreted (Dutton, 1996). Consequently less FB_1 would enter the lymphocytes and that would result in fewer-free sphingoid bases. It is difficult to establish the factor(s) that determine if the sphingoid bases are growth stimulatory, growth inhibitory or cytototoxic.

However previous studies have shown that FB_1 is more cytotoxic at low concentrations. The cell viability of a rabbit kidney cell line (RK13) was reduced to 22% by treatment with 0.5μM FB_1 (Rumora *et al.*, 2002).

Although both FB_1 and AFB_1 are secondary metabolites of fungi, they have totally distinct chemical properties and structures, which lead to their different toxicological mechanisms (Yoo *et al.*, 1992). Generally, AFB_1 shows much higher cytotoxicity and carcinogenicity than FB_1 in either *in vivo* or *in vitro* systems (Kuiper-Goodman, 1995; Dutton, 1996). However in this case FB_1 was just as cytotoxic to human lymphocytes in treated blood as AFB_1.

Aflatoxin B_1 also had a very different cytotoxic profile when the lymphocytes were isolated from treated blood as compared to treated cultured normal lymphocytes. When normal human lymphocytes were treated with 5μM AFB_1, it was shown to be extremely cytotoxic with a cell mortality rate of 40 %. However in lymphocytes extracted from whole blood, the cell mortality is approximately 31%. This reduced cytotoxic effect is possibly due to the presence of red blood cells in the blood, which would absorb some of the AFB_1, thereby reducing the amount of AFB_1 taken up by the lymphocytes.

Once activated, AFB_1 has been shown to form protein adducts. Aflatoxin B_1-lysine has been identified as a major adduct in rat albumin and has also been identified in human plasma (Guengerich *et al.*, 2002). As a result when treated in whole blood, less AFB_1 would be absorbed into the lymphocytes and the cytotoxic effect would be reduced.

Another explanation for the reduced cytotoxic effect exerted by the lower concentrations of AFB_1 could be the presence of macrophages in whole blood. Macrophages are primarily the first line of defence against infectious agents (Skamene and Gros, 1983) and studies have shown that aflatoxin inhibits macrophage functions such as phagocytotic, chemotactic, microbiocidal and adherence activity (Neldon-Ortiz and Quereshi, 1991; Jakab *et al.*, 1994; Cusumano *et al.*, 1995). In the presence of 1.5μg of AFB_1, the cell viability of primary swine alveolar macrophages was reduced to 41% (Liu *et al.*, 2002). Dugyala and Sharma, (1996), showed that AFB_1 had a marked effect on peritoneal macrophage produced cytokines in mice. The levels of interleukin-1α (IL-1α) secreted proteins were significantly suppressed at all dosages and those of interleukin 6 (IL-6) and TNF at the high dose. However a low dose of AFB_1 only slightly decreased the protein levels expressed by the lymphatic IL2, IL3 and interferon γ. This would suggest that AFB_1 preferentially affects macrophage functions.

Having overcome the first line of defence, the macrophages, the AFB_1 could exert greater cytotoxic effects on the lymphocytes, which would possibly account for the reduced cell viability observed after treatment with the 10μM and 25μM AFB_1 concentrations. A saturation point appeared to be reached after the 10μM AFB_1 treatment with a cell mortality of approximately 63% possibly due to the saturation of the cell receptors.

The combination treatment of blood was shown to be cytotoxic in a dose dependent manner with a threshold point reached at 25μM, with a cell mortality rate of approximately 74%. It was suggested previously that during the combination treatment of cultured human lymphocytes at higher concentrations, the cellular proliferating properties of FB_1 negated the cytotoxic effects caused by AFB_1. Now both toxins appear to be working simultaneously to reduce cell viability by different mechanisms.

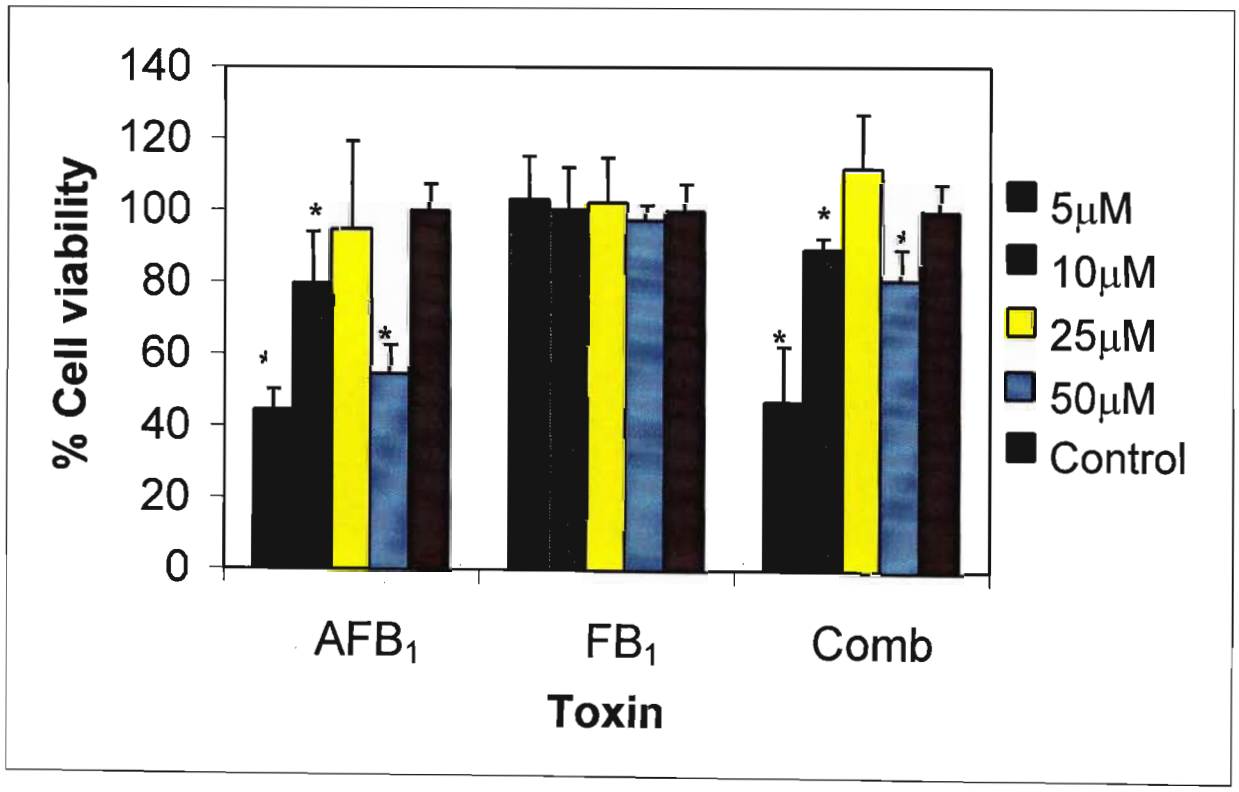

Figure 3.6: Bar graph showing the individual and combined effect of Aflatoxin B_1 and Fumonisin B_1 on normal human lymphocytes after an incubation period of 24 hours.

* = significant difference from the control ($p < 0.05$) using Student's t test.

After 24 hours incubation AFB_1 exerted a similar cytotoxic effect on normal human lymphocytes when compared to the 12 hours treatment. The 5μM AFB_1 was the most toxic with a percentage cell mortality of 45%. However, while the 10μM and 25μM AFB_1 concentrations were slightly cytotoxic with a cell viability of 80% and 95% respectively, the 50μM had a percentage cell mortality of 45% (Figure 3.6). AFB_1 has been shown to have significant mitogenic effects on human T lymphocytes (Griffiths *et al.*, 1996), which could account for the increase in cell viability after the 25μM AFB_1 treatment.

The other factor could be the carcinogenicity of AFB_1. Based on the Solt-Farber method model of chemical carcinogenesis in the rat liver (Solt and Farber, 1976), carcinogen-initiated cells develop into preneoplastic foci upon addition of a strong growth stimulus. Cytotoxicity following initiation can stimulate cell division and hence promotion of preneoplastic foci. It can be speculated that as AFB_1 is both a potent cytotoxin and as well as an initiator, it acts as a "complete carcinogen".

When compared with the 12 hours incubation time, there appears to be a balance between the cell proliferating and cytotoxic effects of FB_1. There are no or very little cytotoxic effects. This would seem to indicate that FB_1 exerts its cytotoxic effects chronically not acutely. Cytotoxicity is dependent on the ability of a molecule to bind cellular receptors and to penetrate the cell, which in turn is reliant on the size, structural conformation and polarity of the molecule (Myburg *et al.*, 2002). Fumonisin B_1 is a strongly polar molecule (Diaz and Boermans, 1994) and its polarity appears to play an important role in its cytotoxic behaviour. In general, less polar molecules have higher cytotoxicity (Gelderblom *et al.*, 1993), which could explain the low cytotoxic response observed on treatment with FB_1.

The cytotoxic profile of the combination and AFB_1 were very similar after 12 hour and 24 hour incubation. This would indicate that AFB_1 is the more dominant toxin especially at the lower concentrations. This is to be expected because AFB_1 when treated individually was significantly more cytotoxic than FB_1 on treated human lymphocytes. A study by Liu *et al.*, 2002 on primary swine alveolar macrophages found that AFB_1 was at least ten fold more cytotoxic than FB_1. The cell viability of the alveolar macrophages was reduced to 80% by 5μg/ml FB_1, whereas 0.5μg AFB_1 was required for the same cytotoxic effect.

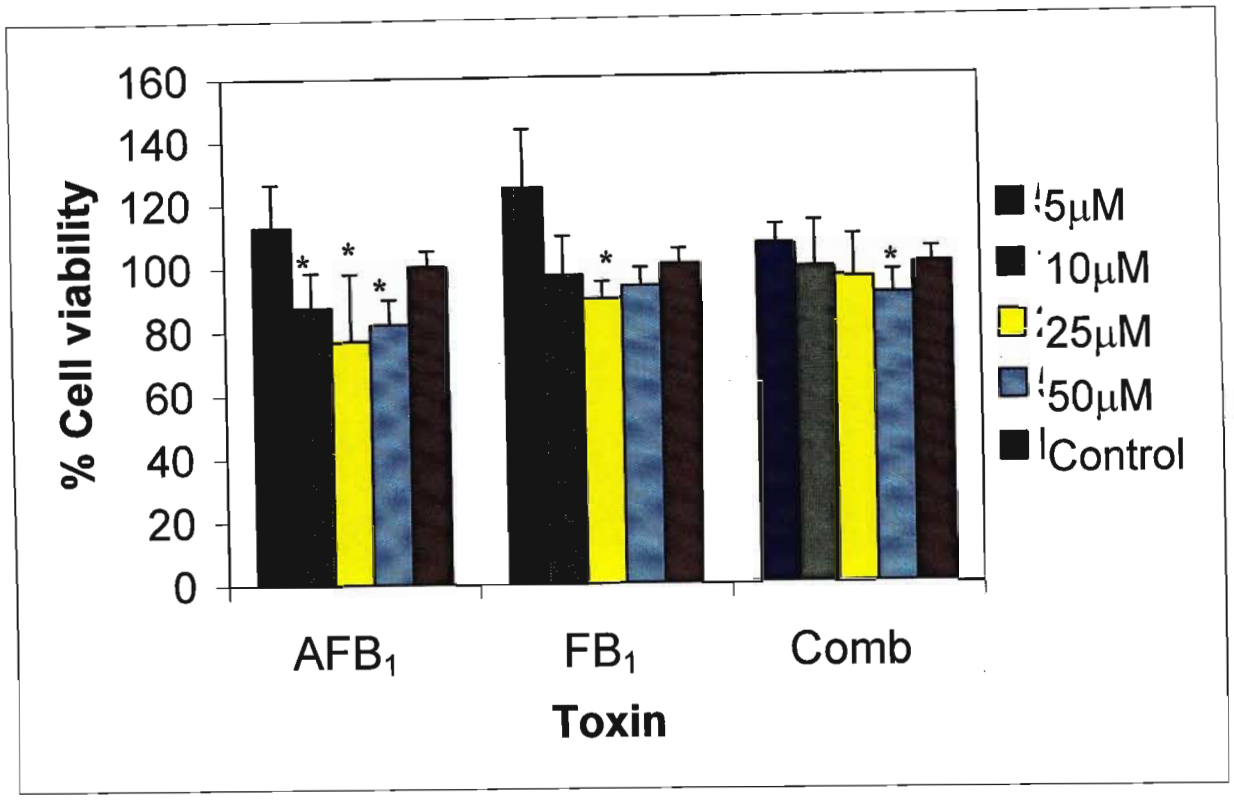

Figure 3.7: Bar graph showing the individual and combined effect of Aflatoxin B_1 and Fumonisin B_1 on normal human lymphocytes after an incubation period of 48 hours.

* = significant difference from the control ($p < 0.05$) using Student's t test.

Aflatoxin B_1 was cytotoxic to normal lymphocytes in a dose dependent manner after 48 hours exposure. A saturation point at 23% cell mortality appears to be reached at 25μM concentration. At 24 hours exposure, the 5μM AFB_1 treated cells have a cell viability of 45% whereas at the same concentration after 48 hours the cell viability was greater than 100%. It is possible that the cell is now able to detoxify the activated AFB_1 using glutathione S-Transferases (GST).

Although the necessity for AFB_1 activation to AFB_1-8.9 epoxide prior to DNA binding and carcinogenesis is well accepted, the levels of activating enzymes are not the sole determinates of sensitivity to AFB_1 toxicity. Activities of detoxifying biotransformation pathways are also critical and the GST catalyzed conjugation of activated AFB_1 is thought to be the most important detoxification system of the cells. The GST's are a group of cytosolic and microsomal enzymes that catalyse the conjugation of reduced glutathione. Since conjugation of the electrophilic AFB_1-8, 9-epoxide with the reduced glutathione is an alternate fate to binding to nucleophilic sites in cellular macromolecules, GST isoenzymes play a key role in the protection of cells from AFB_1 toxicity (Neal and Green, 1983).

After 48 hours exposure Fumonisin B_1 is very slightly cytotoxic with the highest cell mortality rate of 11% being reached after treatment with the 25μM concentration which, may suggest that the cytotoxic effects of FB_1 are chronic. The low cytotoxic response is in agreement with other studies. Myburg *et al.* (2002) showed minimal cytoxicity in the human oesophageal carcinoma cell line, SNO, after 48 hours treatment with FB_1 at similar concentrations. The combination was cytotoxic in a dose dependent manner with only the 50μM treatment being significantly cytotoxic. It appears that the cytotoxic effects exerted by AFB_1 were partially countered by the mitogenic properties of FB_1.

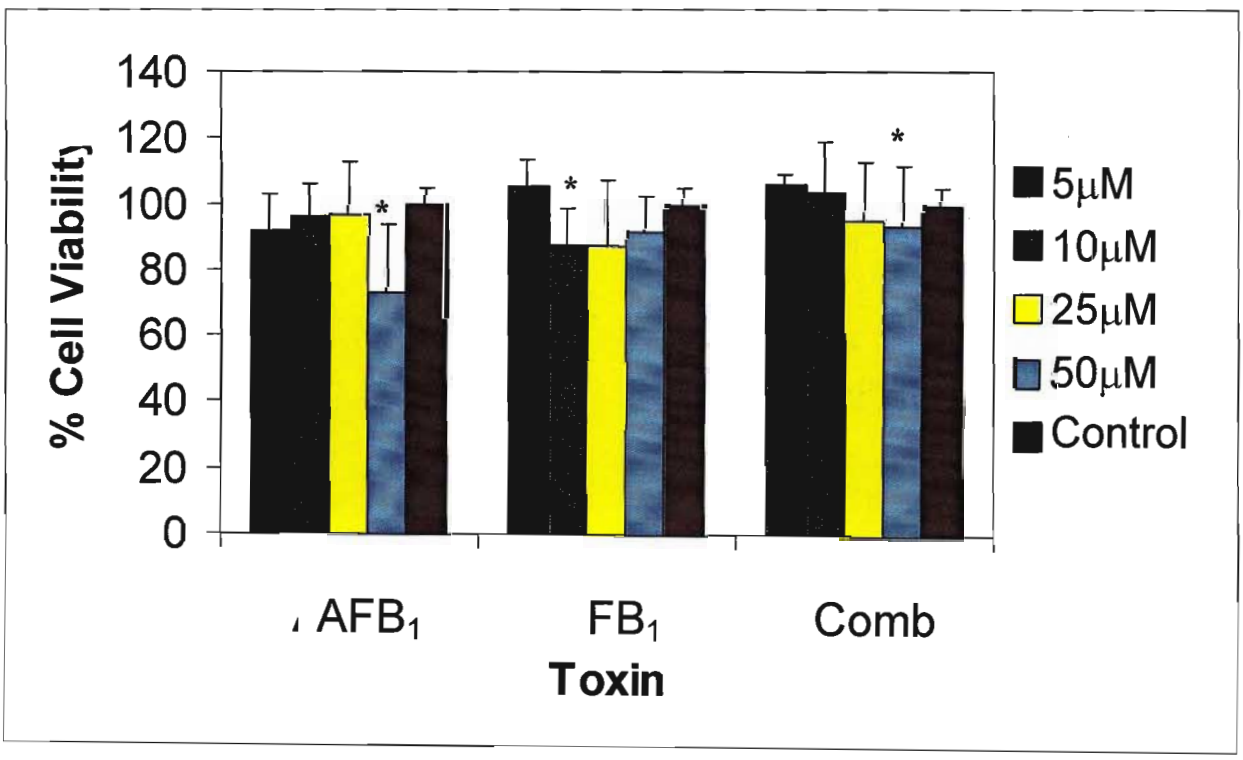

Figure 3.8: Bar graph showing the individual and combined effect of Aflatoxin B_1 and Fumonisin B_1 on normal human lymphocytes after an incubation period of 72 hours.

* = significant difference from the control ($p < 0.05$) using Student's t test.

The cytotoxic profile of AFB_1 treated cells after 72 hours exposure is similar to that after 24 hours exposure with cell mortality rate of the 10μM and 25μM concentrations being lower than the 5μM and 50μM concentration. It is possible that at the lower concentrations, the majority of the AFB_1 has formed conjugates with GSH and thereby prevented from binding to protein and DNA, reducing its cytotoxic effect. However at the 50μM concentration all the free GSH present in the lymphocytes had undergone conjugation with AFB_1 and there was still excess toxin present, resulting in greater cytotoxicity.

Similarly to the 48 hours exposure results, FB_1 and the combination were shown to be slightly cytotoxic to the isolated human lymphocytes in a dose dependent manner. However the only significantly cytotoxic concentration of FB_1 was the 10μM concentration with a cell mortality rate of 13%.Several processes are known to play a role in the molecular events leading to cell damage: lipid peroxidation, covalent binding of xenobiotics or their metabolites to biomolecules and inhibition of the synthesis of cellular molecules. Once lipid peroxidation is initiated, it can easily be propagated in the cellular environment by radical mediated chain reactors (Abado-Becongree *et al.*, 1998). Malondialdehyde (MDA) and a wide range of oxidation products are formed as a result of this chemical process. Formation of MDA and other aldehydes is regarded as significant as these compounds are toxic (Ennamany *et al.*, 1995). Fumonisin B_1 has been shown to be a potent inducer of MDA (Abado-Becongree *et al.*, 1998), which indicate that lipid peroxidation is a very sensitive cellular response to FB_1, at concentrations lower than that required to inhibit cellular synthesis of macromolecules, protein and DNA. This could be a potential mechanism of FB_1 cytotoxicity.

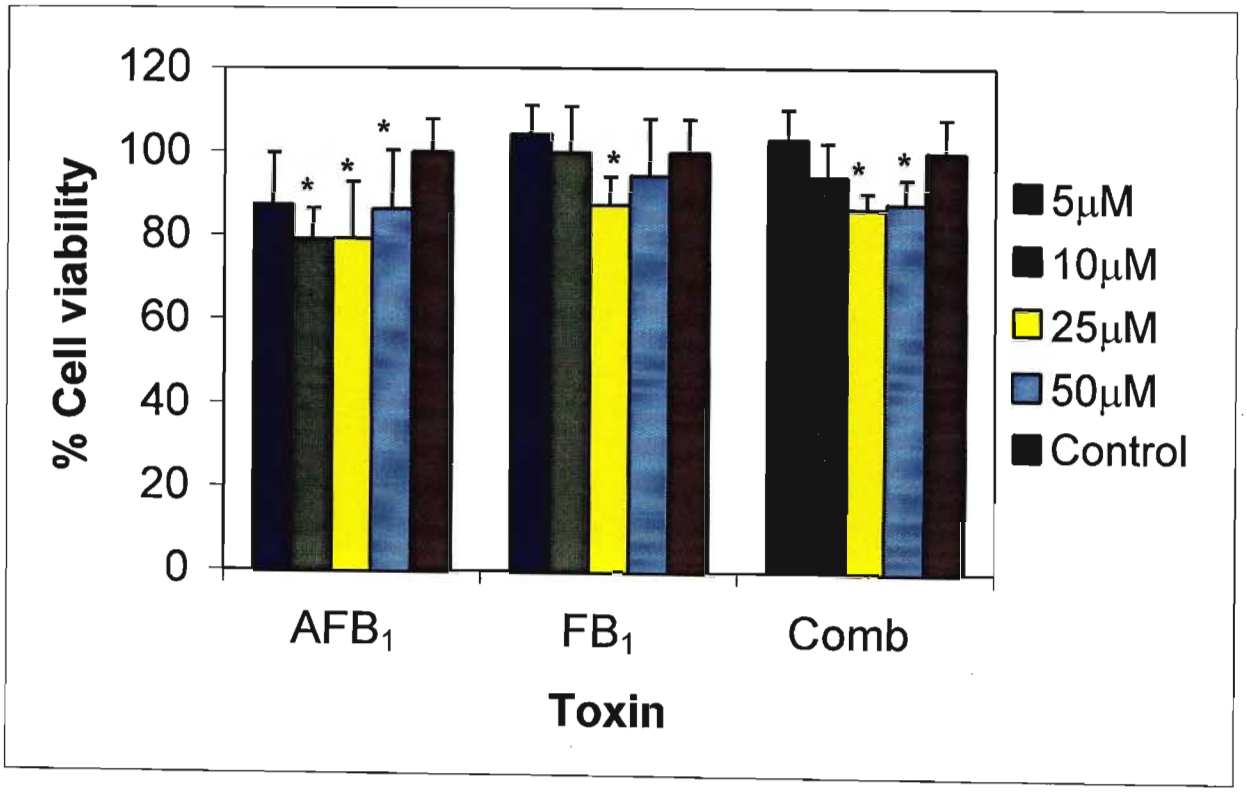

Figure 3.9: Bar graph showing the individual and combined effect of Aflatoxin B_1 and Fumonisin B_1 on normal human lymphocytes after an incubation period of 96 hours.

* = significant difference from the control ($p < 0.05$) using Student's t test.

After 72 and 96 hours exposure of normal human lymphocytes to FB_1, there were very limited cytotoxic effects. This is in agreement with a study by Galvano *et al*, (2002a) on human fibroblasts, when it was found that FB_1 at concentrations of 10, 50 and 100μM did not affect cellular viability after 72 and 96 hours exposure. It also correlates with another study of Galvano *et al.*, (2002b), which indicated that FB_1 treatment of astrocytes for 48 hours, 72 hours and 6 days *in vitro*, at 10, 50, 100 μM concentrations, does not affect cell viability.

Aflatoxin B_1 was significantly cytotoxic to normal human lymphocytes at low concentrations after 96 hours, which is to be expected. However it was not as cytotoxic as the lower concentrations at 12 hours and 24 hours suggesting that after 48 hours the lymphocytes have the ability to convert the epoxide to less potent metabolites primarily by the glutiathione S-transferases.

The cell viability of the combination treated cells decreases as the concentration of toxin increases. This would indicate that AFB_1 and FB_1 are working in conjunction to reduce cell viability. In a study by Gelderblom *et al.*, (2002) it was shown that when rats were treated in a sequential manner with AFB_1 and FB_1, it induced far more toxic effects than the individual treatments. It was hypothesised that in addition to the cancer promoting activity of FB_1 of AFB_1 initiated hepatocytes, the AFB_1 pre treatment enhanced the FB_1 initiating potency, presumably by rendering the liver more susceptible to the toxic effects of FB_1. It is possible that AFB_1 and FB_1 interacted in a similar manner to induce cytotoxic effects in normal human lymphocytes.

Although both FB_1 and AFB_1 are secondary metabolites of fungi, they have totally distinct chemical properties and structures, which lead to the different toxicological mechanisms (Yoo *et al.*, 1992). This is turn will determine how sensitive a cell is to the mycotoxin. It was difficult to determine the potential cytotoxicity of the combination as the mitogenic properties of FB_1 appeared to partially counteract the cytotoxic effect exerted by AFB_1.

A summary of the cytotoxic effects of AFB_1 and FB_1 is shown in the Table 3.1 below.

Table 3.1: Summary of the cytotoxic effects of Aflatoxin B_1 and Fumonisin B_1, individually and in combination on isolated normal human lymphocytes and lymphocytes extracted from treated whole blood using the Methyl tetrazolium assay.

	Whole blood	Isolated normal human lymphocytes				
	12 hours	12 hours	24 hours	48 hours	72 hours	96 hours
5μM AFB_1	68.57%*	60.20%*	45.57%*	112.93%	91.82%	87.30%*
10μM AFB_1	34.68%*	58.91%*	79.81%*	87.48%*	95.84%	79.20%*
25μM AFB_1	67.15%*	92.48%	94.74%	76.77%*	96.41%	79.27%*
50μM AFB_1	89.60%*	148.71%	54.73%*	81.92%*	73.39%*	86.31%
5μM FB_1	62.80%*	116.42%	103.08%	124.62%	105.16%	104.35%
10 μM FB_1	24.38%*	184.38%	100.27%	97.08%	87.09%*	100%
25μM FB_1	70.06%*	197.48%	102.19%	89.57%*	87.46%	87.46%*
50μM FB_1	56.87%*	196.35%	97.47%	93.18%	91.68%	94.39%
5μM combination	103.31%	53.33%*	46.98%*	106.26%	105.88%	103.12%
10 μM combination	37.48%*	168.67%	89.30%*	98.75%	103.64%	94.13%
25μM combination	26.03%*	175.10%	111.73%	95.83%	95.41%	86.44%*
50μM combination	38.99%*	201.34%	81.08%*	90.26%*	93.83%*	87.74%*

*=significant difference from the controls ($p< 0.005$) using Student's t test

3.4 Conclusion

Generally AFB_1 showed much higher cytotoxicity to isolated human lymphocytes than FB_1 especially at lower concentrations after 12 and 24 hours incubation. However when whole blood was treated with the same concentration and ratio of toxin FB_1 was even more toxic to the lymphocytes than AFB_1. The MTT assay has been shown to be a functional cytotoxicity assay, which is highly valuable because of its objective evaluation. It has also been shown to be a fast and efficient colorimetric assay for testing the cytotoxicity of AFB_1 and FB_1 on normal human lymphocytes and for the comparison of their individual and combined cytotoxic potential. However the use of the MTT assay as described here can only provide information on the cytotoxic properties, which are characteristic of certain, but not all compounds.

CHAPTER 4

The effect of Aflatoxin B_1 and Fumonisin B_1 on apoptosis in cultured human lymphocytes

4.1 Flow Cytometry

Cytometry refers to the measurement of physical and chemical characteristics of cells, or, by extension, of other biological particles. Flow Cytometry is a process in which such measurements are made while the cells or particles pass, preferably in single file, through the measuring apparatus in a fluid stream. These measurements are made at a routine rate of 500 – 4000 cells per second in a moving fluid stream (Shapiro, 1994).

4.1.1 Properties Measured by Flow Cytometry

Most flow cytometers can measure two kinds of light from cells, light scatter and fluorescence.

4.1.1.1 Light Scatter

Light scatter is the interaction of light and matter. All materials, including cells, will scatter light. Light scatter is composed largely of light that is reflected or refracted. The position from which an object is viewed often determines what can be concluded about it. In the flow cytometer, light scatter detectors are usually located opposite the laser (relative to the cell) and to one side of the laser, in-line with the fluid-flow/laser beam intersection. The measurements made by these detectors are called forward light scatter and side light scatter, respectively (Shapiro, 1994).

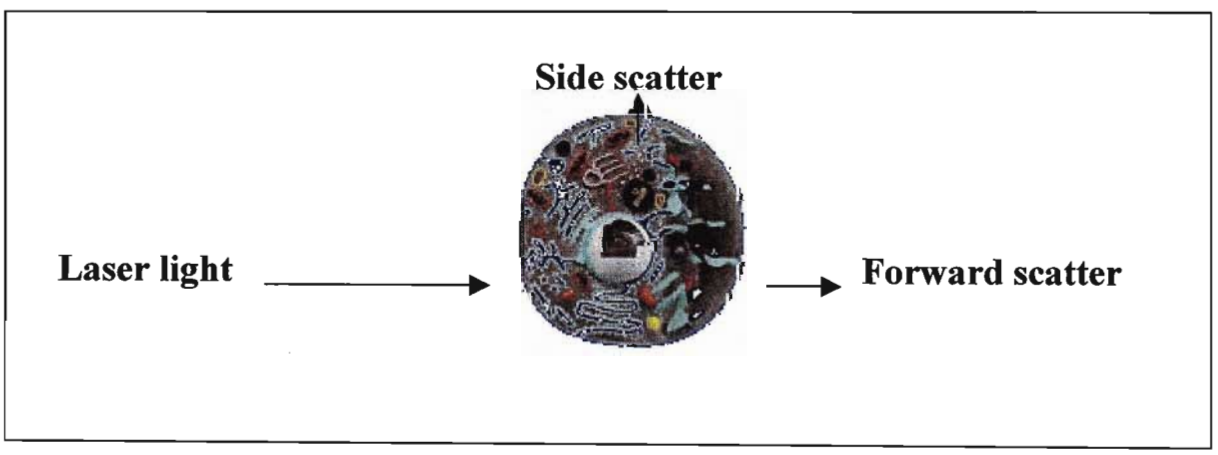

Figure 4.1: Properties of forward light scatter and side light scatter (BD biosciences, 2003).

Forward light scatter provides some information on the relative size of individual cells, whereas side light scatter provides some information on the relative granularity of individual cells. They are often used in combination to distinguish the different major categories of white cells in unseparated mammalian blood, but are useful in a wide variety of other assays as well (Shapiro, 1994).

4.1.1.2 Fluorescence

Fluorescence is the property of a molecule to absorb light of a particular wavelength and re-emit light of a longer wavelength. The wavelength change relates to an energy loss that takes place in the process. It is a characteristic that makes fluorescence extremely useful: filters may be used to exclude the excitation light from the light detector or the viewer. Thus, the only light measured or seen originates from the dye molecules. Interference from background or stray light striking the detectors is extremely low (Shapiro, 1994).

There are many fluorescent dyes that are useful for flow cytometry. They bind to a variety of cytochemical components, such as nucleic acids; proteins; specific cell-membrane, nuclear and cytoplasmic receptors; intracellular ion molecules; and many more. A key property of a fluorescent dye, which determines its potential for use in a flow cytometric assay, is the excitation wavelength: it must match the available wavelengths of the light source. In flow cytometers with lasers, this is most often 488 nm (Shapiro, 1994).

4.1.2 Basic Stages of Flow Cytometry

Flow cytometry involves six basic stages

- sample extraction and preparation,
- labelling,
- 'single file flow',
- laser illumination,
- fluorescence collection and detection and
- data analyses (Radcliff and Jaroszeski, 1998).

4.1.2.1 Sample Extraction and Preparation

Flow cytometry can analyse any cell type provided that the cells are freely suspended in a particle-free saline solution. Several different techniques are applicable depending upon the sample type (Radcliff and Jaroszeski, 1998).

4.1.2.2 Labelling

Flow cytometry ultimately depends upon the presence of specific chemicals in or on the surface of cells. Cells are small and do not naturally have enough molecules with strong fluorescence. For this reason, the cells are specifically labelled with dyes, which are efficient emitters of fluorescence. In order to obtain specific information about cells, the dyes are attached to biological molecules, which have specific affinity to a cell's molecules located on the surface or even within the cells. The range of labelling techniques currently available is extensive and is growing daily (Radcliff and Jaroszeski, 1998).

4.1.2.3 "Single File" Flow

When dilute solutions of the labelled cells are forced to flow through a narrow stream, the cells are forced to pass in "single file" fashion. This is typically done by using narrow capillary tubes or (more conventionally) through the use of hydrodynamic focusing – surrounding the sample stream with another saline liquid (cell free) at higher pressure, which controllably constricts the sample stream (Radcliff and Jaroszeski, 1998).

4.1.2.4 Laser Illumination

A laser is focused onto the sample stream in an effort to illuminate one cell at a time. The dye labels attached to cells are optically excited and fluorescence is emitted. A molecule typically emits a number of times depending upon the speed of the cell and the size of the focused spot (Radcliff and Jaroszeski, 1998).

4.1.2.5 Fluorescence Collection and Detection

The fluorescence light generated is emitted in all directions. It is attempted to collect as much of it as possible, filter out the scattered laser light and detect the rather weak signal using a fast and sensitive detector. Forward scatter detectors are photovoltaic photoiodides while fluorescence detectors are normally photomultiplier tubes (PMT). Light falling on the detector creates current, however PMT generates much more current per incident photon than a photovoltaic photoiodide (Radcliff and Jaroszeski, 1998).

4.1.2.6. Data Analysis

Fast detectors and signal processing equipment are critical for flow cytometry; as many as 100 000 cells/minute are typically analysed. Since not all cells of a particular type will have an identical signal, it is important to employ well-designed equipment, statistics and visualisation techniques to make sense of the data. In practice, a number of different parameters are investigated simultaneously including the use of more than one fluorescent label (Radcliff and Jaroszeski, 1998).

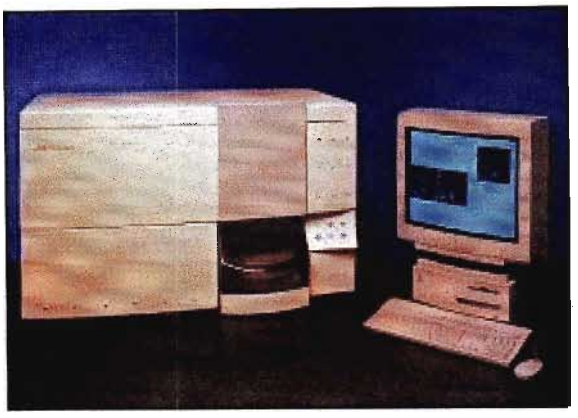

Figure 4.2: BD FACS Calibur benchtop cytometer (BD bioscience, 2003).

4.1.3 Detection of Apoptosis *in vitro* using Flow Cytometry

Apoptosis is a fundamental mode of cell death, which performs a regulatory function during normal development, in tissue homeostasis and in some disease processes (Wyllie, 1997). Cells undergoing apoptosis display typical changes in their morphological and physical properties (cell shrinkage, condensation of chromatin and cytoplasm), which are well measurable by flow cytometry. Thus, several methods have been developed for measuring apoptotic cell death through evaluation of light scattering parameters of cells (Darzynkiewicz *et al.*, 1992).

In viable cells, phosphatidylserine (PS) (Figure 4.3) is located on the cytoplasmic surface of the cell membrane. Upon induction of apoptosis, rapid alterations in the organisation of phospholipids in most cell types occurs leading to exposure of PS on the cell surface (Fadok *et al.*, 1992; Martin *et al.*, 1995). *In vitro* detection of externalised PS can be achieved through interaction with the anticoagulant annexin V (Boersma *et al.*, 1996; Koopman *et al.*, 1994; Frey, 1997). Annexin V is a Ca^{2+} -dependent phospholipid-binding protein with high affinity for PS (Vermes *et al.*, 1995). This protein can thus be used as a sensitive probe for PS exposure upon the outer leaflet of the cell membrane and is therefore well suited to detect apoptotic cells as shown in Figure 4.3 (Vermes *et al.*, 1995; Koopman *et al.*, 1994; Homburg *et al.*, 1995; Verhoven *et al.*, 1995) in cell populations but not on tissue sections.

Since necrotic cells also expose PS according to the loss of membrane integrity, apoptotic cells have to be differentiated from these necrotic cells. The simultaneous application of a DNA stain, propidium iodide (PI), which is used for dye exclusion tests, allows the discrimination of necrotic cells from the Annexin V positively stained cell cluster. In the presence of calcium, rapid high affinity binding of Annexin V to PS occurs. Phosphatidylserine translocation to the cell surface precedes nuclear breakdown, DNA fragmentation and the appearance of most apoptosis-associated molecules making annexin V binding a marker of early-stage apoptosis.

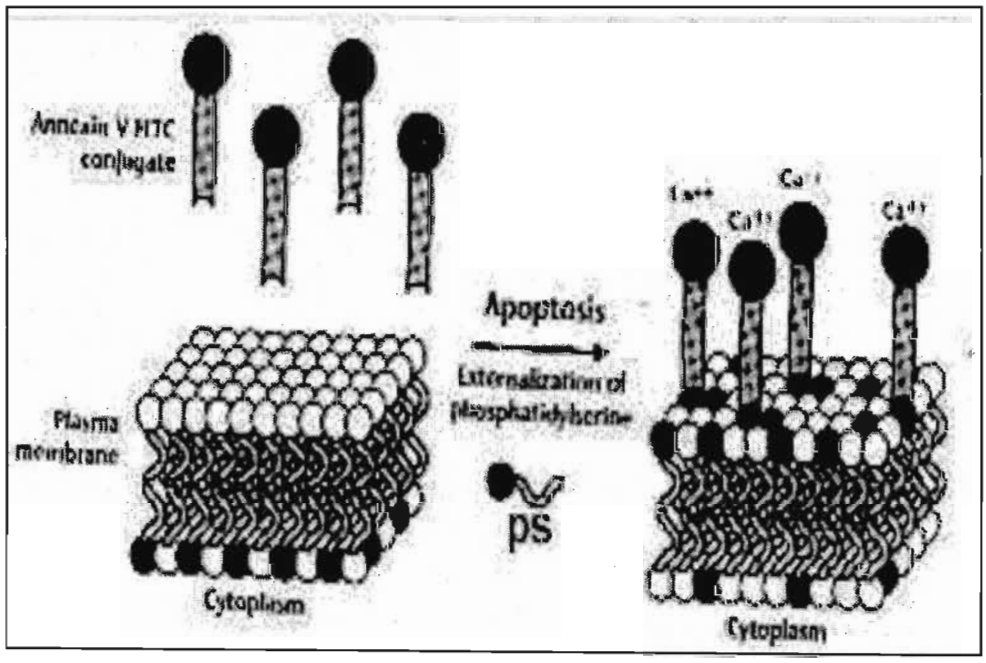

Figure 4.3: Principle of the Annexin V Fluos Staining kit (Roche, 2002).

The aim of this study was to determine whether AFB_1 and FB_1 induce apoptosis or necrosis in normal human lymphocytes, using the Annexin V Fluos staining kit.

4.2 Materials and Methods

4.2.1 Materials

The Annexin-V-Fluos Staining Kit was purchased from Roche Diagnostics, SA. 24 well microtitre plates were obtained from Adcock Ingram, while slides and coverslips were purchased from Shalom, SA. All other reagents and consumables used were obtained from the sources mentioned previously.

4.2.2 Methods

4.2.2.1 Isolation of Lymphocytes

Whole venous blood was collected and normal human lymphocytes were extracted using the procedure outlined previously in Section 3.2.3.5 to obtain a final concentration of 1X 10^6 cells/ml.

4.2.2.2 Treatment of Lymphocytes

Aliquots of the cell suspension (2mls) were dispensed into the individual wells of the 24 well microtitre plate to give a final cell count of 2 X 10^6 cells/well. After vortexing, a 1ml aliquot of the 5μM AFB_1, 50μM AFB_1, 5μM FB_1 and 50μM FB_1 serial dilutions were transferred to individual wells of the microtitre plate using a micropipette. This procedure was repeated with the ethanol and CCM controls. Combinations of the toxins were prepared as a 1:1 mixture of AFB_1 and FB_1 to the same well. The plates were then incubated at 37°C for 12 hours. After incubation the treated cells were transferred aseptically using a 3ml sterile pasteur pipette to a sterile sterilin tube. These cells were washed in HBSS (5ml; 37°C) by centrifugation (400xg; 20 minutes; room temperature). The HBSS washing step was repeated twice, all supernatants were discarded and finally the pelleted cells were resuspended in 400μl HBSS.

4.2.2.3 Treatment of Whole Blood

Whole venous blood (60ml) was collected in 5ml sterile lithium heparin tubes. A 2ml aliquot of the blood was treated with a 1ml aliquot of 5μM AFB_1, 50μM AFB_1, 5μM FB_1 and 50μM FB_1 in sterile tubes. A 500μl aliquot of the 5μM and 50μM concentration of AFB_1 and 500μl of the same concentrations of FB_1 were added to the same well to evaluate the apoptotic or necrotic inducing effects of the combination of mycotoxins. Complete culture medium and ethanol treatments were also prepared. These tubes were then incubated for 12 hours at 37°C in a controlled environmental incubator shaker at 150 rpm. The lymphocytes were then extracted as outlined in 3.2.3.5. The isolated pellets from the individual and combined treatments were then resuspended in 400μl HBSS.

4.2.2.4 Preparation of Staining Solution and Staining Procedure

Annexin-V fluorescein labelling reagent (20μl) was diluted in 1000μl Hepes buffer and a 20μl aliquot of Propidium iodide added. A 100μl aliquot of staining solution was added to each sample and the cells incubated in the dark for 15 minutes at room temperature.

4.2.2.5 Fluorescent Microscopy

The background to fluorescent microscopy and it value as an investigative technique will be discussed further in Chapter 6. For analysis by fluorescent microscopy a 20µl aliquot of the stained cells was placed on a glass slide and a coverslip placed on top. Apoptosis was visualised by viewing cells stained with Annexin V and PI using an inverted research microscope, Olympus I × 51 (Wirsam Scientific, SA) using an excitation wavelength in the range of 450-500nm and detection in the range of 515- 565nm. Images were then captured using the digital camera using a microscope digital camera system, an Olympus DP12 (Wirsam Scientific, SA).

By staining cells with a combination of fluoresceinated Annexin V and PI, it is possible to detect non-apoptotic live cells (Annexin V negative, PI negative), which appear a dull green, early apoptotic cells (Annexin V positive, PI negative), fluoresce a bright green and late apoptotic or necrotic cells (PI positive), which appear orange-red.

4.2.2.6 Flow Cytometry

4.2.2.6.1 Instrumentation

Flow cytometric analysis was preformed using a FACS Calibur (BD Bioscience) benchtop cytometer with standard argon ion laser settings (488nm) and filter sets. Data from the FACS Calibur were acquired and analysed by using Cell Quest (BD Bioscience).

4.2.2.6.2 Flow Cytometry Methodology

A 0.4ml aliquot of binding buffer provided in the Annexin V fluorescein kit was added to the stained treated cell suspensions. An excitation wavelength of 488nm and a 515nm bandpass filter was used for fluorescein detection and a filter with a wavelength greater than 600nm for PI detection.

4.2.2.6.3 Data Analysis

Four-parameter list mode data were acquired for analysis. Light scatter parameters were used to gate on single cells, with an acquisition threshold set on the forward scatter (FS) signal. Irradiated cells tended to have more small cell debris that was included above the FS threshold so as not to include them in the sort window and thus contaminate the sort population. Fluorescence signals were logarithmically amplified and fluorescence compensation was determined from unlabeled and single-labeled cell controls. Samples were run for several seconds before data acquisition to allow dye equilibration in the FACS Calibur sample tubing.

Cell populations differentially stained with Annexin V and PI were routinely gated as follows. Population 1 (Annexin negative, PI negative) was identified by gating on cells exhibiting a low FS. Populations 2 (Annexin positive, PI negative) and 3 (Annexin positive, PI positive) were identified by gating on cells exhibiting higher FS. Population 3 was distinguished from population 2 by its higher side scatter. Cellular debris, erythrocytes and platelets (very low scatter) were excluded from analysis gates when possible.

4.3 Results and Discussion

Aflatoxin B_1 and FB_1 are fungal metabolites widely present in feed and food crops all over the world. As long-term exposure to low levels of these mycotoxins cannot be completely avoided, they are likely to be a potential concern of occupational and public health. Few studies have looked at the effect of AFB_1 and FB_1 on the levels of apoptosis in human lymphocytes, which could be directly and indirectly affected through systematic administration. This study aimed to compare the apoptosis-inducing ability of AFB_1 and FB_1 at high/low concentrations and to determine their combined effect on the levels of apoptosis in human lymphocytes.

In live cells plasma membrane phospholipids are asymmetrically distributed between inner and outer leaflets of the plasma membranes. It has been recently shown that the loss of phospholipids asymmetry, leading to the exposure of PS on the outer surface of the plasma membrane, is an early event of apoptosis. The anticoagulant Annexin V preferentially binds to negatively charged phospholipids such as PS. Apoptotic cells react with Annexin V after the onset of chromatin condensation but prior to the loss of the plasma membranes ability to exclude PI as shown in Figure 4.4.

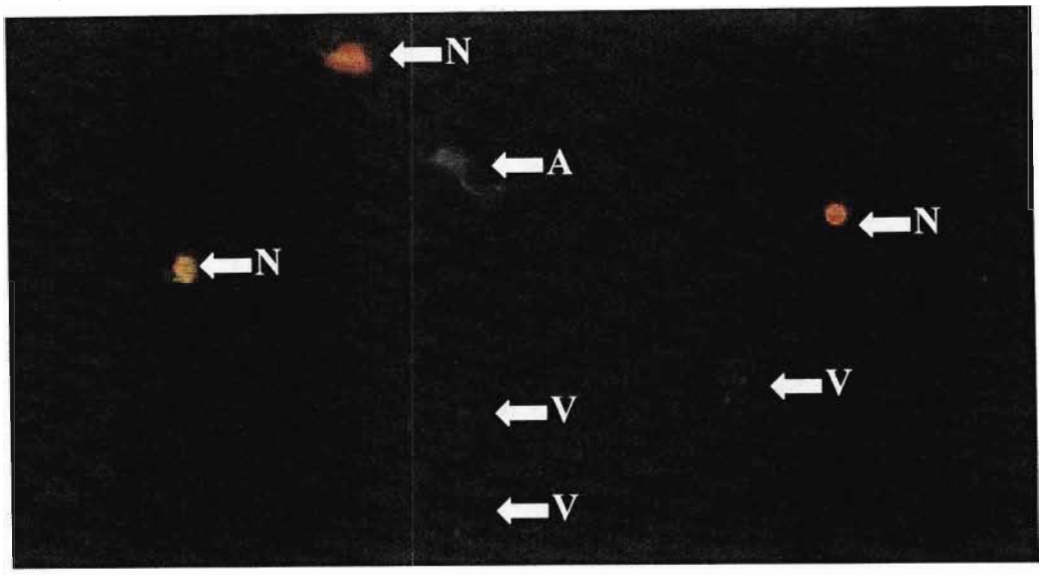

Figure 4.4: Picture of 5μM Combination treated lymphocytes stained with Annexin and Propidium Iodide (400X). A represents an apoptotic cell, N a necrotic cell and V, a viable cell.

The aim to determine differences in staining between apoptotic, necrotic and viable cells when viewed using a fluorescent microscope, was achieved. The level of apoptosis in the controls and untreated sample was accurately quantified using flow cytometry. The scatter plot and histogram obtained from the samples analyzed are shown below in Figure 4.5 and 4.6.

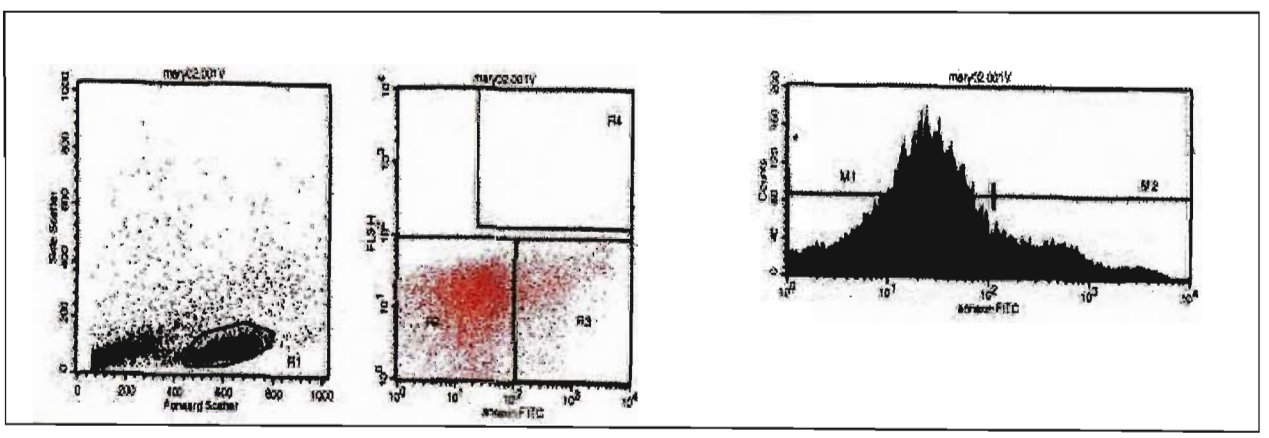

Figure 4.5: Annexin Fluorescien Isothiocyanate scatter plot and histogram of untreated control.

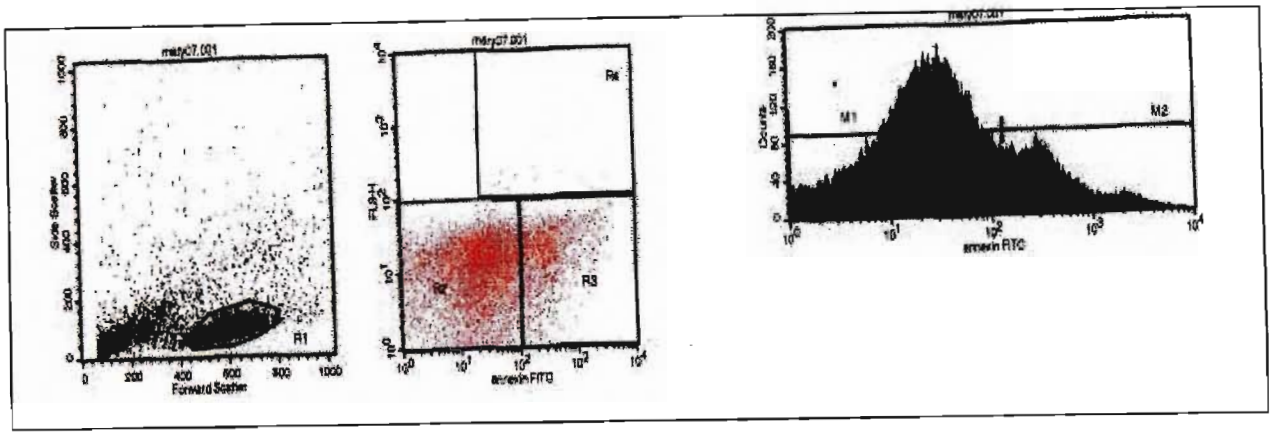

Figure 4.6: Annexin Fluorescien Isothiocyanate scatter plot and histogram of 50μM Combination treatment.

The scatter plot obtained was analysed, the various regions gated according to the procedure outlined in Section 4.2.4 and the results correlated into a table shown below.

Table 4.1: The percentage viable, apoptotic and necrotic cells in untreated and treated cultured human lymphocytes determined using flow cytometry.

Toxin	% Viable Cells gated	% Apoptotic Cells gated	% Necrotic Cells gated
Control	86.47	8.21	0.19
5μM AFB_1	82.25	17.09	0.17
50μM AFB_1	77.10	22.14	0.28
5μM FB_1	78.17	20.98	0.31
50μM FB_1	81.54	17.91	0.09
5μM Combination	77.28	22.17	0.13
50μM Combination	77.71	21.54	0.24

The 5μM FB_1 treatment resulted in increased levels of apoptosis, approximately 12.7% when compared to the control (Table 4.1). Numerous *in vivo* studies have demonstrated that FB_1 induces apoptosis in rat kidney and liver (Lim *et al.*, 1996; Tolleson *et al.*, 1996). It has also been shown that FB_1 induces apoptosis of several different human cell types.

When primary human keratinocyte cultures and transformed human oesophageal epithelial cells (HET-1A cells) were treated with FB_1, it was shown to inhibit cell growth and increase apoptosis (Tolleson 1996), demonstrating that FB_1 treatment can lead directly to apoptosis. Increased apoptosis after exposure to FB_1 *in vitro* has been reported in a number of cell lines such as turkey lymphocytes (Dombrink-Kurtzman *et al.*, 1994), HT29 human colonic cell line (Schmeltz *et al.*, 1998) and C_6 glioma cells (Mobio *et al.*, 2000). It was therefore expected that FB_1 would induce higher levels of apoptosis in the treated human lymphocytes.

It is thought that FB_1 inhibits the enzyme ceramide synthase, (Riley *et al.*, 2001) and therefore interrupts the ability of the cells to synthesise complex sphingolipids, resulting in the accumulation of intracellular sphingosine and sphinganine (Wang *et al.*, 1991), each of which has likewise been shown to down regulate protein kinase C (Ciacci-Zanella and Jones, 1999). As a result there should be a simultaneous decrease in cellular ceramides and complex sphingolipids, each of which has been shown to participate in signal transduction pathways involved in cellular apoptosis. Therefore regardless of whether the apoptotic mechanism involves the elevations of sphinganine or sphingosine, changes in ceramide or sphingolipids, or other yet identified cellular changes, the end result is the induction of apoptosis and a resulting compensation in cell proliferation.

As apoptosis can play a critical role in the development of cancer or disease, the ability of FB_1 to induce apoptosis appears to be important with respect to its toxicological effects (Tolleson 1996). Disruption of sphingolipid metabolism and regulatory function are therefore likely to critical for cytotoxicity, apoptosis and carcinogenicity resulting from FB_1 exposure. There is also evidence that other factors are also involved, including the cytokine, TNFα, which is responsible for the regulation of apoptosis and cellular replication.

It has also been shown that FB_1 is more cytotoxic at lower concentrations in various studies on human cell lines, Myburg *et al.*, 2002 showed minimal cytoxicity in a human esophageal carcinoma cell line, accounting for the increase in necrosis shown in the 5μM FB_1 treatment. In the 50μM FB_1 treated lymphocytes, higher levels of apoptosis were induced but the levels of necrosis had decreased. Fumonisin B_1 has been shown to exhibit both proliferation enhancing (Tolleson *et al.*, 1996; Lim *et al.*, 1996); and growth-inhibiting effects (Dombrink –Kurtzman *et al.*, 1994). It is probable that at the higher concentration, FB_1 is acting as a mitogen. This hypothesis is supported by the increase in percentage viable cells when compared to the lower FB_1 concentration.

When cultured human lymphocytes, were treated with AFB_1 both concentrations induced increased levels of apoptosis, when compared to the control. Caspase-3 is one of cysteine proteases, which plays a major role in the execution of apoptosis. A number of genetic and biochemical studies suggest that caspase activation is essential for the occurrence of the apoptotic phenotype of cell death. A variety of caspase substrates are involved in the regulation of DNA structure, repair and replication. Caspase–3 substrate cleavage has been observed under oxidative stress in different pathological conditions.

Aflatoxin B_1 is one of the most potentially dangerous mycotoxins known due to its high toxicity towards both animals and humans. One of the manifestations of AFB_1-induced toxicity is oxidative stress, free radicals etc. It has been accepted that oxidative stress is an apoptosis inducer (Chandra *et al.*, 2000). Many agents that induce apoptosis are either oxidants or stimulators of cellular oxidative metabolism apoptosis. Conversely, many inhibitors of apoptosis have antioxidant activities or enhance cellular antioxidant defences (Freeman *et al.*, 1982). The glutathione (GSH) redox cycle is an important component of the antioxidant machinery in cells. In normal cells, a primary defence against oxidative damage is provided by antioxidants such as GSH and the onset of apoptosis is associated with a fall in intracellular GSH in numerous cellular systems (Raton *et al.*, 1994). This is a potential mechanism whereby treatment of cultured human lymphocytes with AFB_1 will result in increased levels of apoptosis.

There was an increase in the percentage of necrotic cells gated in the 50μM AFB_1 concentration (Table 4.1). It is a well known fact that AFB_1 is extremely cytotoxic therefore it was to be expected that the levels of necrosis would be increased (Corrier *et al.*, 1991).

It can be seen that in both combination treatments, a higher level of apoptosis was induced thereby indicating that FB_1 and AFB_1 have the potential of working in conjunction to increase levels of apoptosis, using different mechanisms. The levels of necrosis were low in the 5μM combination treatment but increased by almost double at the higher concentration, probably due to the higher concentrations of toxin. As both mycotoxins are common contaminants this could be a potential area of concern.

Treatment of cultured human lymphocytes with mycotoxin and determination of their apoptotic inducing ability will help clarify their mechanism of toxicity. However it does not always reflect what happens when mycotoxins are absorbed into the blood stream of humans. This is because human blood is made up of many different types of cells for example red blood cells, lymphocytes, neutrophils and macrophages. As a result it is extremely difficult to re-create the complex mechanisms and interactions between the different cells which may determine if the toxin is harmful to normal human lymphocytes or not. In an attempt to gain clearer understanding of the apoptotic inducing ability of the AFB_1 and FB_1, whole blood was treated in the same ratio and at the same concentrations of toxin and incubated for the same time period, 12 hours, as treatment of normal human lymphocytes. Following incubation, the lymphocytes were extracted and the level of apoptosis determined. The scatter plot and histogram obtained from some of the samples analyzed using flow cytometry are shown below in Figure 4.7 to 4.8.

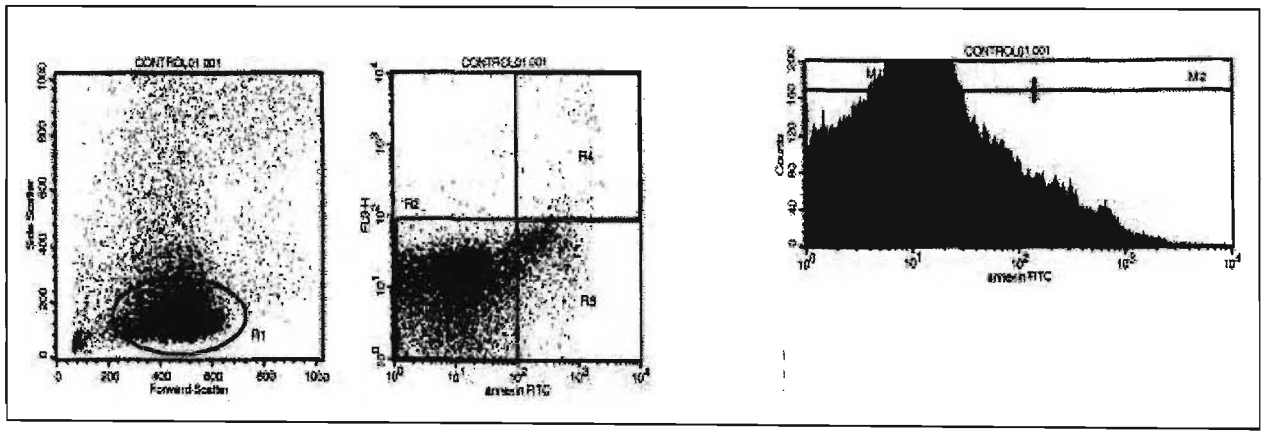

Figure 4.7: Annexin Fluorescien Isothiocyanate scatter plot and histogram of untreated control

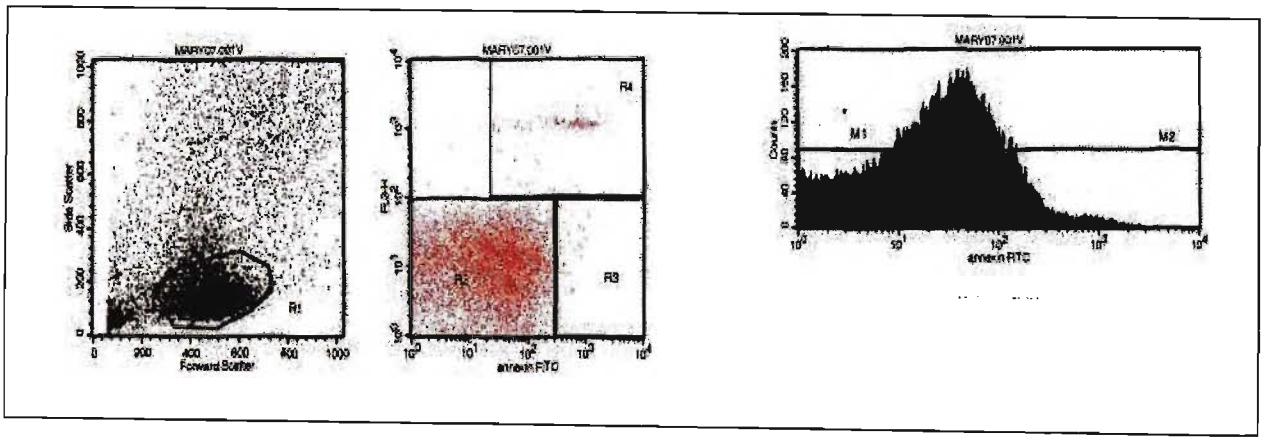

Figure 4.8: Annexin Fluorescien Isothiocyanate ITC scatter plot and histogram of 50μM Combination treatment.

The scatter plot obtained was analysed, the various regions gated and the results correlated into a table shown below.

Table 4.2: The percentage viable, apoptotic and necrotic cells in untreated and treated whole blood, followed by extraction of lymphocytes determined using flow cytometry.

Toxin	% Viable Cells gated	% Apoptotic Cells gated	% Necrotic Cells gated
Control	97.63	1.50	0.52
5μM AFB_1	97.44	1.74	0.43
50μM AFB_1	98.97	0.57	0.27
5μM FB_1	97.75	0.92	0.94
50μM FB_1	98.91	0.56	0.29
5μM Combination	96.74	1.72	1.10
50μM Combination	96.42	0.68	2.60

When comparing these results to those obtained from the treated lymphocytes, the percentage of viable cells in all treatments had increased and the corresponding percentage apoptotic cells decreased. However the levels of necrosis had increased in whole blood when compared to treated lymphocytes. There are a number of different factors that could be responsible for this. In whole blood, not only lymphocytes, other types of cells would also be present. As a result the complex interactions between the cells, used to protect them from foreign particles would be intact. Cellular mediators such as cytokines also play an important role in the regulation of the cell's defence.

Another factor could be the complex transport mechanisms between cells. For example in the AFB_1 treated lymphocytes: albumin (Wild *et al.*, 1990). Aflatoxin B_1-lysine has been identified as a major adduct in rat albumin and has also been identified in human plasma (Guengerich *et al.*, 2002). As a result when treated in whole blood, less AFB_1 would be absorbed into the lymphocytes and therefore less apoptosis induced. It could be seen that in the treated lymphocytes, the higher concentration of AFB_1 caused an increase in apoptotic and necrotic cells when compared to the lower concentration.

However in the treated whole blood the opposite trend was observed with the levels of apoptosis and necrosis decreasing. It is hypothesised that in the whole blood, the levels of AFB_1 absorbed into the cells and its bioconversion is controlled as discussed previously by the formation of conjugates with serum albumin. This could explain why the levels of necrosis were decreased in the higher concentration of AFB_1 treated whole blood.

In both treated lymphocytes and whole blood, the trend was for FB_1 to have lower apoptosis and necrosis at the higher concentration when compared to the lower concentration. The results also showed that the levels of apoptosis in whole blood were decreased in comparison to the treated lymphocytes, however the percentage necrotic cells was increased. As discussed previously FB_1 is a strongly polar molecule (Diaz and Boermans, 1994), which will affect the way it is absorbed into the cells and its mechanism of inhibiting sphingolipid biosynthesis. In whole blood FB_1 would probably be absorbed faster than in culture medium, therefore exerting greater cytotoxic effects and thereby increasing the levels of necrosis at lower concentrations. However at the higher concentration there were lower levels of apoptosis and necrosis. It is suspected that in response to increased levels of apoptosis, a cellular defence mechanism kicked in, possibly the excretion of the toxin and there was an increase in cell proliferation.

The combination treatment of AFB_1 and FB_1 at both concentrations resulted in higher levels of necrosis and lower percentage viable cells thereby suggesting that the toxins are working in conjunction via different mechanisms, to reduce cell viability. As both toxins are common contaminants and have been known to co-exist (Ueno *et al.*, 1997), this could be a potential area of concern for public health.

4.6 Conclusion

The detection of apoptosis using the Annexin V Fluos Staining Kit was shown to be a viable procedure for the determination of apoptotic inducing ability of AFB_1 and FB_1 in treated human lymphocytes. It was shown that FB_1 and AFB_1 working simultaneously resulted in increased levels of apoptosis. Possibly due to the cellular defence mechanisms the levels of apoptosis were reduced in whole blood lymphocytes when compared to isolated lymphocytes.

CHAPTER 5

Assessment of the potential genotoxic effects of Aflatoxin B_1 and Fumonisin B_1 on normal human lymphocytes

5.1. DNA Damage

Techniques which permit the sensitive detection of DNA damage have been shown to be very useful in the studies of environmental toxicology (Hartman and Speit, 1994), carcinogenesis (Lewensohn *et al.*, 1982) and ageing (Niedermuller *et al.*, 1985). Since the effects of environmental toxicants, cancer and ageing are often tissue and cell specific, it is important to develop techniques which can detect DNA damage in specific cells. However, until recently, the most frequently used methods involved either the detection of DNA repair synthesis (so-called unscheduled DNA synthesis or UDS) in individual cells, or the detection of DNA single stranded breaks (SSB) and alkali liable sites (ALS) in pooled cell populations using the alkaline elution assay. The UDS technique is based on the replication of DNA during the excision repair of certain types of DNA lesions, as demonstrated by the incorporation of tritiated thymidine into the DNA repair sites. While providing information at the level of the individual cell, the technique is technically cumbersome, requires the use of radioactivity and is limited in sensitivity. The alkaline elution assay ignores the critical importance of intercellular differences in DNA damage and requires relatively large numbers of cells. A more useful approach for assessing DNA damage is the single-cell gel electrophoresis (SCGE) or Comet assay.

5.1.1 The SCGE Assay

Ostling and Johanson (1984) were the first to develop a microgel electrophoresis technique for detecting DNA damage at the level of the single cell. In their technique, cells embedded in agarose were placed on a microscope slide, detergents and high concentration salt were used to lyse the cells and the liberated DNA electrophoresed under neutral conditions. Cells with an increased frequency of DNA double-strand breaks (DSB) displayed increased migration of DNA toward the anode. The migrating DNA was quantified by staining with ethidium bromide and by measuring the intensity of fluorescence at two fixed positions within the migration pattern using a microscope photometer. However, the neutral conditions used greatly limited the general utility of the assay.

Subsequently, Singh *et al.* (1988) introduced a microgel technique involving electrophoresis under alkaline (pH 13) conditions for detecting DNA damage in single cells. At this pH, increased DNA migration is associated with increased levels of frank SSB, SSB associated with incomplete excision repair sites and ALS. Because almost all genotoxic agents induce orders of magnitude more SSB and/or ALS than DSB, this version of the assay offered greatly increased sensitivity for identifying genotoxic agents.

Since the introduction of the alkaline (pH 13) Comet assay in 1988, the numbers of applications and investigators using this technique have increased almost exponentially. Compared with other genotoxicity assays, the advantages of the technique include:

- Demonstrated sensitivity for detecting low levels of DNA damage
- Small numbers of cells per sample required
- Flexibility
- Low costs
- Ease of application
- Ability to conduct studies using relatively small amounts of a test substance
- Relatively short time period (a few days) needed to complete an experiment.

5.1.2 Applications of SCGE

Although the comet assay is used extensively in *in vitro* and *in vivo* genetic toxicology and many different types of cell lines and practically any type of cell from any target organs can be tested, there are several other applications for the comet assay. By looking at the removal of DNA damage over a period of time, comet assay can reflect the DNA repair that is taking place in the damaged cells including SSB repair (Singh *et al.*, 1988) and DSB repair (Evans *et al.*, 1993). It can also be used to detect excision (Tice *et al.*, 1990) and cross link repair (Pfuhler and Wolf, 1996). Another application of SCGE includes the detection of apoptosis. Kizilian *et al.*, (1999) has used the silver staining comet assay for detecting apoptosis. The 'Halo' assay or 'Tear drop' assay can also be used to detect apoptosis. The comet assay has also been used for the visual quantification of DNA DSB breaks in bacteria (Singh *et al.*, 1999).

There are also several clinical applications including

Prenatal diagnosis (Alapetite *et al.*, 1997);

DNA repair deficiency syndrome (Alapetite *et al.*, 1996);

Cancer Susceptibility (Jaiswal *et al.*, 1994)

Cancer therapy (Ostling *et al.*, 1987; Tice *et al.*, 1992; Olive *et al.*, 1993);

Cataract (Kleiman and Spector, 1993)

Diabetes Mellitus (Collins *et al.*, 1998a; Anderson *et al.*, 1998)

Rheumatoid arthritis (McCurdy *et al.*, 1997)

Alzheimer's disease models (Kruman *et al.*, 2002)

Biomonitoring applications include

Aging (Singh *et al.*, 1990)

Exercise (Hartman *et al.*, 1994)

Malnourishment (Betancourt *et al.*, 1995)

Nutrition (Green *et al.*, 1994)

Ozone (Calderon-Garciduenas *et al.*, 1996)

Population based (Betti *et al.*, 1995)

The comet assay can be used to study the DNA damage in cells in different cell cycle phases. The rationale of this application is similar to that of flow cytometry - the total comet fluorescence is proportional to the DNA content in cell. Cells in G1, S and G2 cell cycle phases are distinguished on the basis of different total comet fluorescence. Using data on the cell cycle distribution of the particular cell line obtained by flow cytometry, one can divide cells into the cell cycle phases. Alternatively, it is possible to use this approach for an unknown cell line on the basis of distribution the comet total fluorescence as measured by the comet assay. Of course, this application is useful only in the case of a homogenous cell population, preferentially - cells in culture. For multilineage samples, like human blood, it is necessary to distinguish the cells of interest by other means, e.g. antibody coated microbeads (Kruszewski *et al.*, 1998).

During the last decade, this assay has developed into a basic tool for use by investigators interested in research areas ranging from human and environmental biomonitoring to DNA repair processes to genetic toxicology. The aim of this study was to determine whether AFB_1 and FB_1, treated individually or in combination induce DNA damage in normal human lymphocytes and lymphocytes extracted from treated whole blood, using the comet assay.

5.2 Materials and Methods

5.2.1 Materials

Low melting point agarose was purchased from Saarchem, SA. Slides and coverslips were obtained from Shalom, SA. NaCl, EDTA, Na_2EDTA and NaOH pellets were acquired from Merck. Tris, 1% and Triton X100 were also purchased from Merck. All other reagents and consumables used were obtained from the sources mentioned previously.

5.2.2 Methodology

5.2.2.1 Isolation of Lymphocytes

Whole venous blood was collected and normal human lymphocytes were isolated as described in sections 3.2.3.4 and 3.2.3.5.

5.2.2.2 Treatment of the Lymphocytes

An aliquot of 2mls of lymphocytes containing approximately 1 X 10^6 cells/ml was added to individual wells of a 24 well microtitre plate. A 1ml aliquot of 5µM AFB_1, 50µM AFB_1, 5µM FB_1 and 50µM AFB_1 was added to each well. Individual ethanol and CCM controls were also prepared. Combinations of the toxins were prepared by adding 500µl aliquot of each concentration of AFB_1 and 500µl of the same concentration of FB_1 to the same well. The plates were then incubated at 37°C for 12 hours.

5.2.2.3 Treatment of Whole Blood

A 2ml aliquot of the blood was treated with a 1ml aliquot of 5µM AFB_1, 50µM AFB_1, 5µM FB_1 and 50µM FB_1 in sterile tubes. A 500µl aliquot of the 5µM and 50µM concentration of AFB_1 and 500µl of the same concentrations of FB_1 were added to the same well to evaluate the potential genotoxic effects of the combination of mycotoxins. CCM and ethanol treatments were also prepared. The suspension was incubated for 12 hours at 37°C in a controlled environmental incubator shaker at 150 rpm. Some of the treated whole blood was then used to prepare the second layer of the mini gels as described in 5.2.2.4. The remainder of the treated whole blood was then used for the extraction of lymphocytes as outlined in 3.2.3.5.

5.2.2.4 Single Cell Gel Electrophoresis Assay

The procedure of the SCGE assay was carried out using aseptic techniques. Contact with hazardous chemicals was minimised by using sterile gloves and a mask. Low melting point agarose (LMPA) (1%, 0.5g) was melted in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) (50ml) by heating in a microwave (60°C) until a clear solution was obtained. The molten agarose was cooled to 35°C. As shown in Figure 5.1, a first layer of LMPA (1%) was placed towards the frosted end of a microscopic glass slide (400µl) (Lasec, 76 × 26mm). The slides were then covered with a coverslip (60×20mm) and maintained at 4°C for 20 minutes to allow the agarose to solidify. The first layer on the slide was used to promote even and firm attachment for the second and third layers. For the second layer 0.5 % LMPA was prepared.

Following the careful removal of the coverslips, a second layer of molten LMPA (90µl) was mixed with lymphocytes or whole blood (20µl) and the mixture rapidly pipetted onto the first solidified layer of LMPA on the microscope glass slides. The slides were covered with a coverslip and maintained at 4°C for 10 minutes. The coverslips were then gently removed after solidification of the second layer. A third layer of the LMPA (0.5%, 90µl) was then placed on the previous two solidified layers on the microscope slides, covered with a coverslip and maintained at 4°C for 10 minutes. The coverslips were carefully removed and the slides then gently immersed in a freshly prepared lysis solution (250ml) consisting of 2.5M NaCl, 100mM EDTA, 10mM Tris (pH 10), 1% Triton X100, 10% DMSO for 1 hour at 4°C to lyse the cells and permit DNA unwinding.

After 1 hour, the slides were removed, drained and placed side by side in a horizontal electrophoresis tank (Amersham SA) with the preset gel ends closest to the anode. The tank was filled with fresh electrophoresis buffer made up of 1mM Na_2EDTA and 300mM NaOH (pH 13), to a level approximately 0.25cm above the slides. The primary role of the buffer is to impose some constant number of charges on the cell, provide good conditions of solubility and to pass some current. The pH and the ionic strength of the buffer will affect the migration of the DNA during electrophoresis. If the electrophoresis buffer is omitted, electrical conductance is minimal and the DNA will migrate slowly, if at all (Sambrook *et al.* 1989). The slides were left in the buffer for 20minutes to allow for unwinding of DNA before electrophoresis. Electrophoresis was then conducted at room temperature for 35 minutes at 25V using a Biorad powerpac 300.

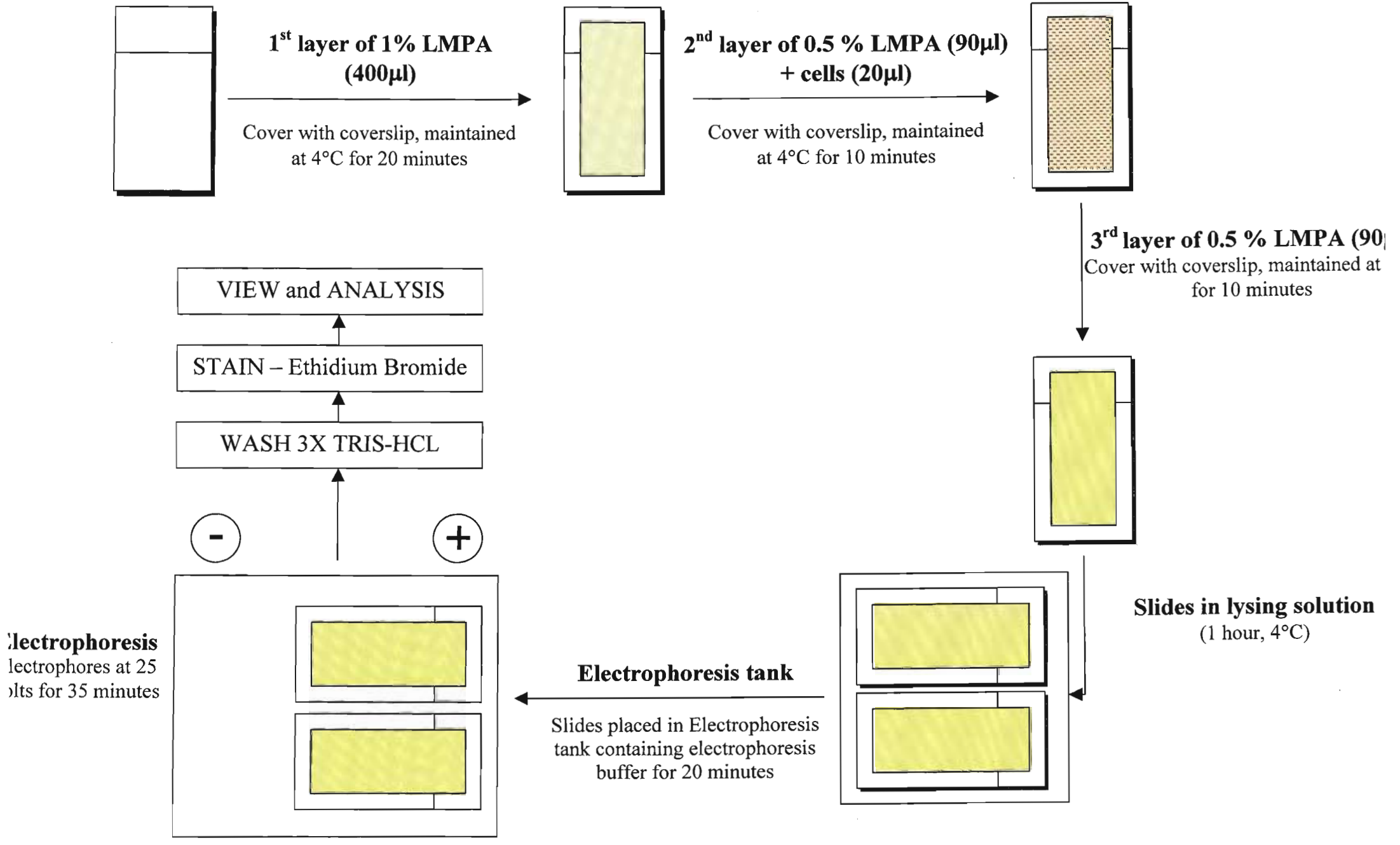

Figure 5.1: Schematic drawing of the SCGE assay.

After electrophoresis, the slides were removed from the tank and were washed gently to remove alkali and detergents which would interfere with ethidium bromide staining, by placing them on a tray and flooding them slowly with 3 changes of 0.4 M Tris (3ml), pH 7.5 for 5 minutes.

5.2.2.5 Viewing and Analysis of Slides

After neutralisation the slides were stained with 50µl of ethidium bromide (20µg/ml) and covered with a coverslip. Ethidium bromide is a dye that intercalates between stacked base pairs of the DNA molecules, extending the length of linear and nicked DNA and making them more rigid. All the steps were conducted in the dark to prevent any additional DNA damage. The slides were then viewed using an inverted research microscope, Olympus I X 51 with an excitation filter of 515-560nm and a barrier filter of 590nm. Images of 50 randomly selected lymphocytes (25 from each of the two replicate slides) were identified on the basis of cell size and location of the nucleus.

The images were then captured using a microscope digital camera system, an Olympus DP12 camera. Scion Image Analysis software was then used to analyse the images. Tail distance, the length from the beginning of the head to the end of the tail was used to determine the extent of DNA damage.

5.2.2.6 Statistical Analysis

All data were presented as means ± standard deviations (SD). The results of the single cell gel electrophoresis test were analysed using the Student's t test. A probability (p) value less than 0.05 was considered statistically significant.

5.3 Results and Discussion

Aflatoxin B_1 and FB_1 have been shown to be cytotoxic to human lymphocytes and to induce nuclear fragmentation. In an attempt to quantify the DNA damage, freshly isolated human lymphocytes and whole blood were treated with the mycotoxins, both individually and in combination for 12 hours and evaluated using the comet assay. The slides were then viewed using fluorescent microscopy (Figure 5.2 to 5.8).

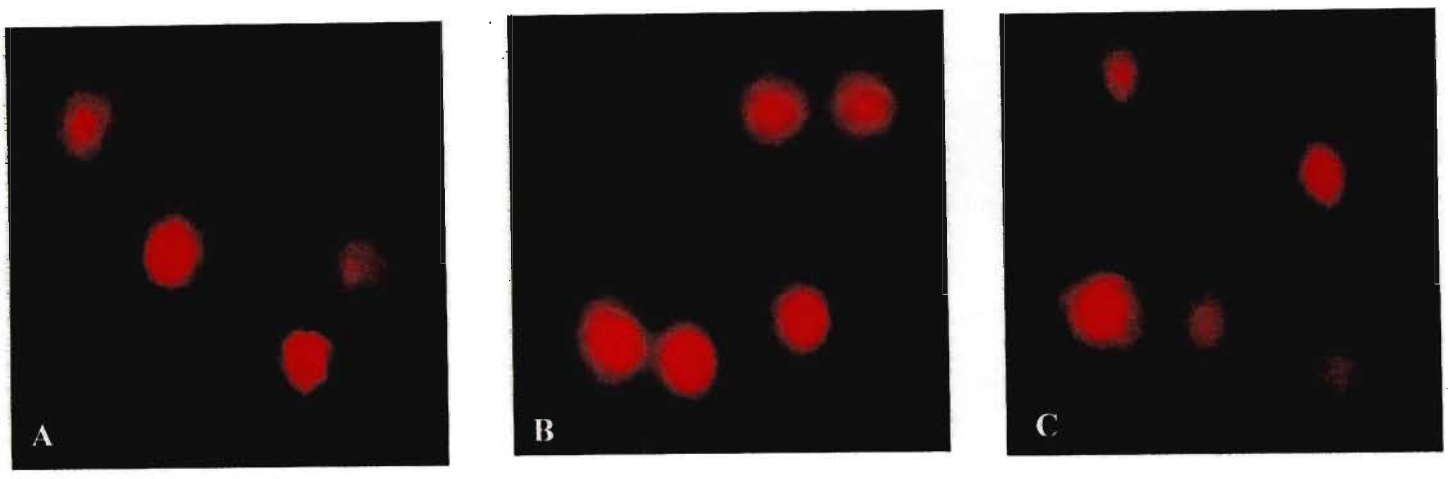

Figure 5.2: Comet assay of untreated normal human lymphocytes (A), untreated whole blood (B) and lymphocytes extracted from untreated whole blood (C).

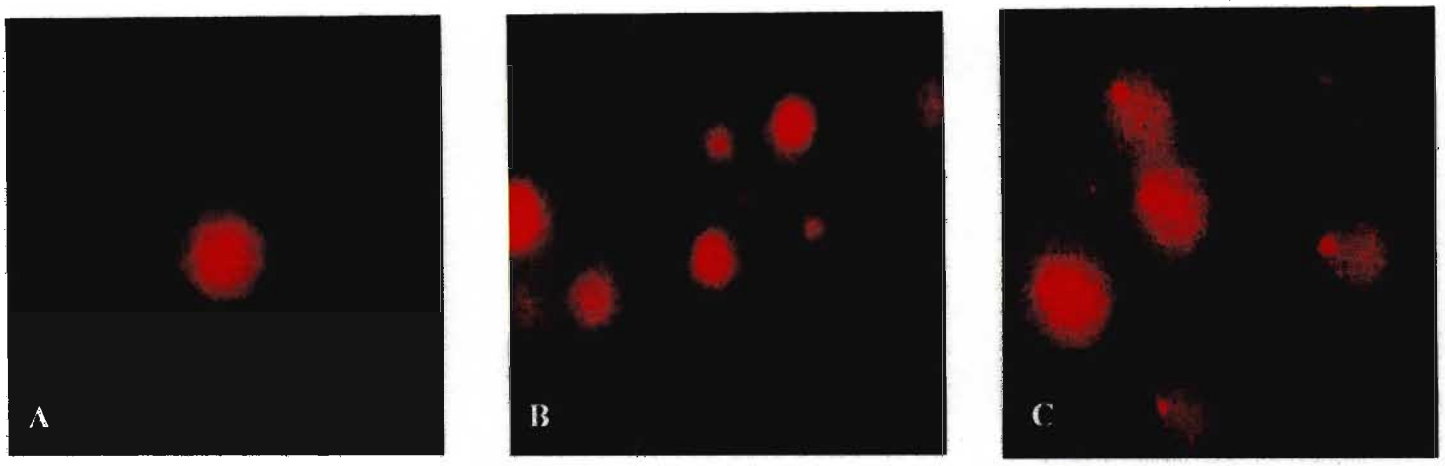

Figure 5.3: Comet assay of 5μM Aflatoxin B_1 treated normal human lymphocytes (A), 5μM Aflatoxin B_1 treated whole blood (B) and lymphocytes extracted from 5μM Aflatoxin B_1 treated whole blood (C).

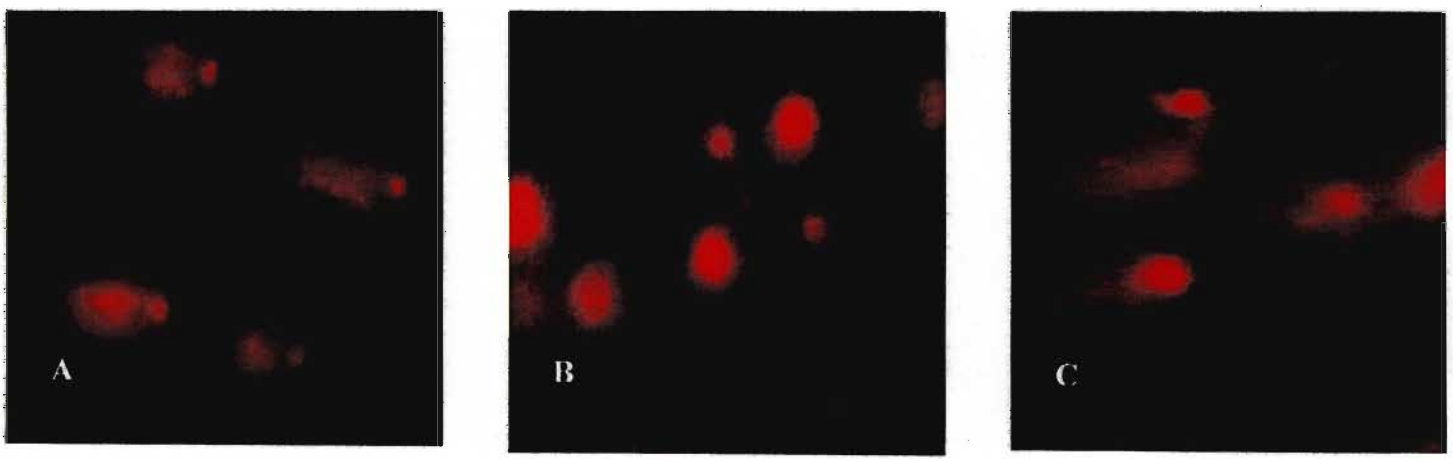

Figure 5.4: Comet assay of 50μM Aflatoxin B_1 treated normal human lymphocytes (A), 50μM Aflatoxin B_1 treated whole blood (B) and lymphocytes extracted from 50μM Aflatoxin B_1 treated whole blood (C).

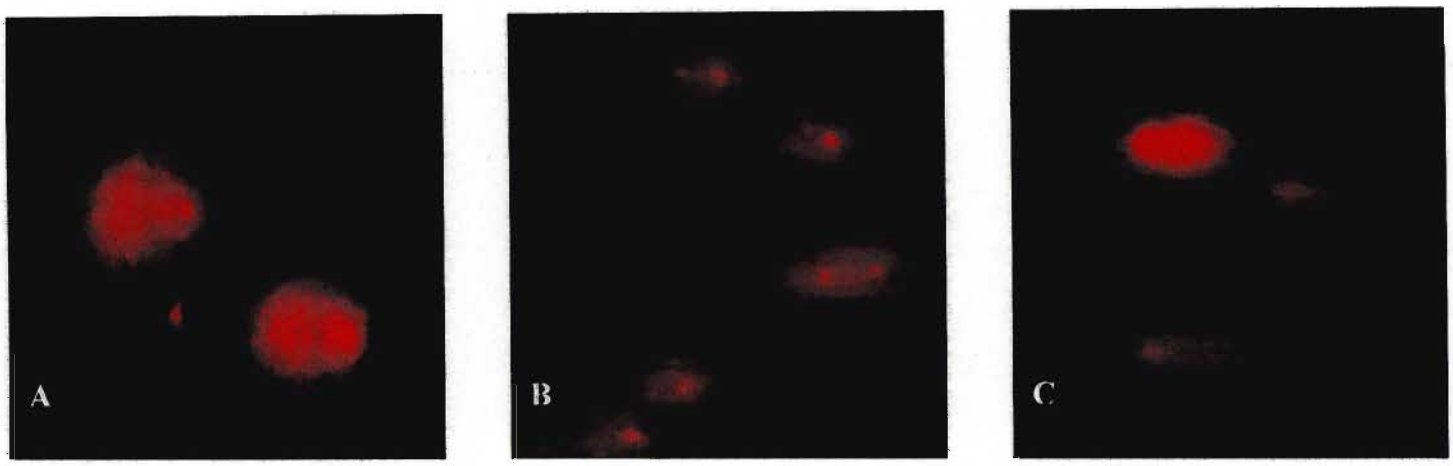

Figure 5.5: Comet assay of 5μM Fumonisin B_1 treated normal human lymphocytes (A), 5μM Fumonisin B_1 treated whole blood (B) and lymphocytes extracted from 5μM Fumonisin B_1 treated whole blood (C).

Figure 5.6: Comet assay of 5μM Fumonisin B_1 treated normal human lymphocytes (A), 5μM Fumonisin B_1 treated whole blood (B) and lymphocytes extracted from 5μM Fumonisin B_1 treated whole blood (C).

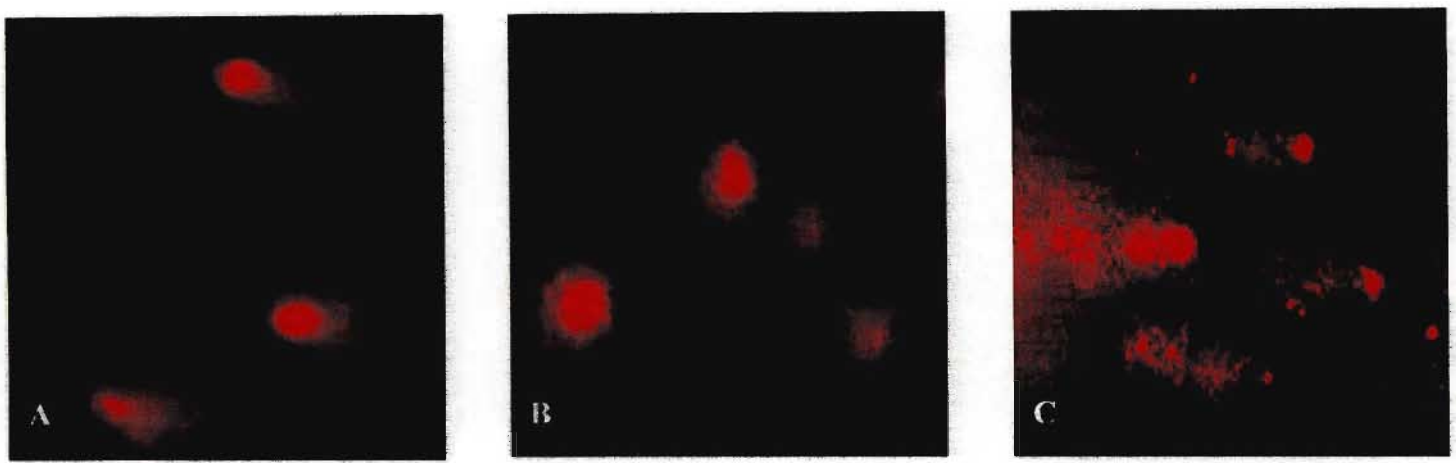

Figure 5.7:Comet assay of 5μM combination treated normal human lymphocytes (A), 5μM combination treated whole blood (B) and lymphocytes extracted from 5μM combination treated whole blood (C).

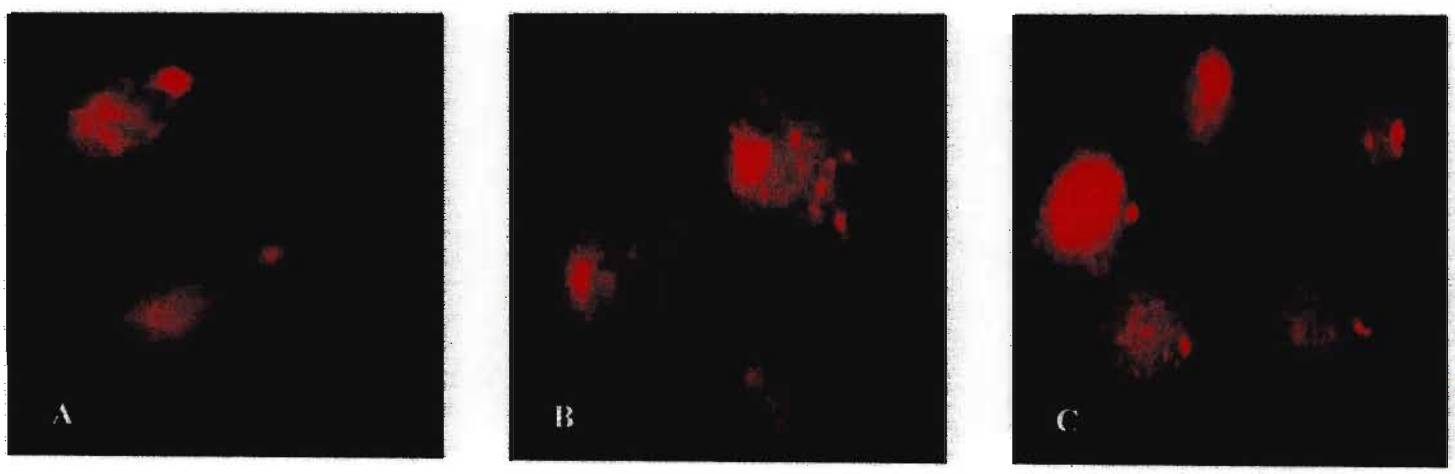

Figure 5.8: Comet assay of 50μM combination treated normal human lymphocytes (A), combination treated whole blood (B) and lymphocytes extracted from combination treated whole blood (C).

Increased DNA migration accompanies the DNA fragmentation associated with cytotoxicity arising through necrosis or apoptosis. Apoptosis results in the extensive formation of single stranded breaks (Marks and Fox, 1991). Similarly, the DNA of necrotic cells also undergoes extensive degradation due to the induction of single stranded breaks. These cells can be detected using the comet assay. Several researchers have thought it possible based on the appearance of the comet, to distinguish between apoptotic and necrotic cells (Olive *et al.*, 1993). Apoptotic cells were concluded to form comets with large fan like tails and small heads, while necrotic cells were shown to form comets with relatively large heads and narrow tails of varying lengths. However this characterisation may not always be completely accurate (Tice *et al.*, 2000).

Tail distance was measured using Scion Image analysis software to assess the potential genotoxic effects of AFB_1 and FB_1 on normal human lymphocytes (Table 5.1).

Table 5.1: Comet assay of normal human lymphocytes, whole blood and lymphocytes extracted from whole blood, which have been treated with Aflatoxin B_1 and Fumonisin B_1, individually and in combination.

	Treated lymphocytes tail distance (μm)	Lymphocytes of treated whole blood tail distance (μm)	Lymphocytes extracted from treated whole blood tail distance (μm)
Ethanol control	7.64 ± 1.35	7.56 ± 1.72	11.96 ± 2.36
5μM AFB_1	7.77 ± 2.24	7.62 ± 2.11	15.78 ± 2.52*
50μM AFB_1	17.21 ± 3.41*	7.63 ± 1.19	16.11 ± 2.29*
5μM FB_1	13.99 ± 3.56*	12.07 ± 2.95*	19.77 ± 4.51*
50μM FB_1	14.73 ± 3.82*	15.66 ± 2.57*	16.18 ± 2.01*
5μM Combination	7.9 ± 5.92	12.25 ± 2.84*	12.31 ± 2.61
50μM Combination	20.00 ± 3.80*	18.04 ± 2.94*	15.93 ± 3.14*

* = significant difference from the control ($p < 0.05$) using Student's t test.

Normal human lymphocytes are not the sole component of blood. The human blood stream also consists of erythrocytes, cytokines, leukocytes, neutrophils, macrophages and other cells. As a result it is very difficult to get a realistic idea of what occurs when mycotoxins are absorbed into the human blood stream by just evaluating their effect on isolated normal lymphocytes. This fails to take into account the transport systems, carrier systems and complex interactions between the different types of cells, which will play a role in the defence mechanism of the immune system. Another factor that should be taken into account is that in an *in vivo* situation, mycotoxins such as FB_1, which are poorly absorbed, would be rapidly excreted compared to an *in vitro* situation (Dutton, 1996). As a result less toxin would be taken up.

It was decided to evaluate the DNA damaging potential of FB_1 and AFB_1 by three different methods of treatment, the treatment of freshly isolated normal lymphocytes, whole blood and the extraction of lymphocytes from treated whole blood. There was no discernable difference between the controls of the three different methods of treatment. The DNA formed normal round solid balls, with no visible DNA migration or damage (Figure 5.2).

When isolated normal human lymphocytes were treated with FB_1, it caused DNA damage in a dose dependent manner (Figure 5.5A and 5.6A). This correlates with recent studies that have reported that FB_1 has DNA damaging potential (Tolleson *et al.*, 1996; Knasmuller *et al.*, 1997; Sahu *et al.*, 1998). In a study by Galvano *et al.* (2002a), it was found that treatment with FB_1 induced DNA damage to human fibroblasts when the cells were exposed to the toxin at high concentrations for prolonged periods of exposure. It was thought that this damage was induced by apoptosis as it was preceded by an increase in caspase 3-activity as well as in the levels of poly (ADP-ribose) polymerase fragments. Under increasing adverse cellular conditions and subsequent DNA damage, the capacity of cellular repair has been shown to be impaired (Galvano *et al.*, 2002a). The altered metabolic conditions could be representative of apoptotic pathways.

In the 5μM AFB_1 treated isolated normal lymphocytes, tail distance was shorter when compared to the control (Figure 5.3A). Many of the cytotoxic effects of AFB_1 require its bioactivation to the 2,3 epoxide metabolite by a cytochrome P450 (P450) dependent polysubstrate monooxygenase enzyme. It has been shown that cytochrome P450 is expressed in human peripheral blood lymphocytes (Haufroid *et al.*, 2003). Some of the epoxide will covalently bind to macromolecules to form protein and DNA adducts (Eaton and Gallagher, 1994). Possibly this would cause the nuclei to become more compact and therefore the tail distance would shrink.

DNA damage was induced in the 50μM AFB_1 treated normal isolated lymphocytes (Figure 5.4A). The comets formed had relatively large heads and narrow tails, which as mentioned previously, could indicate DNA damage caused by necrosis. Aflatoxin B_1 has been shown to be a potent cytotoxic agent so the probability of DNA damage induced by necrosis is very high.

Aflatoxin B_1 and FB_1 have been found in several cases to coexist. This combination of toxins could prove to be a serious health hazard for humans. However, the 5μM combination treatment of isolated normal human lymphocytes did not induce any significant DNA damage (Figure 5.7A). Fumonisin B_1 has been shown to be both mitogenic (Tolleson *et al.*, 1996; Lim *et al.*, 1996); and cytotoxic (Dombrink –Kurtzman *et al.*, 1994; Tolleson *et al.*, 1996). It can therefore be speculated that at this concentration the effects of FB_1 were mitogenic rather than cytotoxic thereby resulting in no DNA damage. This hypothesis is supported by the fact that the tail distance profile is almost identical to the 5μM AFB_1 treated lymphocytes.

Fumonisin B_1 treatment of whole blood resulted in DNA damage at both the high and low concentrations (Figure 5.5B and 5.6B). Previously FB_1 was considered less genotoxic than other mycotoxins, such as AFB_1, as it is believed to induce cancer by altering the signal transduction pathways of the target cells (Wang and Groopman, 1999). However a study by Mobio *et al.* (2000) on C6 glioma cells showed that FB_1 at low concentrations induced comets with increased tail lengths. Under these conditions FB_1 induced DNA fragmentation and laddering and many apoptotic bodies.

Data from the literature indicates the ability of FB_1 to selectively block the cell cycle and play an important role in inducing apoptosis, through the TNF pathway and caspase activity (Ciacci-Zanella *et al.*, 1998). The elevated concentrations of free highly bioactive sphingoid bases must contribute to the cellular effects of FB_1 (Wang and Groopman, 1999; Yoo *et al.*, 1996); although the specific targets are partially unclear, any system affected by free sphingolipid bases and/or their metabolites is a potential target.

None of the AFB_1 treatments of whole blood induced any DNA damage, which could be due to several factors (Figure 5.3B – Figure 5.4B). Red blood cells are present in blood and therefore would absorb some of the AFB_1, thereby reducing the amount of toxin taken up by the lymphocytes. Another explanation could be the presence of macrophages in whole blood.

Macrophages are primarily the first line of defence against infectious agents (Skamene and Gros, 1983) and studies have shown that aflatoxin inhibits macrophage functions such as phagocytotic, chemotactic, microbiocidal, an adherence activity (Neldon-Ortiz and Quereshi, 1991; Jakab *et al.*, 1994; Cusumano *et al.*, 1995). In the presence of 1.5μg of AFB_1, the cell viability of primary swine alveolar macrophages was reduced to 41% (Liu *et al.*, 2002). Dugyala and Sharma, (1996), showed that AFB_1 had a marked effect on peritoneal macrophage produced cytokines in mice. The levels of interleukin-1α (IL-1α) secreted proteins were significantly suppressed at all dosages and those of interleukin 6 (IL-6) and TNF at the high dose. However low dose of AFB_1 only slightly decreased the protein levels expressed by the lymphatic IL2, IL3 and interferon γ. This would suggest that AFB_1 preferentially affects macrophage functions.

The 5μM combination treatment of whole blood was shown to induce DNA damage to lymphocytes (Figure 5.7B). It can be hypothesised that this damage was induced by the FB_1, as the 5μM AFB_1 when treated individually did not cause DNA damage. From the shape of the comets, it appears the damage was apoptotic, which could also be indicative of FB_1 being responsible for inducing DNA damage.

The lymphocytes extracted from FB_1 treated whole blood, resulted in the 5μM FB_1 inducing more DNA damage than the 50μM FB_1 treatment (Figure 5.5C and 5.6C). It should also be noticed that this method of treatment with FB_1 had the longest tail distance at both the high and the low concentration. At the 5μM treatment, distinct comets were formed, with small heads and large fan-like tails, which as discussed previously, could indicate DNA damage induced by apoptosis. Similar results were shown with the 50μM concentration.

Only one method of treatment showed that the 5μM AFB_1 had the potential to induce DNA damage to human lymphocytes, the lymphocytes extracted from treated blood (Figure 5.3C). The level of DNA damage at a particular time will reflect the rate of DNA damage versus the rate of repair (Galvano *et al.*, 2002a). Therefore it may be considered as a biomarker of both DNA damage and different stress conditions. It is possible that the level of DNA damage induced by the 5μM AFB_1 in lymphocytes extracted from treated blood may be the result of different stress conditions rather than indicating apoptosis or necrosis. The 50μM AFB_1 treatment also induced DNA damage possibly by necrosis (Figure 5.4C).

There was no DNA damage induced by the 5μM combination treatment of lymphocytes extracted from whole blood (Figure 5.7C). Aflatoxin B_1 and FB_1 have very different mechanisms of action; AFB_1 is absorbed into the cell, where it is converted to a potent epoxide, which forms conjugates with proteins and DNA, while FB_1 is thought to interfere with sphingolipid biosynthesis. It is possible that the different mechanisms of action could have interfered with each other to reduce the toxic effect.

In all three different methods of treatment, the 50μM combination induced the most DNA damage. This would appear to indicate that the mycotoxins at higher concentrations work in conjunction, either synergistically or additively, to induce greater DNA damage than if treated individually.

5.4 Conclusion

The comet assay was shown to be a viable technique for assessing the level of DNA damage induced by AFB_1 and FB_1. Fumonisin B_1 induced DNA damage in normal human lymphocytes at both a low and high concentration, indicative of its potential as a genotoxic agent.

It was seen that although treatment of isolated lymphocytes and isolating lymphocytes from treated blood, will give a fairly accurate idea of the toxins effect on the immune system, the most realistic way of analysing a toxins effect on the immune response is by assessing its effects on whole blood. Unfortunately, due to experimental procedure, this is not always possible.

CHAPTER 6

The effects of Aflatoxin B_1 and Fumonisin B_1 on the nuclei of normal human lymphocytes

6.1 Introduction

6.1.1 Fluorescent Microscopy

Fluorescence is the luminescence of a substance when it is excited by radiation. In microscopy, fluorescence is used as a means of preparing specific biological probes. Some biological substances like chlorophyll and some oils and waxes have primary fluorescence; that is, they autofluorescence. But most biological molecules or structures do not fluorescence on their own, so they must be linked with fluorescent molecules (fluorochromes) in order to create specific fluorescent probes (Prescott *et al.*, 1996).

Fluorescence of a substance is seen when the molecule is exposed to a specific wavelength of light (excitation wavelength or spectrum) and the light it emits (the emission wavelength or spectrum) is always of a higher wavelength. To view this fluorescence in the microscope, several light filtering components are needed. Specific filters are needed to isolate the excitation and emission wavelengths of a fluorochrome and therefore filter sets must be made to correspond to the excitation and emission characteristics of a given fluorochrome. Examples of fluorochromes include acridine orange, hoechst 33342 and ethidium bromide. Some sets are made to allow visualisation of two or even three fluorochromes simultaneously (Herman, 1998).

A bright light source with proper wavelengths for excitation is also needed. For normal fluorescence applications, this is a mercury vapour arc burner. Mercury arc burners are very bright lamps with a limited lifetime and require some maintenance and care to make sure that they are producing the brightest possible light beam for fluorescence excitation. For fluorescence confocal microscope applications, where up to 95% of the emission light is filtered out, specific wavelength lasers are used as these are extremely bright (Herman, 1998).

One other component is required: a dichroic beam splitter or partial mirror which reflects lower wavelengths of light and allows higher wavelengths to pass. A beam splitter is required because the objective acts as a condenser lens for the excitation wavelength as well as the objective lens for emission (Prescott *et al.*, 1996).

It is important that only the light emitted from the fluorochrome is seen and not any of the excitation light and the beam splitter isolates the emitted light from the excitation wavelength. This epi-illumination type of light path is required to create a dark background so that the fluorescence can be easily seen. The wavelength at which a beam splitter allows the higher wavelengths to pass must be set between the excitation and emission wavelengths of any given fluorochrome so that excitation light is reflected and emission light is allowed to pass through it (Herman, B. 1998). A typical fluorescence filter setup is shown in the diagram below (Figure 6.1)

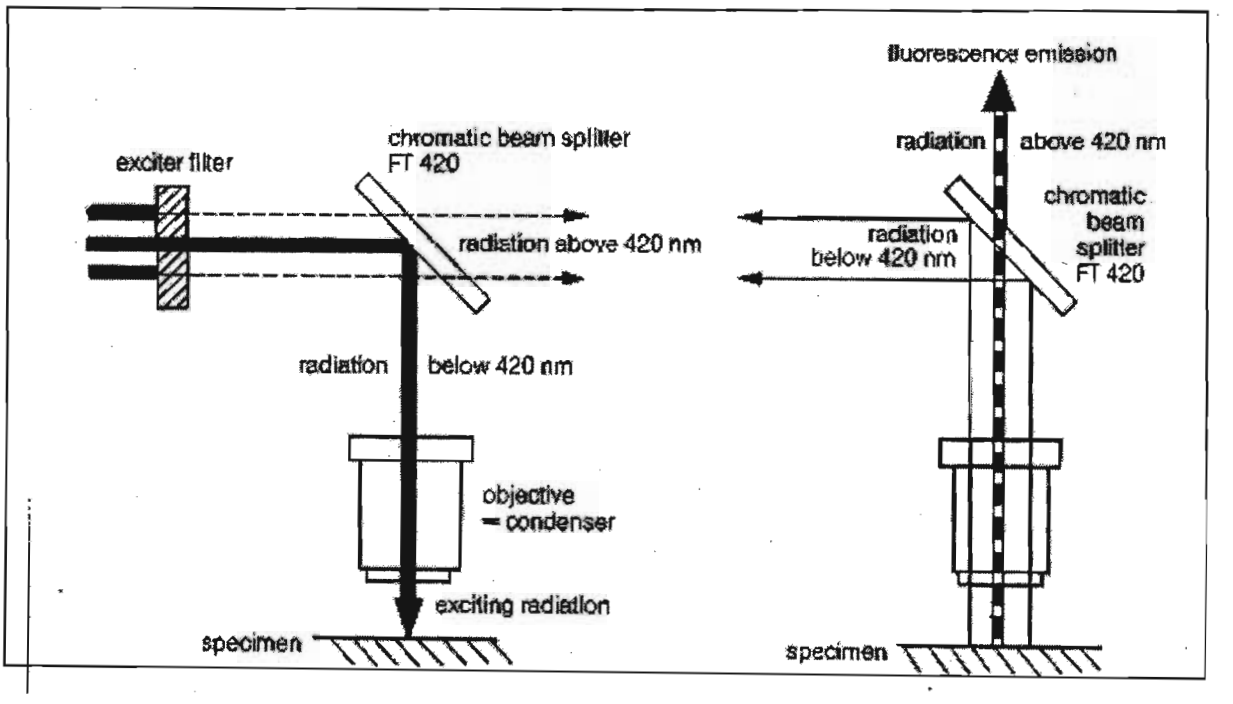

Figure 6.1: A schematic diagram of a typical fluorescence filter setup (Carl Zeiss Inc., 2003).

6.1.2 Use of Fluorescent Microscopy in the Detection of Apoptosis

The characteristic breakdown of the nucleus during apoptosis is comprised of the collapse and fragmentation of the chromatin, degradation of the nuclear envelope and nuclear blebbing, resulting in the formation of micronuclei (Wyllie, 1997). Consequently, nucleic acid stains can be useful tools for identifying even low numbers of apoptotic cells in cell populations. Several nucleic acid stains have been used to detect apoptotic cells by fluorescent microscopy.

One of the most common nucleic acid stains are the bisbenzimide dyes. The bisbenzimide dyes, 4-[5-(4-methyl-1-piperazinyl) [2,5'-bi- 1H-benzimidazol]-2'-yl]-, (Hoechst 33258), 2,5'-Bi-1H-benzimidazole (Hoechst 33342) and Hoechst 34580, are cell membrane-permeant, minor groove-binding DNA stains that fluoresce bright blue upon binding to DNA.

Hoechst 33342 has slightly higher membrane permeability than Hoechst 33258 (Arndt-Jovin and Jovin, 1989) but both dyes are quite soluble in water, up to 2% solutions can be prepared. The relatively non-toxic Hoechst 34580 dye has somewhat longer wavelength spectra than the other Hoechst dyes when bound to nucleic acids (Shapiro and Perlmutter, 2001).

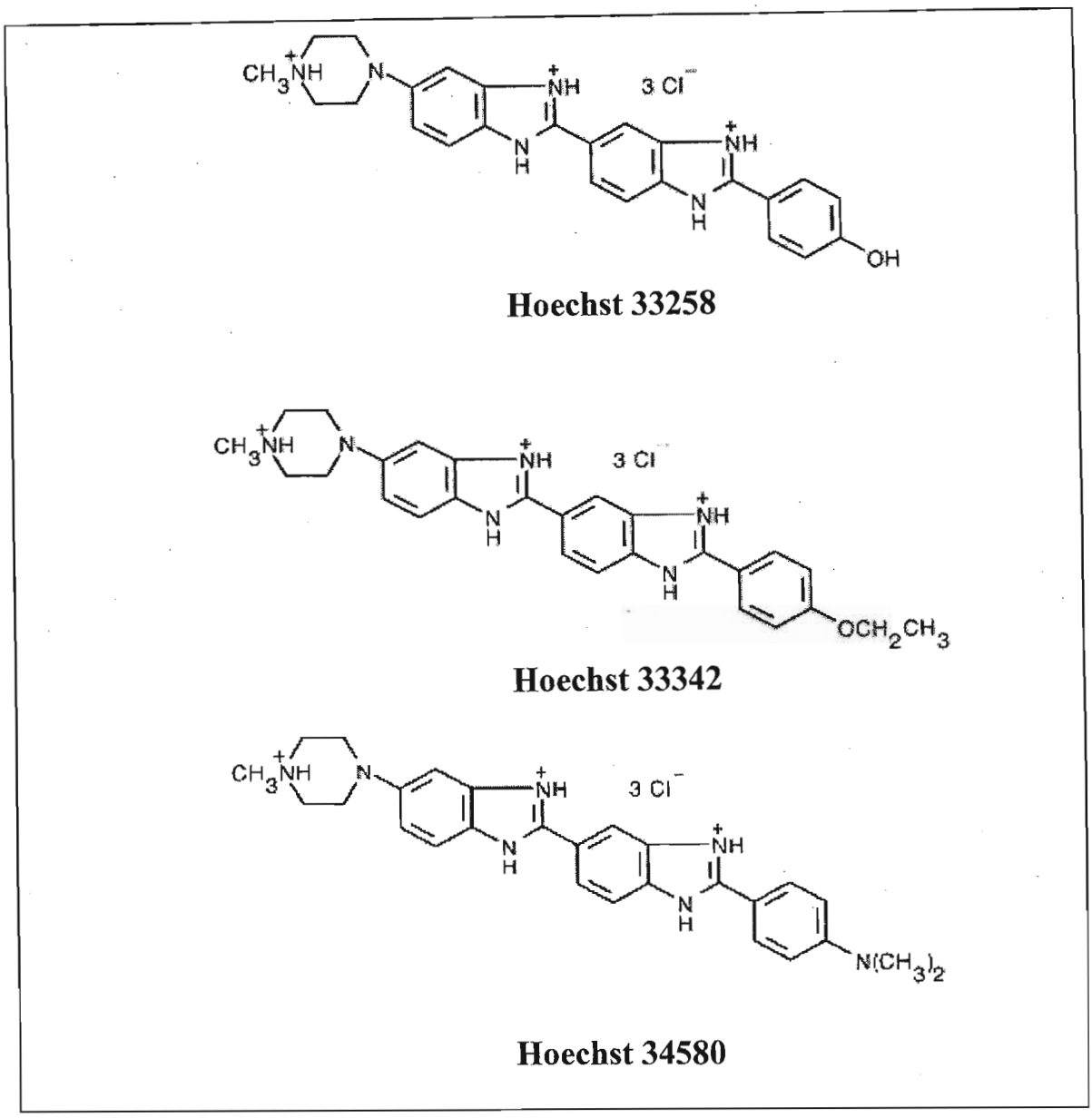

Figure 6.2: Molecular structure of three different Hoechst stains (Molecular probes, 2003).

The Hoechst dyes (Figure 6.2), which can be excited with the UV spectral lines of the argon-ion laser and by most conventional fluorescence excitation sources, exhibit relatively large Stokes shifts (excitation/emission maxima ~350/460 nm), making them suitable for multicolour labelling experiments. The Hoechst 33258 and Hoechst 33342 dyes have complex, pH-dependent spectra when not bound to nucleic acids, with a much higher fluorescence quantum yield at pH 5 than at pH 8. Their fluorescence is also enhanced by surfactants such as sodium dodecyl sulfate (SDS) (Gorner, 2001).

These dyes appear to show a wide spectrum of sequence-dependent DNA affinities and bind with sufficient strength to poly (d (A-T)) sequences that they can displace several known DNA intercalators (Loontiens *et al.*, 1990). They also exhibit multiple binding modes and distinct fluorescence emission spectra that are dependent on dye:base pair ratios (Stokke and Steen, 1985). Hoechst dyes are used in many cellular applications, including in cell-cycle and apoptosis studies and they are common nuclear counterstains.

The aim of this study was to determine whether AFB_1 and FB_1, treated individually or in combination induce nuclear fragmentation in normal human lymphocytes and lymphocytes extracted from treated whole blood, using a nuclear stain.

6.2 Materials and Methods

6.2.1 Materials

Hoechst 33258 was purchased from Capital, SA. Paraformaldehyde and phosphate buffer saline tablets were acquired from Merck. All other reagents and consumables used were obtained from the sources mentioned previously.

6.2.2 Methodology

6.2.2.1 Isolation of Lymphocytes

Whole venous blood was collected and normal human lymphocytes extracted as described in section 3.2.3.5.

6.2.2.2 Treatment of the Lymphocytes

An aliquot of 2mls of lymphocytes containing approximately 1 X 10^6 cells/ml was added to individual wells of a 24 well microtitre plate. The cells were then treated by adding a 1ml aliquot of AFB_1 (5μM, 50μM) and FB_1 (5μM, 50μM) to individual wells. CCM and ethanol controls were also prepared. Combinations of the toxins were evaluated by adding 500μl aliquot of each concentration of AFB_1 and 500μl of the same concentration of FB_1 to the same well. The plates were then incubated at 37°C for 12 hours. The cells were then removed aseptically using a sterile 3ml pasteur pipette and placed in sterile sterilin tubes.

6.2.2.3 Treatment of Whole Blood

A 2ml aliquot of the blood was treated with a 1ml aliquot of 5μM AFB_1, 50μM AFB_1, 5μM FB_1 and 50μM FB_1 in sterile tubes. A 500μl aliquot of the 5μM and 50μM concentration of AFB_1 and 500μl of the same concentrations of FB_1 were added to the same well to evaluate the potential of the combination of mycotoxins to cause nuclear fragmentation. Ethanol and CCM treatments were also prepared. The sterile blood tubes were then incubated for 12 hours at 37°C in a controlled environmental incubator shaker at 150 rpm. The treated whole blood was used for the extraction of lymphocytes as outlined in 3.2.3.5.

6.2.2.4 Preparation of 10% Paraformaldehyde and Hoechst 33258

A 10% paraformaldehyde solution was prepared by dissolving 1g paraformaldehyde in 10mls PBS. The solution was heated for 30 minutes at 60°C and the pH adjusted to 7.2. Paraformaldehyde is routinely used for the fixation of cells and tissues especially cells labelled by fluorochrome-conjugated antibodies to membrane antigens as it will stabilize the light scatter and labelling.

A stock solution of Hoechst 33258 was prepared by dissolving 4mg in 2mls DMSO and vortexing thoroughly. The mixture was stored at 4°C. A working solution was obtained by adding 1.4mls of the stock solution to 1ml PBS.

6.2.2.5 Staining Procedure

The normal human lymphocytes were pelleted by centrifugation using a bench top centrifuge at 400g for 15 minutes at room temperature (RT) and then resuspended in PBS. The supernatents were discarded and the cells washed twice in PBS (2ml; 37°C) by centrifugation (400xg; 15 minutes; RT).

The cells were then resuspended in 10% paraformaldehyde and fixed for 5 minutes. The washing step in PBS was repeated twice and finally the pelletted cells were resuspended in 500μl PBS. An equal volume of Hoechst 33342 was added and the cells were incubated in sterile sterilin tubes for 15 minutes at 37°C. A 20μl aliquot of the cell suspension was placed on a slide and coverslipped.

6.2.2.6 Viewing of slides

The slides were viewed with an excitation wavelength in the range of 350 nm and detection wavelength in the range 450nm using an inverted research microscope, Olympus I × 51 (Wirsam Scientific, SA). Images were then captured using the digital camera using a microscope digital camera system, an Olympus DP12 (Wirsam Scientific, SA).

6.3 Results and Discussion

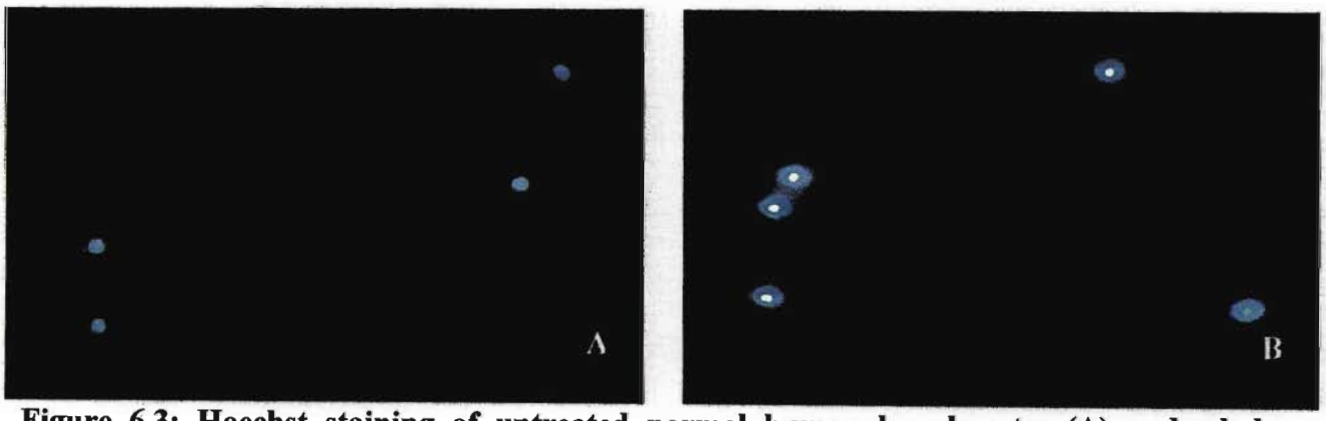

Figure 6.3: Hoechst staining of untreated normal human lymphocytes (A) and whole blood (B).

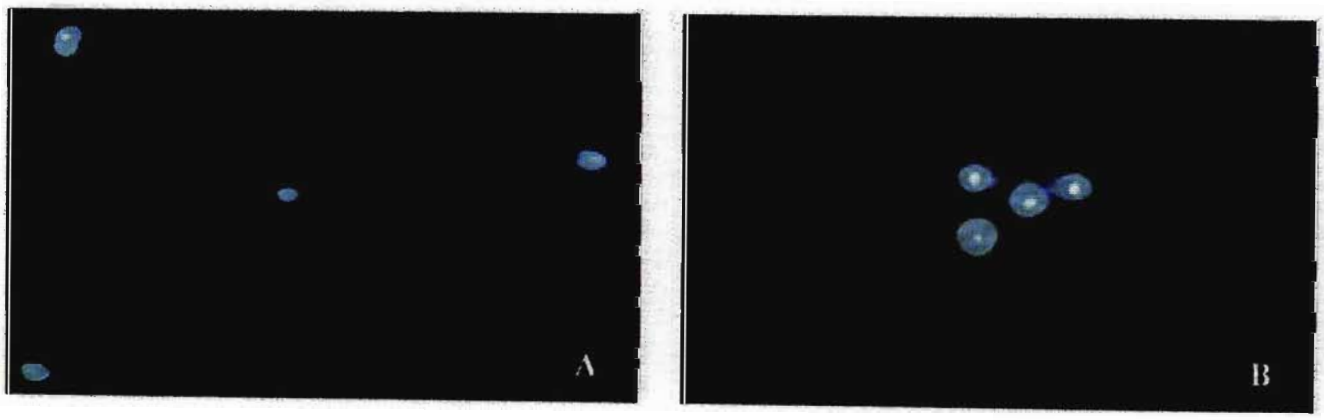

Figure 6.4: Hoechst staining of 5μM Aflatoxin B_1 treated normal human lymphocytes (A) and whole blood (B).

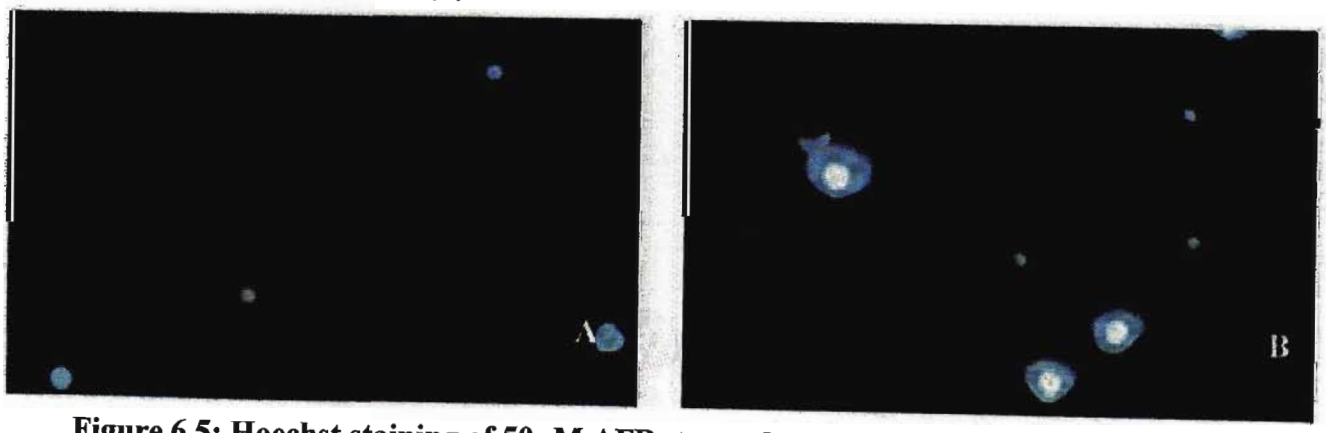

Figure 6.5: Hoechst staining of 50μM AFB_1 treated normal human lymphocytes (A) and whole blood (B).

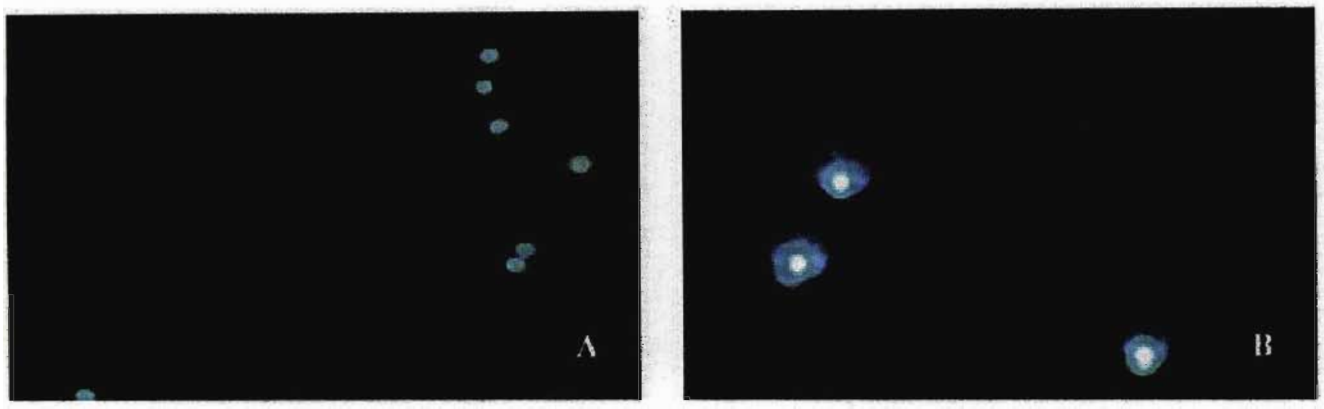

Figure 6.6: Hoechst staining of 5μM Fumonisin B_1 treated normal human lymphocytes (A) and whole blood (B).

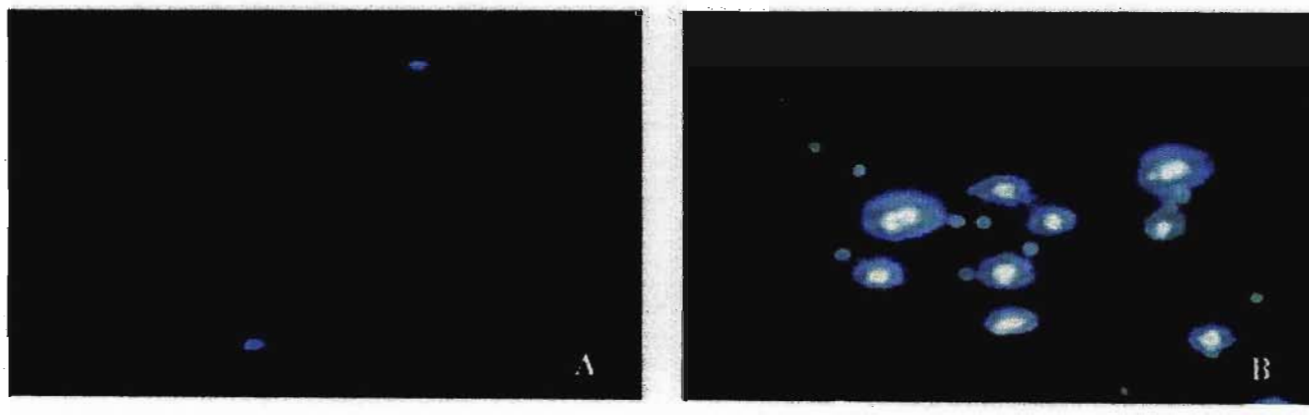

Figure 6.7: Hoechst staining of 50μM FB_1 treated normal human lymphocytes (A) and whole blood (B).

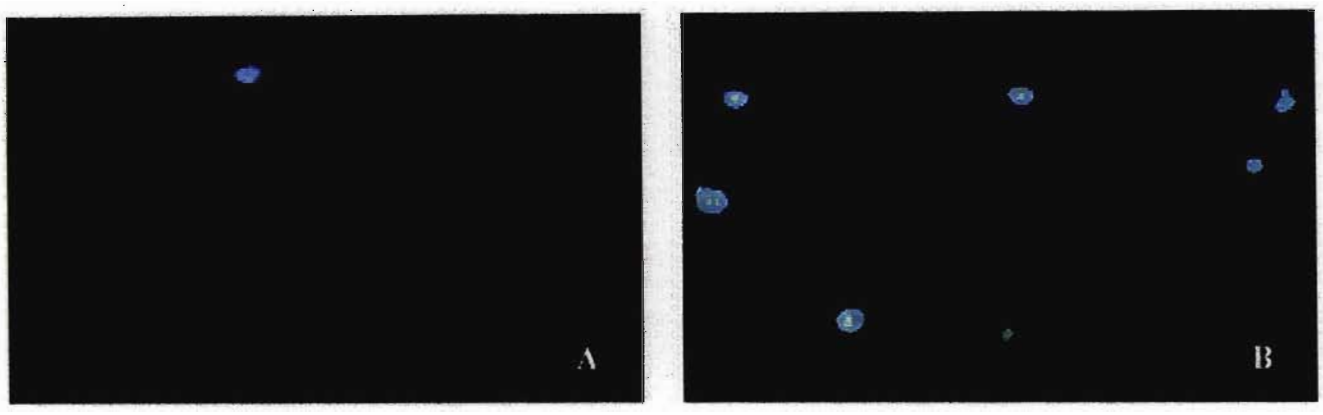

Figure 6.8: Hoechst staining of 5μM combination treated normal human lymphocytes (A) and whole blood (B).

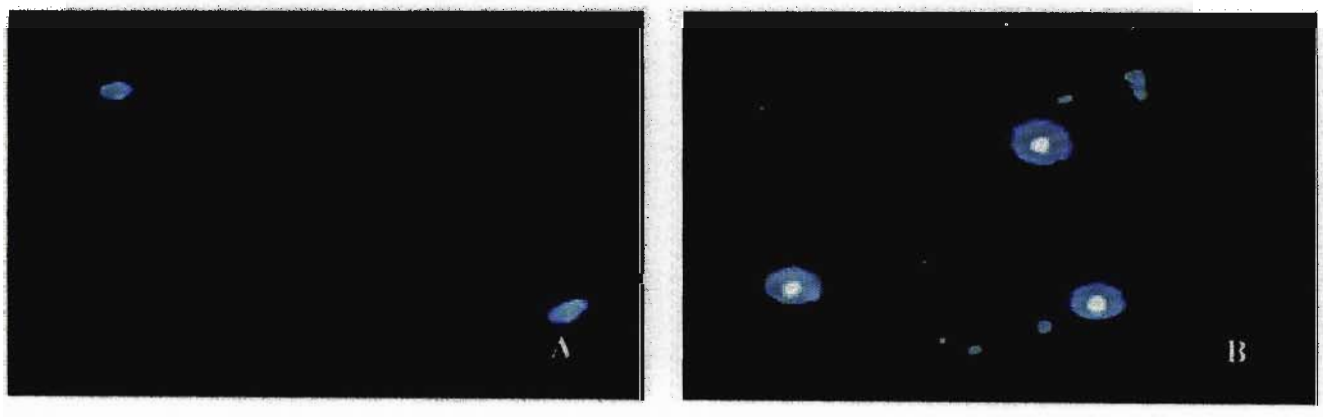

Figure 6.9: Hoechst staining of 50μM combination treated normal human lymphocytes (A) and whole blood (B).

In the untreated normal human lymphocytes (Figure 6.3A) , the stained cells appear as small dull blue–green round compact balls, with no nuclear fragmentation. The nuclei of the lymphocytes extracted from untreated whole blood were larger, with more detail visible probably due to being present in their natural environment (Figure 6.3B). However the nuclei still appeared as blue round balls with solid white centres.

The nuclei of the 5μM AFB_1 treated lymphocytes were larger than the untreated controls (Figure 6.4B). Instead of a circular shape, the nuclei were elongated. A possible hypothesis could be that the cells are undergoing necrosis. During necrosis, the dying cells swells, both cytosolic and nuclear structures alter, but the general disposition of hetero- and euchromatin is maintained, as are the nuclear pores. The plasma membrane ruptures and internal materials reach the extracellular space, where some of them induce an inflammatory reaction (Wyllie, 1997). The nuclei also appeared to have a stronger fluorescence which could be indicative of necrosis as once cells have undergone necrosis, their membranes become more permeable to the uptake of stains, such as Hoechst (Wyllie, 1997). Aflatoxin B_1 has been shown to be a potent cytotoxic agent (Corrier *et al.*, 1991) so the probability of nuclear damage induced by necrosis is very high.

Many of the cytotoxic effects of AFB_1 require its bioactivation to the 2,3 epoxide metabolite by a cytochrome P450 (P450) dependent polysubstrate monooxygenase enzyme. Cytochrome P450 is expressed in human peripheral blood lymphocytes (Song *et al.*, 1990; Haufroid *et al.*, 2003). Once the epoxide has been activated, some of it will covalently bind to macromolecules to form protein and DNA adducts (Eaton and Gallagher, 1994), possibly resulting in nuclear damage.

The nuclei of the lymphocytes extracted from 5μM AFB_1 treated whole blood were slightly larger than nuclei of lympohocytes extracted from untreated whole blood (Figure 6.4B). It was also observed that the white centres were bigger in diameter when compared to the control. The nuclei of the treated whole blood lymphocytes were still circular in shape. However the nuclei of lymphocytes extracted from 50μM AFB_1 treated whole blood were much larger than the control and also appeared to be more elongated. As mentioned previously, these signs could be indicative of cells undergoing cell death by necrosis or nuclear damage caused by the conjugation of the epoxide to the DNA.

There are several key structural features associated with apoptosis. The dying cell membrane separates from its neighbours, usually with loss of specialised membrane structures such as microvilli and desmosomes. The membrane undergoes a period of blebbing and contortion. The blebs are membrane-invested extensions of cytosol that are usually devoid of organelles and are reversibly extruded and reabsorbed (Wyllie, 1997). This is followed by rapid, irreversible condensation of cytoplasm, accompanied by an increase in cell density, compaction of cytoplasmic organelles and condensation of the nuclear chromatin to form dense granular caps or torodal structures underlying the nuclear membrane.

Nuclear pores disappear from the membrane subjacent to these chromatin condensations whilst, within the nucleus, the proteinaceous centre of the nucleolus separates from its surrounding shell of osmiophilic transcription complexes (Wyllie, 1997). Around this time, the cell splits into a cluster of membrane bounded bodies, each containing a variety of organelles. The 50μM AFB_1 treated lymphocytes appeared in some cases to be undergoing nuclear fragmentation (Figure 6.5A) which could be due to cells undergoing apoptosis. However this is in direct contrast to a previous study by Galvano *et al.* (2002a) which showed AFB_1 did not induce nuclear fragmentation in primary swine alveolar macrophages. A possible explanation could be that AFB_1 affects different cell lines in various ways.

The 5μM FB_1 treated lymphocytes showed little or no change from the controls (Figure 6.6A). The only difference being that the nuclei were slightly unevenly shaped. It has been shown that FB_1 can exhibit both proliferation enhancing (Tolleson *et al.*, 1996; Lim *et al.*, 1996); and growth-inhibiting effects (Dombrink –Kurtzman *et al.*, 1994; Tolleson *et al.*, 1996) in a panel of cultured cell lines. It is possible that at this concentration the cellular effects of FB_1 were mitogenic not cytotoxic, thereby resulting in no nuclear damage. There were signs of nuclear fragmenation starting to occur in the 50μM FB_1 treated lymphocytes (Figure 6.7A).

FB_1 has been shown to induce apoptosis in human cell lines (Tolleson *et al.*, 1996) and as mentioned previously fragmentation is one of the structural changes of a cell undergoing apoptosis. Fumonisin B_1 structurally resembles sphingoid bases such as sphinganine, an intermediate in the biosynthesis of complex sphingolipids. As a result FB_1 acts as an inhibitor of ceramide synthetase (N-Acyltransferase), a key enzyme involved in *de novo* sphingolipid biosynthesis and in the reacylation of free sphingoid bases derived from sphingolipid turnover. These highly bioactive molecules play key roles in protein kinase activity, cell growth and differentiation, carcinogenicity and programmed cell death.

This results in disturbances in cellular processes such as cell growth, cell differentiation, endothelial cell permeability and apoptosis. Inhibition of biosynthesis of sphingolipids is seen at different levels of the process and is reflected in changes of the ratio sphinganine/sphingosine (Wang *et al.*, 1999). Similar results were obtained from the lymphocytes extracted from FB_1 treated whole blood. In the lymphocytes extracted from 5μM FB_1 treated whole blood, the cells were larger and unevenly shaped but no nuclear fragmentation could be seen (Figure 6.6B). After treatment of whole blood with 50μM FB_1, nuclear fragmentation in the extracted lymphocytes was clearly seen (Figure 6.7B). A study by Galvano *et al.* (2002a) showed that treatment of primary swine alveolar macrophages with 25 μg/ml FB_1 induced nuclear fragmentation, which was visualized by staining with Hoechst 33258. The 50μM FB_1 treatment of whole blood appeared to induce the most nuclear damage, which could indicate that FB_1 potential to bring about cytotoxic effects by inducing apoptosis has been underestimated.

The 5μM combination treatment of normal human lymphocytes, was similar to the 5μM FB_1 treated cells. However there were signs of nuclear fragmentation possibly starting to occur (Figure 6.8A). After the 50μM combination treatment, the nuclear fragmentation can be clearly seen (Figure 6.9A). Possibly the AFB_1 and the FB_1 are working in conjuction to induce apoptosis via different mechanisms.

The lymphocytes extracted from 5μM combination treated whole blood appear condensed when compared to the control (Figure 6.8B). This could be due either to the the condensation of cytoplasm, a classic feature of apoptosis or the cells could have undergone secondary necrosis, resulting in the formation of cluster of membrane bounded bodies, each containing a variety of organelles. Either which is indicative of cells undergoing apoptosis. The 50μM combination treatment of whole blood does not appear to induce DNA fragmentation (Figure 6.9B). However there is swelling and the size of the nucleus is larger, which could be the result of cellular death via the necrotic pathway.

6.4 Conclusion

Staining normal human lymphocytes with Hoechst 33258 has shown that both AFB_1 and FB_1 are capable of inducing nuclear fragmentation, which could be indicative of apoptosis. Treatment with FB_1, a known apoptotic-inducing agent (Tolleson *et al.*, 1996), resulted in the greater degree of fragmentation at both concentrations.

CHAPTER 7

Conclusion

Aflatoxin B_1 and FB_1 are fungal metabolites widely present in feed and food crops all over the world. As long-term exposure to low levels of these mycotoxins cannot be completely avoided, they are likely to be a potential concern of occupational and public health.

The results of the MTT cytotoxicity assay showed that routinely AFB_1 induced higher levels of cytotoxicity in normal isolated human lymphocytes than FB_1, especially at lower concentrations after 12 and 24 hours incubation. It was difficult to determine the potential cytotoxicity of the combination treatment, as the mitogenic properties of FB_1 appeared to partially counteract the cytoxic effect exerted by AFB_1. However this does not necessarily mean that the combination of AFB_1 and FB_1 is not potentially harmful to normal human lymphocytes.

When whole blood was treated with the same concentration and ratio of toxin, FB_1 was shown to be more toxic to the lymphocytes than AFB_1. This was thought to be the result of the presence of other cells such as erythrocytes and macrophages. Another factor could have been that FB_1 is strongly polar and poorly absorbed and as a result is rapidly excreted in an *in vivo* situation. The combination treatment of blood was shown to be cytotoxic in a dose dependent manner. Both toxins appear to be working simultaneously to reduce cell viability by different mechanisms and induced a greater cytotoxic effect, treated in combination than individually at the higher concentrations.

The high concentration of AFB_1 induced higher levels of apoptosis and necrosis in isolated normal human lymphocytes, than the control. It is a well known fact that AFB_1 is extremely cytotoxic therefore it was feasible that the levels of necrosis would be increased. The treatment of isolated lymphocytes with FB_1 with a low concentration resulted in increased levels of apoptosis. Previous studies have shown that FB_1 is more cytotoxic at low concentrations (Rumora *et al.*, 2002). It was shown that FB_1 and AFB_1 working simultaneously resulted in increased levels of apoptosis.

Possibly due to the cellular defence mechanisms and the rapid excretion of the toxins, the levels of apoptosis were reduced in whole blood lymphocytes when compared to isolated lymphocytes. However there was a similar trend with the treatment of AFB_1 and FB_1 resulting in increased levels of apoptosis.

It was shown that both AFB_1 and FB_1 are capable of inducing nuclear fragmentation, which could be indicative of apoptosis. Treatment with FB_1, a known apoptotic-inducing agent (Tolleson *et al.*, 1996), resulted in the greater degree of fragmentation at both concentrations. Treatment with the combination of mycotoxins induced more nuclear fragmentation than individual treatments. As both toxins are common contaminants and have been known to co-exist (Ueno *et al.*, 1997), this could be a potential area of concern for public health.

The 50μM combination treatment of normal human lymphocytes induced the most DNA damage. This would appear to indicate that the mycotoxins at higher concentrations work in conjunction, either synergistically or additively, to induce greater DNA damage than if treated individually.

Further investigation needs to be done to quantify the levels of mycotoxins taken up by normal human lymphocytes to gain better understanding of their potential cytotoxicity. Fumonisin B_1 is known to be a competitive inhibitor of sphingosine and sphinganine *N* acetyltransferase, which are key components in the pathways for *de novo* biosynthesis of sphingolipids (Wang *et al.*, 1991) resulting in free sphingoid bases. Further research is needed to establish the factor(s), which determines if the free sphingoid bases are growth stimulatory, growth inhibitory or cytototoxic. It could be important to elucidate the mechanisms whereby AFB_1 and FB_1 induce apoptosis and subsequently DNA damage. A better knowledge of the regulation and execution of apoptosis may be a potentially excellent target for diagnosis and therapeutic intervention in AFB_1 and FB_1 induced toxicoses. These types of studies will also permit more precise biochemical analyses to be applied to assessment of the health risk due to exposure to AFB_1 and FB_1.

The history of medicine has clearly established that preventative strategies offer the best long term means for eliminating morbidity and mortality from infections and diseases. Chemically induced diseases such as mycotoxicoses are likely to be the same.

Opportunities for prevention of mycotoxin induced disease include reducing exposure and interfering with the toxicological processes through pharmacological and nutritional interventions. Aflatoxin B_1 and FB_1 are human health hazards and continuous exposure to even small amounts can be detrimental.

In South Africa, the importance of mycotoxins in public health will increase as the available food supplies decrease due to population growth and other factors. An association between the sources of exposure and disease is essential in disease prevention. The united efforts of researchers from disciplines such as molecular biology, biochemistry, physiology, microbiology, botany and basic science are crucial to provide a scientific basis for regulatory guidelines that will protect the population from health hazards associated with exposure to AFB_1 and FB_1.

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Appendix

Appendix 1.1: RPMI-1640 Medium

Inorganic salts (g/l)	
$Ca(NO_3)_2.H_2O$	0.10000
$MgSO_4$ (anhydrous)	0.04884
KCl	0.40000
$NaHCO_3$	1.50000
NaCl	6.00000
Na_2HPO_4 (anhydrous)	0.80000

Amino acids (g/l)	
L-Arginine (free base)	0.20000
L-Asparagine.H_2O	0.05682
L-Aspartic Acid	0.02000
L-Cystine.2HCl	0.06520
L-Glutamic Acid	0.02000
L-Glutamine	0.30000
Glycine	0.01000
L-Histidine (free base)	0.01500
Hydroxyl-L-Proline	0.02000
L-Isoleucine	0.05000
L-Leucine	0.05000
L-Lysine.HCl	0.04000
L-Methionine	0.01500
L-Phenylalanine	0.01500
L-Proline	0.02000
L-Serine	0.03000
L-Threonine	0.02000
L-Tryptophan	0.00500
L-Tyrosine.2Na.2H_2O	0.02883
L-Valine	0.02000

Vitamins (g/l)	
D-Biotin	0.00020
Choline Chloride	0.00300
Folic Acid	0.00100
Myo-Inositol	0.03500
Nicotinamide	0.00100
p-Amino Benzoic Acid	0.00100
D-Panthothenic Acid (hemicalcium)	0.00025
Pyridoxine	0.00100
Riboflavin	0.00020
Thiamine.HCl	0.00100
Vitamin B-12	0.000005

Other (g/l)	
D-Glucose	4.50000
Glutiathione (reduced)	0.00100
HEPES	2.38300
Phenol Red, Sodium Salt	0.00500
Sodium Pyruvate	0.11000

Appendix 1.2: Hanks' Balanced Salt Solution

KCl	0.40000 g/l
KH_2PO_4	0.06000 g/l
NaCl	8.00000 g/l
$NaHCO_3$	0.35000 g/l
Na_2HPO_4 (anhydrous)	0.09000 g/l
Glucose	1.00000 g/l
Phenol Red, Sodium Salt	0.02120 g/l