

***An Investigation into the Cellular Expression of Genes Involved in Resistance to Aflatoxin B<sub>1</sub>, and the Mode of Cell Death Elicited by this Carcinogen, in a Model System using AFB<sub>1</sub>-resistant and AFB<sub>1</sub>-sensitive Rat Hepatocyte Cell lines.***

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*This thesis is written in memory of*

*Mr. Ernest Arthur Jackson,*

*who died on 25th March, 1997,*

*and is dedicated to my son,*

*Henry William Mills,*

*born on 9<sup>th</sup> October 1998.*



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### Abbreviations list

ADC	analog-to-digital converter
AFAR	aflatoxin B1-aldehyde reductase
AFB <sub>1</sub>	aflatoxin B1
AFB <sub>1</sub> -dhd	AFB <sub>1</sub> -dihydrodiol
AFB <sub>1</sub> -FAPY	8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl formamido)-9-hydroxy AFB <sub>1</sub>
AFB <sub>1</sub> -N <sup>7</sup> -guanine	8,9-dihydro-8-(N <sup>7</sup> -guanyl)-9-hydroxy-AFB <sub>1</sub>
AFG <sub>1</sub>	aflatoxin G1
AFM <sub>1</sub>	aflatoxin M1
AFP <sub>1</sub>	aflatoxin P1
AFQ <sub>1</sub>	aflatoxin Q1
AFL	aflatoxicol
AMV	avine myelo virus
AP	alkaline phosphatase
ARE	antioxidant responsive element
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CM	carboxymethyl
CYP	cytochrome P450 mono- oxygenase
DABCO	1,4-diazobicyclo(2.2.2.)octane
DEA	diethylamine
DEAE	2-(diethylamino)ethyl

## Abbreviations

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DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMF	dimethylfluoride
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylene diaminetetracetate
ELISA	enzyme linked immunosorbant assay
ERCC3	excision-repair-cross complementing 3
FAH	foci of altered hepatocytes
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GDP	guanosine diphosphate
GGT	$\gamma$ -glutamyl transpeptidase
GS <sup>-</sup>	glutathione (reduced)
GSH	glutathione
GST	glutathione-S-transferase
GS-X	glutathione-X
GTC	guanidinium thiocyanate
GTP	guanosine triphosphate

## Abbreviations

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HBV	hepatitis B virus
HBSS	Hanks buffered saline solution
HBX	hepatitis B X
HCC	hepatocellular carcinoma
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
HPLC	high performance liquid chromatography
ISEL	<i>in situ</i> end labelling
LSC	laser scanning cytometer
LTC <sub>4</sub> S	leukotriene C <sub>4</sub> synthase
3-MC	3-methylcholanthrene
mdr1b	multi-drug resistance
MDR	multi-drug resistance
MOAT	multi-specific organic anion transporter
MRC	Medical Research Council
mRNA	messenger RNA
MRP	multidrug resistance-related protein
MTT	3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide)
NADPH	nicotinamide adenine dinucleotide phosphate
OD	optical density

## Abbreviations

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PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pgp	P-glycoprotein
PI	propidium iodide
PS	phosphatidylserine
RNase	ribonuclease
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl-sulphate
TAE	tris-acetate-EDTA buffer
Taq	<i>Thermus aquaticus</i>
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TGF- $\beta$ 1	transforming growth factor $\beta$ 1
TRIS	tris (hydroxymethyl)amino methane
tTG	tissue transglutaminase
UP	ultra pure
UV	ultra violet
XRE	xenobiotic responsive element

## ***Abstract***

The toxicity of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), has been extensively studied in the rat, and its carcinogenic effects have been well documented. Binding of the cytochrome P-450-activated *exo*-8,9-epoxide form of the molecule to DNA and protein to form adducts, forms the basis of the xenobiotic's cytotoxicity and genotoxicity.

In the rat, the major detoxification pathway is its glutathione-S-transferase (GST)-mediated conjugation with glutathione. An alternative route for the AFB<sub>1</sub>-8,9-epoxide is hydrolysis to give the cytotoxic AFB<sub>1</sub>-dihydrodiol (AFB<sub>1</sub>-dhd). The rat GST A5 subunit, exhibits a high catalytic activity for the AFB<sub>1</sub>-*exo*-8,9-epoxide, and its protective effect towards AFB<sub>1</sub> has been shown. In addition, the expression of a novel AFB<sub>1</sub>-aldehyde reductase (rAFAR1) gene has been investigated and found to be expressed in the AFB<sub>1</sub>-sensitive RLE cell line as well as in the AFB<sub>1</sub>-resistant JB1 cell line, along with the GST A3 and the p-glycoprotein (mdr1b) gene. A model system was devised for the study of AFB<sub>1</sub> toxicity and the need for optimisation of all assay conditions is stressed, in particular the concentration of quail liver microsomes (an extrinsic AFB<sub>1</sub> activating system). The toxic effect of the microsomes on the RLE cell line has been demonstrated and a concentration of 1% (v/v) in unsupplemented medium (as an AFB<sub>1</sub> diluent) is recommended.

Cell death in response to AFB<sub>1</sub> toxicity is important since the elimination of individual initiated cells which are induced to undergo apoptosis (programmed cell death) will prevent their clonal expansion and ultimate promotion and progression to tumours. Cell death by necrosis however, leads to replacement division which in itself could prove to have a promoting effect on any initiated cells.

The mode of cell death in AFB<sub>1</sub>-induced cytotoxicity was investigated using annexin V as a marker for apoptosis, by way of flow cytometry, laser scanning cytometry and by a novel ELISA/MTT technique devised in this thesis. Cell death was found to be largely by necrosis, with low levels of apoptosis evident after the time points investigated: 2 and 24 hours.

Chapter 1  
General Introduction

## 1 General Introduction

Risks to human health caused by exposure to toxic chemicals have become of increasing concern in the search into the molecular basis of cancer. This has led to a need for devising reliable methods for predicting human cancer risks as a result of chemical exposures. There is now sufficient evidence to suggest that many carcinogens or their metabolites bind covalently to DNA giving rise to adducts. Unless repaired by the cell, these adducts can lead to secondary genomic alterations which is an important initiating step in the process of carcinogenesis.

One such carcinogen which has been extensively studied in man and the rat is aflatoxin. This study is concerned with the importance of the rat glutathione-S-transferase (GST) A3 and A5 subunits, aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) aldehyde reductase (rAFAR1) and p-glycoprotein (the product of the *mdr1b* gene), in providing protection against AFB<sub>1</sub>-8,9-epoxide, the product of phase I metabolism of aflatoxin B<sub>1</sub>, in two rat hepatocyte cell lines: RLE and JB1. RLE is an undifferentiated, untransformed epithelial cell line arising from cells isolated from an adult male Fischer rat. Under normal conditions, cells do not express the A5 subunit and are sensitive to the cytotoxic effects of AFB<sub>1</sub>. For comparison, JB1 is a tumourigenic epithelial cell line isolated from AFB<sub>1</sub>-induced hepatomas (Power *et al.*, 1987), and expresses a constitutively high level of the GST A5 subunit. Both cell lines exhibit contact inhibition and are grown in monolayer culture (Manson *et al.*, 1981) (Fardel *et al.*, 1997).

The following chapter will give a general background to the toxic effects of AFB<sub>1</sub> in rats and in humans, detoxification mechanisms, and the relevance of differentiating between apoptosis or necrosis as the mode of cell death, in particular in relation to carcinogenicity.

## 1.1 Aflatoxins

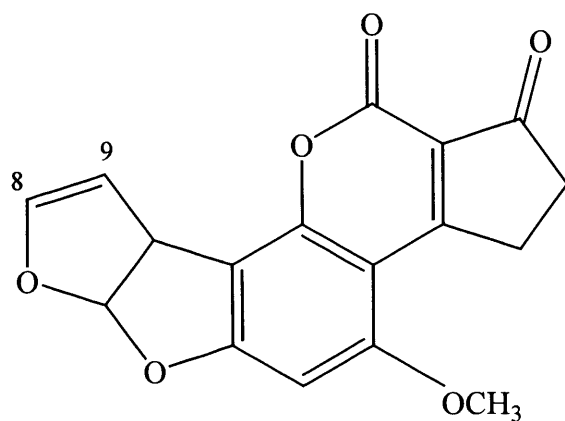
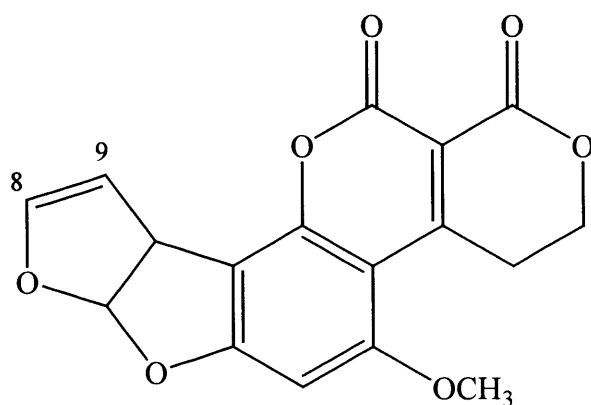
### 1.1.1 Biological Significance of Aflatoxins

Hepatocellular carcinoma (HCC) is a major cause of cancer mortality in areas such as southern China, sub-Saharan Africa and parts of South East Asia where both infection with hepatitis B virus (HBV) and exposure to aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) occurs. Epidemiological studies support an apparent interaction between these viral and chemical factors in hepatocarcinogenesis, and indicate a possible synergism between dietary consumption of AFB<sub>1</sub> and chronic active viral hepatitis (Yu *et al.* 1997; Kirby *et al.* 1996). Further experimental and epidemiological evidence suggests that lung and kidney are also likely target sites for AFB<sub>1</sub> attack after consumption of contaminated food, or inhalation of contaminated grain dusts (Harrison and Garner, 1991; Hayes *et al.*, 1984), and the effects of aflatoxin on the immune system have been discussed by Pestka (2000). The aflatoxins (AFB<sub>1</sub>, AFB<sub>2</sub>, AFG<sub>1</sub>, AFG<sub>2</sub>) are therefore perceived as a health hazard in developing countries. They are found as secondary metabolites of some species of the *Aspergillus* fungal genus: *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*, which naturally contaminate foodstuffs such as corn, peanuts and cottonseed. The problem of aflatoxin contamination however, was first noted in the U.K. An outbreak of poultry deaths occurred in the south east of England in 1960; the principal animal response being liver necrosis. The cause of this 'Turkey-X' disease was traced to a consignment of ground nut meal from Brazil (Blount, 1961). The major factor contributing to the presence of these toxins in the foodstuff was poor storage conditions; the most serious outbreaks since occurring in areas of high temperature and humidity and in crops damaged due to growth under stress conditions e.g. drought or insect damage (Wilson and Payne, 1994).

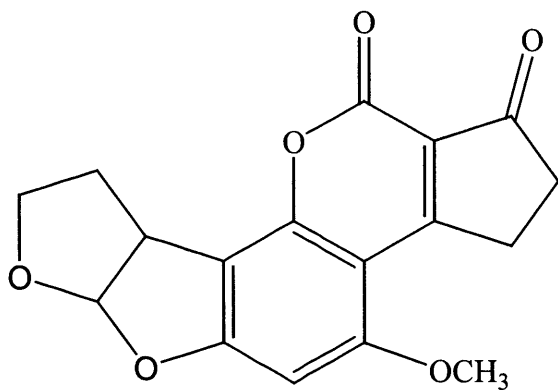


### 1.1.2 Physical Characteristics of Aflatoxins

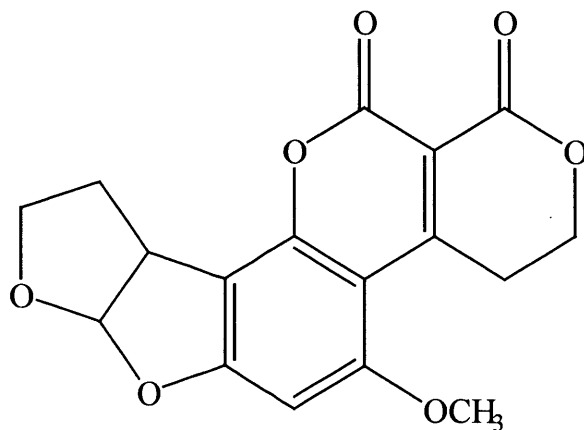
Of the four primary aflatoxins, the order of toxicity is; B<sub>1</sub>, G<sub>1</sub>, B<sub>2</sub>, G<sub>2</sub>. AFB<sub>1</sub>, as well as being the most abundant in nature, is the most biologically active (Butler and Lujinsky, 1971), due to the crucial role played by epoxidation of the 8,9 double bond and also the greater potency associated with the cyclopentenone ring in the B series as opposed to the 6-membered lactone ring in the G series. Structures of the aflatoxins can be seen in figures 1.1 and 1.2. Aflatoxins are stable at high temperatures; the melting point of AFB<sub>1</sub> is 268-269 °C and AFG<sub>1</sub> has a melting point of 244-246°C (Asao *et al.*, 1965). The lactone ring in the structure renders them susceptible to degradation under alkaline conditions and they are freely soluble in apolar solvents such as chloroform, moderately polar solvents such as methanol, and in particular dimethylsulphoxide (DMSO). They can be detected via long-wave ultra violet (UV) absorption e.g. AFB<sub>1</sub> ( $\lambda_{\max}^{\text{ETOH}}$  223, 265 and 362nm) and AFG<sub>1</sub> ( $\lambda_{\max}^{\text{ETOH}}$  243, 257, 264 and 362nm) (Asao *et al.*, 1965). When antibodies to aflatoxin are available, immunochemical methods such as enzyme linked immunosorbant assay (ELISA) can be used to detect and measure aflatoxins in a relatively short time (Truckess and Wood, 1994; McLean and Dutton, 1995).

AFB<sub>1</sub>AFG<sub>1</sub>

**Figure 1.1:** Structure of aflatoxins B<sub>1</sub> and G<sub>1</sub>. Note the 8,9-double bond which renders both aflatoxins open to epoxidation. The cyclopentenone ring in the B series accounts for its greater potency compared to the G series.



AFB<sub>2</sub>

AFG<sub>2</sub>

**Figure 1.2:** Structure of aflatoxin B<sub>2</sub> and G<sub>2</sub>. Note the absence of an 8,9-double bond. The cyclopentenone ring in the B series accounts for its greater potency compared to the G series.

### **1.1.3 Aflatoxin and Hepatocellular Carcinogenesis**

#### **1.1.3.1 Carcinogenesis**

Carcinogenesis is a multistep process. The application of a carcinogen does not lead to the immediate production of a tumour, rather there are a series of changes after the initiation step induced by the carcinogen. These subsequent changes, or tumour promotion, may be produced by the carcinogen or by other non-tumour producing agents (promoting agents). Initiation, the primary and essential step in the process is very rapid and once the initial change has taken place the initiated cells may persist for a considerable time - even perhaps the lifetime of the individual. The genetic material of the cell is the most likely site for the primary event, probably in the stem cell population of the tissue involved, and initiated cells remain latent until acted upon by promoting agents which are not carcinogenic in themselves but which induce cells to divide. It is likely that the effects of promoters are multiple and that different cell types will respond differently to the same agent. Once initiation has occurred via some form of carcinogen-DNA interaction, focal areas, or foci of altered hepatocytes (FAHs), within the carcinogen-treated region of the tissue are seen as a result of clonal expansion of these altered cells. These focal areas of abnormal tissue are intermediate in character between normal and malignant, and in time, after further mutational changes, new cell populations are produced resulting in the appearance of the malignant phenotype. This is the most obvious change seen in promotion and is found consistently in liver carcinogenesis long before the appearance of hepatocellular carcinoma. The third stage of carcinogenesis is progression, the process by which initiated cells acquire more and more aberrant characteristics (for a review see: Farber, 1984).

### 1.1.3.2 Binding of AFB<sub>1</sub> to DNA

Once formed, AFB<sub>1</sub>-8,9-epoxide, if not eliminated by the various detoxification or elimination methods employed by the cell, can cause genotoxic damage by binding predominantly with the N<sup>7</sup> atom of guanine in DNA to form adducts (Essigmann *et al.*, 1977). The principal adduct formed is the 8,9-dihydro-8-(N<sup>7</sup>-guanyl)-9-hydroxy-AFB<sub>1</sub> adduct (N<sup>7</sup>-guanyl) while the second most prevalent adduct is 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimidin-5-yl formamido)-9-hydroxy AFB<sub>1</sub> (FAPY). The N<sup>7</sup>-guanyl adduct represents 80-90% of the total AFB<sub>1</sub> adducts formed (Essigman *et al.*, 1977; D'Andrea and Heseltine, 1978) with maximum levels occurring 2 hours after exposure to AFB<sub>1</sub> in a manner dependent upon AFB<sub>1</sub> dose over the range 0.125-1.0mg/kg (Croy *et al.*, 1978; Croy and Wogan, 1981). It has a life span of 7.5 hours and 70% of the adduct undergoes nonenzymatic depurination during the first 24 hours after exposure to AFB<sub>1</sub>. The second major adduct, FAPY, is formed by alkaline hydrolysis of the remaining N<sup>7</sup>-guanyl adduct by imidazole ring opening to give a more stable product (Essigman *et al.*, 1982; Hertzog *et al.*, 1982)) with a much longer half life than the N<sup>7</sup>-guanyl adduct.

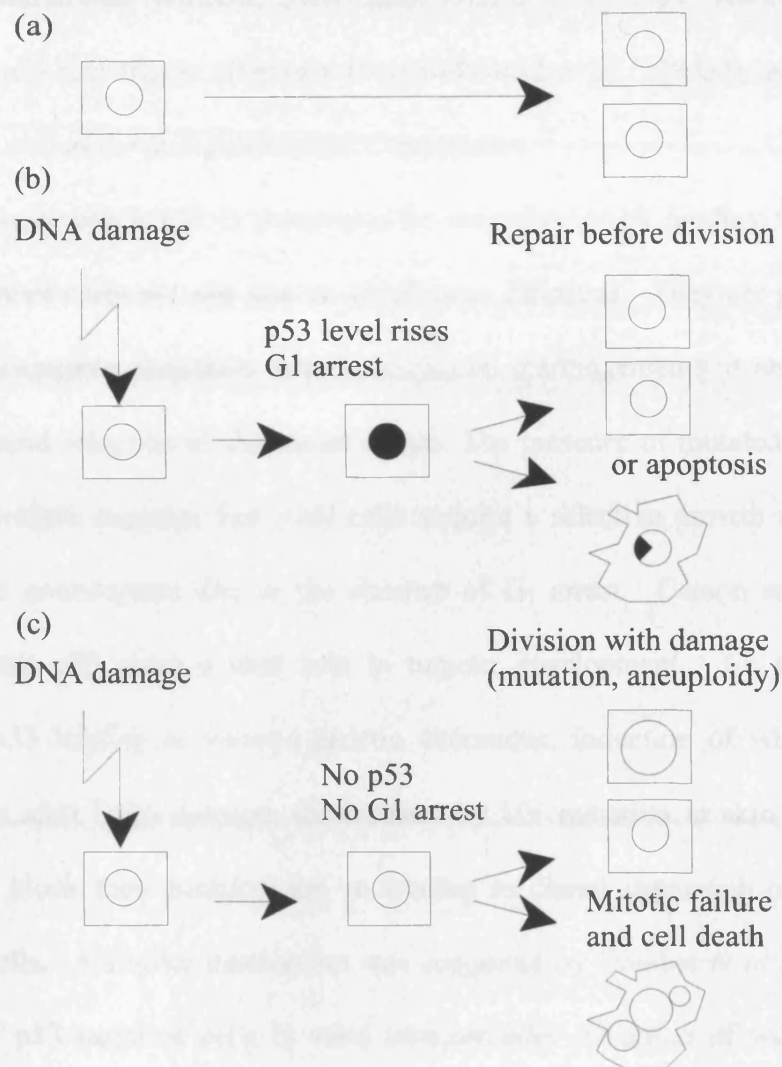
The formation of AFB<sub>1</sub>-DNA adducts is harmful to the cell and it is thought that the accumulation of such products leads to mispairing or error-prone repair during DNA replication resulting in G:C→A:T conversions and is ultimately responsible for the carcinogenic and mutagenic effects of AFB<sub>1</sub> (Croy and Wogan, 1981). A good correlation has been shown between DNA adduct levels and tumour incidence in rats (Otteneeder and Lutz, 1999), and aflatoxin adducts have been proposed as intermediate end points in clinical cancer prevention studies in animals and man (Kensler *et al.*, 1998a; Phillips *et al.*, 1999). However, whilst Denissenko *et al.*, (1999) report that adducts form with a similar sequence-specificity *in vitro* and *in vivo*, Stanley *et al.*,

(1999) report that *in vitro* AFB<sub>1</sub>-induced mutations are not necessarily the same as those seen *in vivo*.

The mutagenicity of AFB<sub>1</sub> has been demonstrated in an Ames test using *Salmonella typhimurium* TA-100 and TA-98 (Wong and Hsieh, 1976; Gurtoo *et al.*, 1978) following activation of the AFB<sub>1</sub> by rat hepatic microsomes. Whilst certain mutations will be lethal to the cell those that involve oncogenes (e.g. *ras* in the rat) or tumour suppressor genes (e.g. *p53* in humans) can result in carcinogenesis (McMahon *et al.*, 1986)

### 1.1.3.3 Mutation of p53 by AFB<sub>1</sub>

The *p53* tumour suppressor gene, located on chromosome 17, is commonly mutated in human cancer (Greenblatt *et al.*, 1994; Greenblatt *et al.*, 1996; Varley *et al.*, 1991) including hepatocellular carcinoma (HCC) (Bressac *et al.*, 1991; Hsu *et al.*, 1991). Recent studies investigating the mechanisms underlying the biological activity of p53 indicate that the 53kDa protein is involved in gene transcription, DNA synthesis and repair and programmed cell death (apoptosis) (Hollstein *et al.*, 1991; Levine *et al.*, 1991; Harris, 1993; Schwartz and Jordan, 1997). This has led to p53 being regarded as the guardian of the genome (Lane, 1992) (figure 1.3). In most cell types the levels of wild type p53 are reported to be low; high levels being found only when cells express mutant forms of p53 (Bjelogrić *et al.*, 1994). It is generally believed that wild type p53 is rapidly degraded, whereas conformational changes in mutant p53 confer stability and therefore protection upon these forms. Expression of p53 is thought to be an important response to DNA damage, causing accumulation of wild-type p53 which is involved in the transcriptional control of other genes by sequence-specific DNA-binding



**Figure 1.3:** Model for the function of p53. a) Normal cell division which does not require p53. b) Induction of p53 in response of a normal cell to DNA damage. c) Cells in which p53 is inactivated by mutation, or by host or viral oncoproteins, replicate damaged DNA, resulting in aneuploidy, mitotic failure and cell death. These genetically less stable cells accumulate mutations and chromosomal rearrangements at an increased rate leading to rapid selection of malignant clones (from Lane, 1992).

(Kern *et al.*, 1991). One such gene is WAF1, which codes for p21 expression which in turn switches off replication and results in G<sub>1</sub> arrest (Pines, 1994). This allows time for repair of the damaged DNA (Kastan *et al.*, 1991) in which p53 plays both a direct and indirect role by forming high-affinity complexes with the damaged DNA site, possibly providing a focus for the proteins involved in DNA repair and by inducing Gadd45

expression which may stimulate DNA repair (Smith *et al.*, 1994; Marx, 1994). If the repair fails, p53 may trigger apoptosis (Yonish-Rouach *et al.*, 1991) by activation of the bax gene as well as down-regulating bcl-2 expression.

Tumour cells in which p53 is inactivated by mutation, or by binding to host or viral proteins, cannot carry out any one or all of these functions. They are genetically less stable so accumulate mutations and chromosomal rearrangements at an increased rate leading to rapid selection of malignant clones. The presence of mutated p53 protein in tumours therefore suggests that such cells acquire a selective growth advantage over their normal counterparts due to the absence of G<sub>1</sub> arrest. Carson and Lois (1995) suggested that p53 plays a dual role in tumour development. As well as loss of functional p53 leading to various genetic alterations, induction of wild type p53 in normal cells after DNA damage, for instance by UV radiation in skin (Ziegler *et al.*, 1994), will block their proliferation so leading to clonal expansion of p53 negative (mutated) cells. A similar mechanism was suggested by Greaber *et al.*, (1996) for the selection of p53 negative cells in solid tumours after induction of wild type p53 by hypoxia in surrounding normal cells. Van Gijssel *et al.*, (1997) demonstrated a mechanism of hepatic tumour promotion that involved expression of p53 and inhibition of the cell cycle in normal hepatocytes in the rat, after treatment with various hepatocarcinogens, including AFB<sub>1</sub>. This allowed for the clonal expansion of preneoplastic cells that did not express p53. They also showed that p53 expression in these cells was much less, if at all, increased by DNA damaging compounds such as AFB<sub>1</sub> and proposed that a model of selective inhibition of normal hepatocytes exists as a mechanism for hepatic tumour promotion.

The mutation of the *p53* gene in HCC suggests that loss of normal gene function may be a key step during malignant transformation of hepatocytes and there have been several reports of *p53* mutation in HCC in humans after AFB<sub>1</sub> exposure and subsequent AFB<sub>1</sub>



adduct formation (Ozturk *et al.*, 1991; Mace *et al.*, 1997). It appears that a mutational hotspot exists in the third position of codon 249 of the *p53* tumour suppressor gene and that AFB<sub>1</sub> preferentially induces the transversion of G → T at this position resulting in the insertion of serine instead of arginine in the mutant protein. Aguilar *et al.*, (1994) reported that this codon 249 mutation is also present in apparently normal liver. This suggests either its early involvement in the development of AFB<sub>1</sub>-induced HCC or its non-association. The highest frequency of the mutation is found almost exclusively in HCC from high AFB<sub>1</sub> regions in East Asia and Africa (Hsu *et al.*, 1991; Aguilar *et al.*, 1993; Mace *et al.*, 1997) whereas in Japan, Hong Kong and Europe where there is a low risk of exposure to AFB<sub>1</sub> there is no reported incidence of *p53* mutation at codon 249 in HCC samples (Nose *et al.*, 1993; Hayashi *et al.*, 1993; Ng *et al.*, 1994; Volkmann *et al.*, 1994). This supports the notion that AFB<sub>1</sub> represents a causative carcinogen in hepatocarcinogenesis in certain regions of the world

It has been suggested however, that mutations in *p53* are not necessary in AFB<sub>1</sub>-induced hepatocarcinogenesis in nonhuman primates by Fujimoto *et al.*, (1992) who reported no changes at the third position of codon 249 in nine AFB<sub>1</sub>- induced tumours but identified a point mutation in one hepatocellular carcinoma at the second position of codon 175. Mutations in *p53* have rarely been detected in mouse liver tumours (Chen *et al.*, 1993; Calvert *et al.*, 1995; Sipowicz *et al.*, 1997), but a relatively high incidence of *p53* mutations were found in tamoxifen-induced rat liver tumours (Vancutsem *et al.*, 1994) and in rat liver carcinomas caused by a choline-deficient diet (Smith *et al.*, 1993).

This suggests that under certain circumstances *p53* mutations may contribute to rat liver tumourigenesis. The occurrence of a mutation in codon 249 of the *p53* gene in human hepatocellular carcinomas may therefore indicate involvement of environmental carcinogens other than AFB<sub>1</sub>, or that the presence of the hepatitis B virus (HBV) is

involved in AFB<sub>1</sub> induction of G to T transversion in codon 249 (Denissenko *et al.*, 1999).

#### 1.1.3.4 Hepatitis B Virus and p53

The relative risk of HCC is elevated in viral hepatitis carriers with chronic active viral hepatitis (Beasley *et al.*, 1981) suggesting that cell proliferation and inflammatory response associated with chronic active hepatitis are the critical factors responsible for the neoplastic transformation of the precursor cells of HCC. HBV DNA integrates into host DNA of HCC cells at random sites in the genome, and a possible relationship between hepatitis B virus and p53 protein has been shown by Slagle *et al.*, (1991), who reported integration of the hepatitis B virus into host DNA in human chromosome 17p near to the *p53* gene causing the deletion of at least one allele. In addition, other allele deletions were noted, such as on chromosome 8q near to the *myc* gene.

HBV DNA contains the X gene which codes for the hepatitis B X (HBX) protein which in turn modulates the transactivation of other cellular genes and is a possible viral oncoprotein. Sohn *et al.*, (2000) suggest that by altering the balance between DNA repair and apoptosis, the HBX protein enhances the susceptibility of liver cells to carcinogen-induced mutagenesis. It has been shown to complex with p53, inhibiting its sequence-specific DNA-binding and transcriptional activation properties, partially disrupting p53 oligomerisation and preventing p53 from binding to transcription-repair coupling factor ERCC3 (Wang *et al.*, 1994). These mechanisms of epigenetic p53 inactivation could account for the transactivation properties of HBX and influence a wide range of p53 functions so contributing to the process of carcinogenesis. There have been many reports on the presence of chronic HBV infection and p53 mutations in the same tumours (Bressac *et al.*, 1991; Li *et al.*, 1993; Teramoto *et al.*, 1994; Fujimoto *et*

*al.*, 1994). Results indicate that HBV infection alone does not influence the prevalence of *p53* mutation but that AFB<sub>1</sub> exposure is the most important influence on the rate of such mutations, and an elevated risk factor with HBV and AFB<sub>1</sub> has been reported by Ross *et al.*, (1992).

Frequent induction of *p53* mutations in the presence of hepatitis C infection has also been found (Teramoto *et al.*, 1994), although little work has yet been carried out to determine the role of this virus in hepatocarcinogenesis.

#### **1.1.3.5 Activation of *ras* by AFB<sub>1</sub>**

The *ras* oncogene was originally discovered in two murine retroviruses known as Harvey (Ha-*ras*) and Kirsten (Ki-*ras*) sarcoma viruses and the later discovery of N-*ras* in neuroblastoma DNA added to the *ras* gene family. All three forms of *ras* are ubiquitously found and are located on the inner surface of the plasma membrane of mammalian cells. The Ras protein is part of a cell signalling pathway which begins with cell surface receptors and ends in the cell nucleus with proteins that regulate gene expression. The normal products of the *ras* gene are known to bind guanine nucleotides, functioning like a binary switch; in its OFF position a Ras protein binds the nucleoside diphosphate GDP and sits at its usual location on the inner surface of the cell membrane until it receives an incoming activating signal. It then sheds its GDP and binds GTP, existing briefly in the ON state and releasing growth stimulatory signals into the cell. However, the bound GTP is quickly converted to GDP by the intrinsic GTPase activity of the Ras protein, so returning Ras to its original OFF state (Egan and Weinberg, 1993). The oncogenic forms of Ras found in many cancer cells however, work differently as they possess greatly reduced GTPase activity and so do not get

turned off. They therefore remain switched on for long periods flooding the cell with growth stimulatory signals.

All three *ras* genes appear to be mutated after exposure to AFB<sub>1</sub> but Ki-ras mutations are the most prevalent (Sinha *et al.*, 1988; McMahon *et al.*, 1986). Such mutations have been demonstrated by Soman and Wogan (1993) at codon 12 in AFB<sub>1</sub>-induced hepatocellular carcinomas in Fischer 344 rats in the form of a G:C→A:T transition in the second base of the codon. This type of mutation correlates well with the N<sup>7</sup>-guanyl adducts formed with DNA by AFB<sub>1</sub>. In humans, however, although *ras* mutations are commonly found in many tumour types (Slamon *et al.*, 1984)) such as lung (Gao *et al.*, 1997) and breast (Spandidos and Agnantis, 1984), there is little evidence of their importance in hepatocarcinogenesis (Chao *et al.*, 1999).

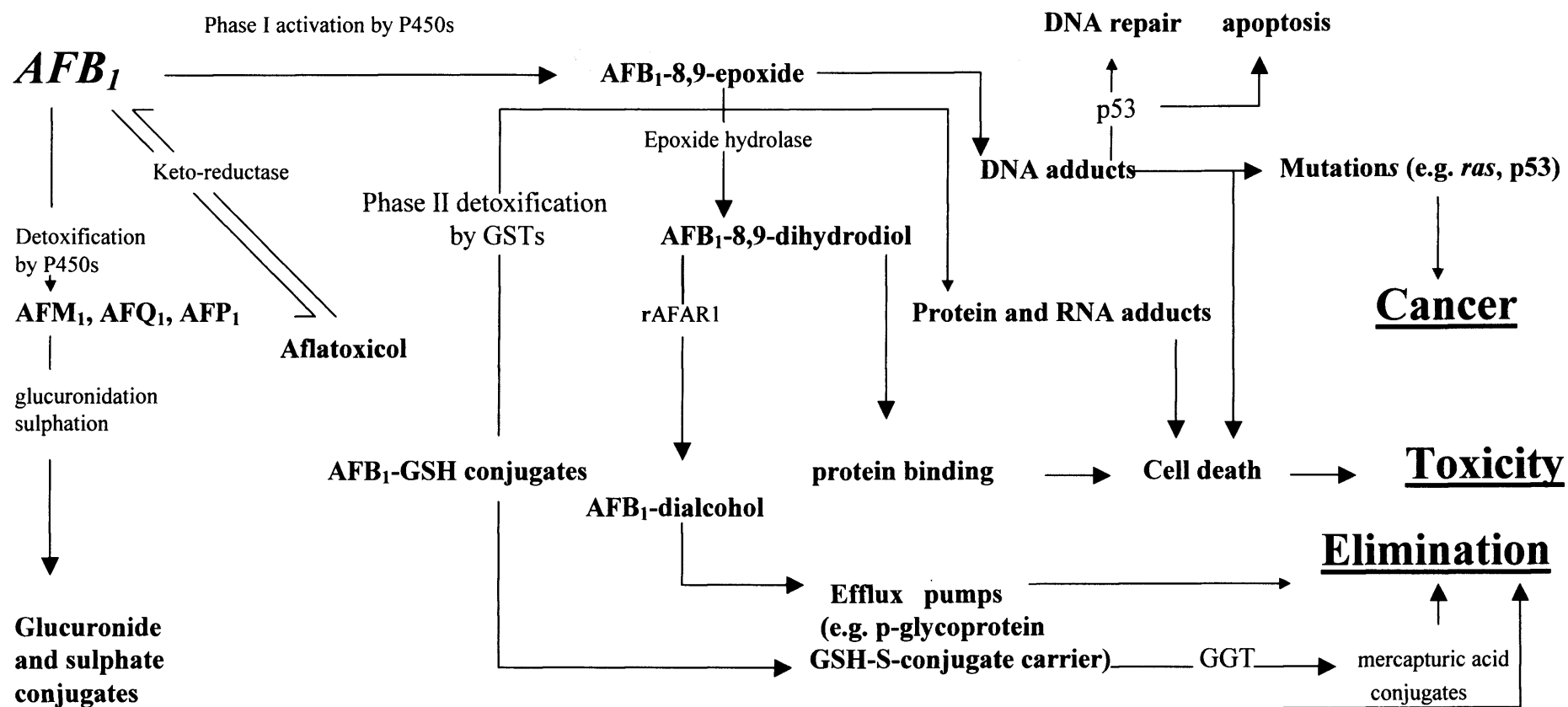
#### 1.1.3.6 Cytotoxic Effects of AFB<sub>1</sub>

As well as causing mutations through binding with DNA, AFB<sub>1</sub> also binds to other macromolecules in the cell, in particular ribosomal RNA (Garner and Wright, 1975), and it may also disturb the integrity of cellular membranes by stimulating phospholipase A and thus facilitating lipid autoxidation (Hassid and Levine, 1977). The presence of the AFB<sub>1</sub>-dihydrodiol (AFB<sub>1</sub>-dhd), formed from the AFB<sub>1</sub>-8,9-epoxide also exerts cytotoxic effects on the cell due to binding with proteins and serum albumin (Sabbioni and Wild, 1991; Eaton *et al.*, 1994), and the involvement of reactive oxygen species (ROS) in AFB<sub>1</sub>-induced cell injury has been reported in cultured rat hepatocytes (Shen *et al.*, 1995). The interaction of AFB<sub>1</sub> with proteins, lipids and RNA is potentially as deleterious to the cell in terms of cytotoxicity as the formation of AFB<sub>1</sub>-DNA adducts since it can lead to loss of cell viability through inactivation of essential macromolecules.

Cytotoxic and genotoxic damage are inter-related as injury to the liver results in replacement division, and rapidly dividing cells are more at risk from mutations than quiescent cells owing to a limited time for DNA repair.

## 1.2 Metabolism and Detoxification of Aflatoxins

A number of enzymes in animal organs, particularly in the liver, are capable of transforming lipid-soluble xenobiotics so as to render them less lipid soluble. This reduces the ability of the metabolite to partition into biological membranes which restricts the distribution of the metabolites to various tissues. This in turn decreases the renal tubular and intestinal reabsorption of the metabolite and ultimately promotes the excretion of the chemical by the urinary and biliary faecal routes. These enzyme reactions are of two types; phase I reactions which involve oxidation, reduction and hydrolysis, and phase II reactions which consist of conjugation or synthetic reactions. Once a foreign compound has undergone phase I metabolism it can then undergo phase II reactions where it is covalently linked to an endogenous molecule to produce a conjugate which is then eliminated from the body by the above mentioned routes (for a review see: Josephy, 1997). Biotransformation is not, however, strictly related to detoxification and in a number of cases the metabolic products are more toxic than the parent compound. This is particularly true for some chemical carcinogens such as AFB<sub>1</sub>, and the toxicity of this mycotoxin is therefore based on a balance between the rate of its primary activation and detoxification of primary metabolites, as well as on the rate of elimination of conjugates from the cell and repair of cellular damage. These factors in turn are determined by the relative activity of enzymes responsible for these reactions. Since AFB<sub>1</sub> is the most potent of the aflatoxins, work has concentrated on its biotransformation pathway rather than that of the other primary aflatoxins. A simplified overview of AFB<sub>1</sub> biometabolism can be seen in figure 1.4.



**Figure 1.4:** Simplified overview of AFB<sub>1</sub> biometabolism, showing main routes for carcinogenesis, cytotoxicity and elimination.

### 1.2.1 Phase I Metabolism of AFB<sub>1</sub>

AFB<sub>1</sub> requires metabolic activation before it can exert its toxic effects and the ultimate carcinogen formed from this is the highly reactive electrophile AFB<sub>1</sub>-8,9-epoxide (Swenson *et al.*, 1974), formed by epoxidation of the 8,9 –vinyl ether bond in the furan ring. The epoxide exists in either the *endo*- or *exo*- stereoisomer form (figure 1.5) (Raney *et al.*, 1992a), and it is the *exo*- stereoisomer (Moss *et al.*, 1983) that is responsible for the mutagenicity of AFB<sub>1</sub> (Baertschi *et al.*, 1988). The chemical synthesis of the *endo*- epoxide has been described, as well as its formation by both human and rat microsomes. It is formed in lesser amounts than the *exo*- stereoisomer and although its reactivity with DNA has not been investigated, it is possible that it may contribute to the overall toxicity of AFB<sub>1</sub> (Raney *et al.*, 1992b).

Formation of the epoxide is catalysed principally by the cytochrome P450 associated mixed function oxidase pathways (figure 1.4) in the presence of O<sub>2</sub> and NADPH and many studies have been carried out to establish the enzymes involved. In humans the proteins expressed by the P450 gene families: CYP 3A and CYP 1A are important in this function. CYP 1A2 is the major human P450 enzyme capable of activating AFB<sub>1</sub> at a low substrate concentration, while CYP 3A4 has more activity at higher substrate concentrations (Gallagher *et al.*, 1994; Kirby *et al.*, 1996). The CYP 1A family does not appear to play a major role in activation of AFB<sub>1</sub> in rat liver. However, the male specific rat CYP 3A2, an orthologue of human CYP 3A4, may be primarily responsible for AFB<sub>1</sub> activation in male rat liver at both high and low substrate concentrations (Buetler *et al.*, 1996). Other cytochrome P450 enzymes which appear to be important in rats are members of the CYP 2C family, in particular the male specific CYP 2C11 form (Shimada *et al.*, 1987). A cytochrome P450 with a particularly high epoxidation activity is found in quail liver microsomes, which is probably responsible for the

remarkable sensitivity of the quail to AFB<sub>1</sub> (Hayes *et al.*, 1991a; Neal *et al.*, 1986).

These differences in ability to bioactivate the aflatoxins in part explain the variation in both inter- and intra-species susceptibility to AFB<sub>1</sub>. An association between overexpression of specific CYP-450 isoenzymes in human hepatocytes, and hepatic inflammation and cirrhosis due to HBV infection, was reported by Kirby *et al.*, (1996), who suggested that this may increase susceptibility to AFB<sub>1</sub> genotoxicity.

### 1.2.2 Alternative Mechanisms for Bioactivation of AFB<sub>1</sub>

AFB<sub>1</sub> may also be reversibly converted to aflatoxicol (AFL), by the NADPH-dependent keto-reductase (figure 1.4). AFL is almost as potent a mutagen and carcinogen as AFB<sub>1</sub>, and is also important as it is oxidised readily back to AFB<sub>1</sub> therefore acting as a reservoir for AFB<sub>1</sub> *in vivo* (Campbell and Hayes, 1976).

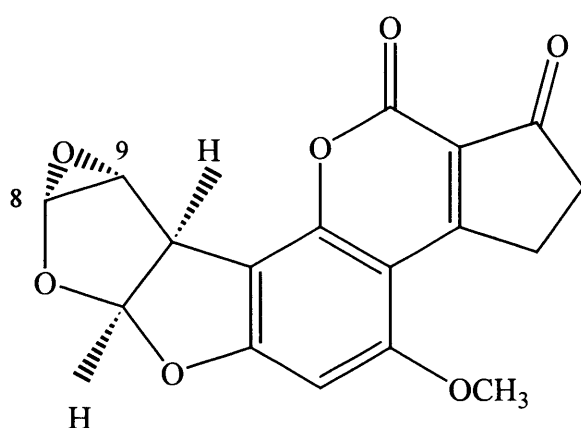
Evidence of bioactivation of AFB<sub>1</sub> by lipoxygenases and prostaglandin H synthase in extrahepatic tissues has also been demonstrated in the guinea-pig (Liu *et al.*, (1992).

### 1.2.3 Primary Detoxification of AFB<sub>1</sub>

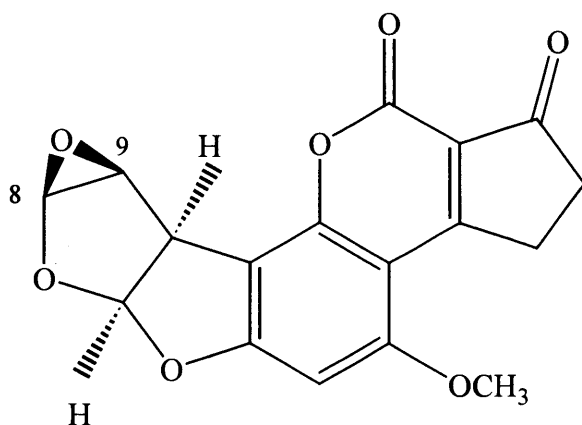
Primary detoxification of AFB<sub>1</sub> can be achieved through hydroxylation of the cyclopentenone ring structure to form AFQ<sub>1</sub>, and this reaction in man appears to be catalysed by members of the P450 3A gene family. This suggests that the P450 gene family is responsible for both the epoxidation of AFB<sub>1</sub> as well as its primary detoxification to form AFQ<sub>1</sub>. This is apparent in the rat where both the activation of AFB<sub>1</sub> and the formation of AFQ<sub>1</sub> are co-induced by phenobarbital treatment. Hydroxylation between the furan rings of aflatoxin results in the formation of AFM<sub>1</sub>, formed in humans by CYP 1A2, and this pathway is induced by treatment with 3-methylcholanthrene (Zarba *et al.*, 1992). *O*-demethylation of AFB<sub>1</sub> is a further primary



detoxification reaction leading to the production of AFP<sub>1</sub>, although this is not a major detoxification pathway in man or the rat (figure 1.4) (see review: Campbell and Hayes, 1976). Activation of, and susceptibility to, AFB<sub>1</sub> will therefore be affected by drugs which alter the levels of cytochrome P450's (Hakkola *et al.*, 1997).



AFB<sub>1</sub> *exo*-epoxide



AFB<sub>1</sub> *endo*-epoxide

**Figure 1.5:** Structure of *exo*- and *endo*-AFB<sub>1</sub> epoxides.

## 1.2.4 Phase II Metabolism of AFB<sub>1</sub>

### 1.2.4.1 Glutathione-S-Transferases

The AFB<sub>1</sub>-8,9-epoxide is short-lived; in many mammalian species, including the rat, it is conjugated with reduced glutathione (GS<sup>-</sup>). This reaction is catalysed by a supergene family of enzymes known as the glutathione-S-transferases (GSTs) (see review: Hayes and Pulford, 1995), which possess the ability to conjugate GSH with compounds containing an electrophilic centre. GSTs are widely distributed in nature. They are ubiquitous, with the greatest activity found in the liver, testis, intestine, kidney and adrenal gland. They are localised both in the cytoplasm and the endoplasmic reticulum (Otieno *et al.*, 1997) but the cytosolic activities are usually 5 to 40 times greater than the microsomal activity. In addition, another membrane-bound GST is Leukotriene C<sub>4</sub> synthase (LTC<sub>4</sub>S). As far as is known at present, LTC<sub>4</sub>S has no role in drug metabolism, but conjugates the epoxide Leukotriene A<sub>4</sub> with GSH to yield Leukotriene C<sub>4</sub>. Neither microsomal GST nor LTC<sub>4</sub>S share any sequence identity with the cytosolic GST, and it is assumed that they therefore have evolved separately (Dejong *et al.*, 1988). Numerous different isoforms of the GSTs exist. On the basis of their primary structures, a minimum of six gene families make up the GST multigene family; alpha, mu, pi, theta, sigma and zeta (Campbell *et al.*, 1989; Morrow *et al.*, 1989; Meyer *et al.*, 1991), and more recently, the class omega GSTs have been identified (Board *et al.*, 2000; Dulhunty *et al.*, 2000). Each gene family consists of separate subfamilies and each species of GST exists as either a homodimer or heterodimer of different subunits (Hseih *et al.*, 1989) (for revised nomenclature see review: Hayes and Pulford, 1995). Heterodimer associations are not random and the various GSTs demonstrate different but overlapping substrate selectivity, with each subunit in the dimeric protein functioning independently (Danielson and Mannervik, 1985). This high multiplicity of

GSTs may be essential to their role in xenobiotics metabolism. The overlapping substrate selectivity may be a factor which accounts for some of the species variation to the toxic effects of AFB<sub>1</sub>; two of the more sensitive species are avian and trout while some strains of mice are some of the most resistant animals to the acute and chronic toxicity of AFB<sub>1</sub> (Hayes *et al.*, 1991a). In most species examined the male is more sensitive than the female and this suggests a hormonal influence on GST enzyme levels (Hayes and Pulford, 1995).

Adult rat liver cytosol has been shown to have a high affinity for the AFB<sub>1</sub>-*endo*-8,9-epoxide and little activity toward the *exo*-stereoisomer. Mouse liver cytosol almost exclusively conjugates the *exo*-epoxide, whereas human liver cytosols are capable of conjugating both forms of the epoxide, but at much lower levels than either the rat or the mouse (Raney *et al.*, 1992a). The rat alpha class GSTs, rather than the mu class have been shown to possess the greatest ability to metabolise AFB<sub>1</sub>-8,9-epoxide (Coles *et al.*, 1985). This is in agreement with Hayes *et al.*, (1991b) who reported that the alpha class GSTs make the most significant contribution to AFB<sub>1</sub>-GSH conjugating ability in rat and mouse liver. The high levels of AFB<sub>1</sub> tolerance seen in some strains of mice, is due to the constitutively expressed alpha class mouse liver GST (a member of the Yc alpha class subfamily), which has a high affinity for the AFB<sub>1</sub>-8,9-epoxide (Hayes *et al.*, 1992). Two GSTs have been characterised from adult rat liver, induced by the antioxidant ethoxyquin, which possess 25-fold greater activity towards AFB<sub>1</sub>-8,9-epoxide than the constitutively expressed alpha class mouse liver GST (Hayes *et al.*, 1991c). The inducible rat GSTs are both heterodimers and share a common subunit which is a member of the alpha class Yc subfamily (designated GST A5). This is distinct from the constitutively expressed rat subunit (redesignated GST A3) (Hayes *et al.*, 1991a, Hayes and Pulford, 1995). The rat A5 subunit is associated with high activity towards the *exo*- form of AFB<sub>1</sub>-8,9-epoxide (Hayes *et al.*, 1994; ) and appears to be

closely structurally related to the constitutively expressed mouse liver alpha-class GST.

The primary structures of these enzymes show approximately 90% homology (Daniel *et al.*, 1988), and furthermore, antibodies raised against the mouse constitutive alpha-class GST show stronger immunochemical cross-reactivity towards rat A5 than rat A3 (Hayes *et al.*, 1991a).

Although the constitutively expressed rat A3 subunit shows 91% homology to the inducible A5 subunit, it lacks the ability to conjugate the AFB<sub>1</sub>-8,9-epoxide effectively (Hayes *et al.*, 1992). Clearly comparisons between the structures and functions of these two subunits, as well as between rat and mouse GST will provide interesting studies into the mechanisms involved when one GST possesses the ability to conjugate AFB<sub>1</sub>-8,9-epoxide over another. McDonagh *et al.*, (1999) have reported that two residues at positions 208 and 108 could be important for determining substrate-specificity for the AFB<sub>1</sub>-*exo*-epoxide among the alpha class GSTs.

In addition, although ROS have been shown to exert an inducing effect upon class mu GST, class alpha and pi GST are reported to be inhibited by ROS (Murata *et al.*, 1990). This could have implications in the detoxification of the AFB<sub>1</sub>-8,9-epoxide since the involvement of ROS in AFB<sub>1</sub>-induced cell injury has been reported in cultured rat hepatocytes (Shen *et al.*, 1995).

#### 1.2.4.1.1 Human GSTs

Human GSTs were first purified by Kamisaka *et al.*, (1975) and at least 20 different isoenzymes exist. Three separate alpha-class isoenzymes, dimers of two distinct subunits (B1 and B2), have been demonstrated in human liver (Stockman *et al.*, 1985, 1987) (for revised nomenclature see review: Hayes and Pulford, 1995), and a class alpha enzyme that appears to comprise polypeptides orthologous to the rat A5 subunit has been identified in human skin (Del Boccio *et al.*, 1987). Besides the class alpha GST, a

class mu enzyme is present in some human liver specimens. This enzyme is highly polymorphic in the population (Matthias, *et al.*, 1998), and as well as the 'null' phenotype, due to a gene deletion, two allelic variants exist that differ in the amino acid present at residue 173 (Seidegard *et al.*, 1988). Two class theta GSTs are present in human liver (Meyer *et al.*, 1991), and the human class pi transferases have been isolated from extra-hepatic organs, (Marcus *et al.*, 1978). (For a review see; Hayes and Strange 2000; Landi, 2000).

However, the role of GSTs in detoxification of AFB<sub>1</sub>-8,9-epoxide in human liver do not appear to play as important a role as rodent GST (Kirby *et al.*, 1993; Hayes *et al.*, 1991b); activity towards AFB<sub>1</sub>-*exo*-8,9-epoxide being two orders of magnitude less than that of mouse liver (Ketterer, 1994). This however remains to be evaluated since studies to date have concentrated on the phase I Cytochrome P450 catalysed reactions rather than on those catalysed by GSTs.

#### 1.2.4.2 $\gamma$ -Glutamyl Transpeptidase

AFB<sub>1</sub>-8,9-epoxide-GSH conjugates, once formed, are subsequently cleaved by enzymes located in the kidney to cysteine derivatives. These are then acetylated to give N-acetylcysteine (mercapturic acid) conjugates which are excreted into urine. An important enzyme involved in this pathway is  $\gamma$ -glutamyl transpeptidase (GGT) (figure 1.4), located on the plasma membrane of many cells, including bile duct epithelial cells and renal epithelial cells. GGT activity has been shown to be induced 5-10-fold in the liver after the administration of AFB<sub>1</sub>; occurring in cells not normally showing detectable levels, such as foci of altered hepatocytes (Moss *et al.*, 1984). Cells containing high levels of GGT have been found to be less sensitive to microsomally-activated AFB<sub>1</sub> than GGT negative cells (Manson and Green, 1982).

#### 1.2.4.3 AFB<sub>1</sub> Aldehyde Reductase (AFAR)

As an alternative to reacting with nucleic acids or GSH, the AFB<sub>1</sub>-8,9-epoxide, once formed, may hydrolyse spontaneously or possibly via epoxide hydrolase, to AFB<sub>1</sub>-8,9-dihydrodiol (AFB<sub>1</sub>-dhd). This may cause the acute toxic effects of AFB<sub>1</sub> by forming adducts with primary amine groups of proteins (Sabbioni and Wild, 1991; Eaton *et al.*, 1994), in particular with albumin (Sabbioni *et al.*, 1987). The importance of this metabolite in the toxic response to AFB<sub>1</sub> has been shown by the parallel between relative production of AFB<sub>1</sub>-dhd by microsomes from various animal species, and the *in vivo* susceptibilities to acute AFB<sub>1</sub> toxicity (Neal *et al.*, 1981). The role of the epoxide hydrolase in catalysing the hydrolysis of AFB<sub>1</sub>-epoxide to the AFB<sub>1</sub>-dhd is not clear. No increase in AFB<sub>1</sub>-dhd formation or DNA binding was seen on addition of epoxide hydrolase inhibitors to microsomal incubations with AFB<sub>1</sub> (Eaton *et al.*, 1994), but a significant association between the presence of DNA adducts and the epoxide hydrolase genotype in humans has been shown (McGlynn *et al.*, 1995). The possibility of species variability of this enzyme may help to explain the inter-species differences seen in susceptibility to AFB<sub>1</sub>.

A novel AFB<sub>1</sub>-aldehyde reductase (rAFAR1) has been reported in the rat, which is inducible in response to dietary ethoxyquin in a co-ordinate manner to the GST A5 subunit. It is responsible for catalysing the reduction of the dialdehyde form of AFB<sub>1</sub>-dhd at physiological pH to AFB<sub>1</sub>-dialcohol (figure 1.4), which is unable to form adducts with protein (Judah *et al.*, 1993). The highest levels of rAFAR1 were found to be present in the kidney, followed by the testis and liver, where it was present at higher levels in foci of altered hepatocytes than in normal parenchyma (Hayes *et al.*, 1993; Judah *et al.*, 1993; McLellan *et al.*, 1994). More recently, a previously unrecognised rat

aldehyde reductase (designated rAFAR2) has been isolated (Kelly, *et al.*, 2000a), which, as well as possessing different electrophoretic and immunochemical properties to rAFAR1, appears to be regulated differently, and does not appear to be induced by ethoxyquin.

A distinct aldo-keto reductase (AKR) has also been isolated and characterised from adult human liver (Knight *et al.*, 1999). Designated AKR7A3, it shares an 80% sequence homology with the rat AKR7A1 (a member of the rat AFB<sub>1</sub>-aldehyde reductases) and may contribute to protection against AFB<sub>1</sub>-induced hepatotoxicity.

#### 1.2.4.4 Production of Glucuronide and Sulphate Conjugates of AFB<sub>1</sub>

As well as the formation of GSH conjugates, alternative secondary metabolites of AFB<sub>1</sub> are formed by conjugation of the primary metabolites AFM<sub>1</sub>, AFP<sub>1</sub> and AFQ<sub>1</sub> with glucuronide and sulphate (figure 1.4). Such conjugation reactions may aid the excretion of AFB<sub>1</sub>. Their toxicological value, however, is unclear since such hydroxylated metabolites are not particularly harmful (Wei *et al.*, 1985; Valsta *et al.*, 1988).

### 1.3 DNA Repair Mechanisms

The induction of mutations in DNA-repair-deficient bacteria after administration of AFB<sub>1</sub> was shown by Garner and Wright (1973). Further studies carried out on fibroblasts from patients with xeroderma pigmentosum showed that they lacked the ability to remove the AFB<sub>1</sub>-N<sup>7</sup>-guanine adduct enzymatically, which resulted in a more rapid accumulation of AFB<sub>1</sub>-FAPY than in normal fibroblasts (Leadon *et al.*, 1981). An inducible, rapid but short-lived, repair response to the AFB<sub>1</sub>-N<sup>7</sup>-guanine adduct in human lymphoblast cells, was proposed by Kaden *et al.* (1987), who suggested that this occurred prior to the slower process of spontaneous depurination of any remaining

aflatoxin-N<sup>7</sup>-guanines. Later studies on the removal of AFB<sub>1</sub>-DNA adducts in cultured tracheal epithelial cells revealed significant interspecies differences in repair capacity (Ball *et al.*, 1990). Such a variation is likely to have important implications with regard to the susceptibility of different species to respiratory cancer.

## 1.4 Transport of AFB<sub>1</sub> by Efflux Pumps

### 1.4.1 P-glycoprotein Efflux Pump

The transport of foreign compounds out of cells can be achieved by two types of efflux pumps, both of which may provide protection against AFB<sub>1</sub> by helping to eliminate it from target cells (figure 1.4). The best characterised of these is the P-glycoprotein (Pgp) pump which is the product of the *mdr 1* gene and has been shown to be over-expressed in several models of rat liver carcinogenesis (Lee *et al.*, 1995). In humans two *mdr* genes have been identified (MDR1 and MDR2) (Ng *et al.*, 1989); in rodents there are three (*mdr1a*, *mdr1b* and *mdr2*) (Silverman *et al.*, 1991).

Expression of *mdr1b* genes can be induced in rats by exposure to such compounds as statins (3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) (Hooiveld *et al.*, 1999) and tamoxifen (Riley *et al.*, 2000). In addition, the expression of *mdr1b* has been shown to be induced in primary rat hepatocytes after exposure to AFB<sub>1</sub>, which suggests the involvement of Pgp in the transport of AFB<sub>1</sub>, and several studies have demonstrated a close association between the overexpression of Pgp and the process of tumour progression in rat liver carcinogenesis (Silverman and Hill, 1995).

Over-expression of Pgp plays an important role in multi-drug resistance in some human cancers when the Pgp functions as an energy-dependent drug efflux pump that decreases the intracellular concentration of the drugs, permitting the cells to survive otherwise lethal doses. This is especially significant for anti-cancer drugs such as



vinblastine, vincristine and adriamycin when multi-drug resistance is a major hindrance to successful chemotherapy (Gottesman and Pastan, 1988).

### 1.4.2 Glutathione-S-Conjugate Efflux Pumps

In addition to the MDR1 gene, another transporter protein, a member of the same ABC family of transporter genes exists. Known as MRP1, it acts as a GS-X pump i.e. it can transport drugs conjugated with GSH (Muller *et al.*, 1994). Its over-expression in human cancer cells has been shown to result in an increased ATP-dependent transport of GSH S-conjugates into isolated plasma membrane vesicles and from intact cells (Muller *et al.*, 1994). Several other GSH pumps are also likely to exist, and one such pump which may contribute to drug resistance is a multispecific organic anion transporter (cMOAT), a homologue of (multidrug resistance-related protein) *MRP1*, and also known as *MRP2*. Paulusma *et al* (1996) showed that a 1-base pair (bp) deletion in the *cmoat* gene in transport deficient mutant rat strains was responsible for the absence of cMOAT. These rats had an autosomal recessive defect in the hepatobiliary excretion of bilirubin glucuronides and GSH S-conjugates. Other *MRP* homologues may be present in the human genome. Kool *et al.*, (1997), found three more *MRP* homologues expressed in humans and called these new homologues *MRP3*, *MRP4* and *MRP5*. They reported elevated cMOAT expression in several cell lines selected for cisplatin resistance and in some human cancer cell lines selected for doxorubicin resistance. However, although *MRP3* and *MRP5* were found to be over-expressed in some resistant cell lines, no clear correlation between drug resistance and the expression levels of *MRP3*, *MRP4* and *MRP5* was noticed in these initial studies.

Although functional relationships exist between the GS-X pump, cMOAT and MRP, it is not certain whether they are all identical, but it is clear that multiple transport mechanisms exist for glutathione-S conjugates in rat liver (Ballatori and Truong, 1995).

## **1.5 Intrinsic and Acquired Resistance to AFB<sub>1</sub>**

Drug resistance can be divided into two main categories; intrinsic and acquired. Intrinsic describes an inherent ability of a cell or organism to tolerate a drug before ever being exposed to it. Such resistance may be expressed constitutively or it may be inducible. With acquired resistance, a cell that was initially sensitive to a drug develops the ability, usually during therapy, to resist it. In some cases of acquired resistance, cells develop the ability to withstand a number of different drugs and if the compounds are related in structure then the phenomenon is termed cross-resistance. If the drugs that the cell can resist are distinct from each other then this is known as multi-drug resistance (see review: Hayes *et al.*, 1991b; Hayes and Pulford, 1995).

### **1.5.1 Constitutive Intrinsic Resistance to AFB<sub>1</sub>**

The ability of an organism to withstand AFB<sub>1</sub> is determined by the levels of enzymes which catalyse the activation of AFB<sub>1</sub> as well as enzymes catalysing detoxification reactions and on DNA repair (Garner and Wright, 1973) and efflux pumps (Silverman and Hill, 1995; Paulusma *et al* (1996)). It is not possible to say if any proteins are associated with resistance to AFB<sub>1</sub> across a wide range of animal species since most studies carried out have only involved rodents. However, C57 mice can tolerate between 7 and 20 times more AFB<sub>1</sub> than male Fischer 344 rats but the mice activate the AFB<sub>1</sub> efficiently, and actually activate the AFB<sub>1</sub> to the epoxide at 1.5 times the rate of the rats. The hepatic cytosol from the C57 mouse possesses a 12-fold greater capacity for GSH conjugation than that of the male Fischer 344 rat and this enhanced GST-mediated detoxification is thought to be responsible for the inherent tolerance of AFB<sub>1</sub> by mice (Neal, 1990). This would suggest that GSTs play a more important role in

providing protection against AFB<sub>1</sub> than other detoxification enzymes and is in accordance with the results of Quinn *et al.* (1990).

### 1.5.2 Inducible Intrinsic Resistance to AFB<sub>1</sub>

Many of the enzymes involved in metabolising AFB<sub>1</sub> are inducible by drugs such as phenobarbital, nifedipine and 3-methylcholanthrene (Pickett *et al.*, 1984) as well as by the antioxidants  $\beta$ -naphthoflavone, ethoxyquin and butylated hydroxyanisole. These chemicals can therefore protect against the effects of AFB<sub>1</sub> (in particular against AFB<sub>1</sub>-induced carcinogenesis) and can be divided into two categories: monofunctional and bifunctional inducers (Prochaska and Talalay, 1988).

Monofunctional inducers elevate the expression of phase II metabolising enzymes such as GSTs and include diphenols and thiocarbamates. Bifunctional inducers however, increase the expression of both phase I and phase II enzymes and include 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and  $\beta$ -naphthoflavone. Two distinct chemoprotective mechanisms exist at the molecular level in the form of two separate genetic elements in the 5'-flanking region of the rat A5 alpha-class GST gene (McLellan *et al.*, 1994). These elements have been designated the antioxidant responsive element (ARE) (Rushmore *et al.*, 1991) and the xenobiotic responsive element (XRE) (Rushmore and Pickett, 1990a; Rushmore *et al.*, 1991) and allow respectively the inducible expression of the GST A5 gene by *tert*-butylhydroquinone (a monofunctional inducer) and TCDD (a bifunctional inducer). In general, monofunctional inducers, which avoid possible increases in cytochrome P450s, are thought to represent a safer class of chemoprotector than bifunctional inducers, which lead to an increase in cellular levels of phase I activating enzymes (which could increase the activation of carcinogens such as AFB<sub>1</sub>). However, in the case of AFB<sub>1</sub> which is activated as well as

detoxified by the cytochrome P450s, the bifunctional inducer indol-3-carbinol has been shown to have a protective effect against hepatocarcinogenesis (Manson *et al.*, 1998). It is not possible, however, to state categorically that bifunctional inducers will protect against AFB<sub>1</sub>.

### 1.5.3 Induction of Rat GST A5 Subunit and rAFAR1 in AFB<sub>1</sub> Resistance

There have been many reports recently of both naturally occurring or synthetic chemical agents which elevate the expression of genes that code for known cytoprotective enzymes. The Fischer 344 rat is sensitive to AFB<sub>1</sub> toxicity, but develops tolerance when fed anti-carcinogenic agents such as the antioxidant ethoxyquin (Cabral and Neal, 1983), butylated hydroxyanisole,  $\beta$ -naphthoflavone (Gurtoo *et al.*, 1985; McLellan *et al.*, 1991) phenobarbital (Kalinyak, 1982) and oltipraz (Buetler *et al.*, 1996). It has been shown that ethoxyquin treatment results in the elevation of both alpha (Ya and Yc) and mu-class (Yb) GST, along with rAFAR1 in rat liver (Pearson *et al.*, 1983; Manson *et al.*, 1997), and that Indole-3-carbinol, has an inhibitory effect on AFB<sub>1</sub>-induced tumourigenesis, due, in part, to the induction of GST A5 and rAFAR1 (Manson *et al.*, 1998). In addition, Fischer 344 rats fed on a selenium-deficient diet exhibit an enhanced capacity to conjugate the AFB<sub>1</sub>-8,9-epoxide. This can be accounted for by a 5-fold increase in hepatic levels of GST A5 subunit, and by an 11-fold increase in levels of rAFAR1 (McLeod *et al.*, 1997).

Induction of GST A5 and rAFAR1 is of interest therefore, with regard to chemoprevention of aflatoxin B<sub>1</sub>-induced carcinogenesis, and many chemicals capable of the induction of these enzymes are found as naturally occurring dietary constituents (Manson *et al.*, 1997). Most notably however, oltipraz, an antischistosomal drug, has been proposed and investigated recently as a potential chemoprotective agent against AFB<sub>1</sub>-

induced hepatocarcinogenesis in humans (Kensler, 1994, Zhang *et al.*, 1997, Jacobson *et al.*, 1997, Kensler *et al.*, 1998b, Wang *et al.*, 1999).

#### 1.5.4 Acquired Resistance to AFB<sub>1</sub>

Following exposure to genotoxic carcinogens, hepatic nodules are often found to be present in rat liver (Kleman *et al.*, 1989; Oesterle *et al.*, 1989; Hayes *et al.*, 1990a). These nodules, or foci of altered hepatocytes (FAH), display a broad cross-resistance to many xenobiotics, including AFB<sub>1</sub>. In all nodules the drug resistance appears to be achieved by changes in gene expression that result in the down regulation of cytochrome P450, the over expression of phase II detoxification enzymes, including GSTs (Roomi *et al.*, 1985) and induction of P-glycoprotein (Moscow *et al.*, 1989; Lee *et al.*, 1995). The nodules appear to originate from individual hepatocytes, possessing the drug-resistant phenotype, which are scattered throughout the liver and are selected for in rats treated chronically with carcinogens by clonally outgrowing their sensitive counterparts. The majority of nodules are short-lived with over 95% disappearing after 6 weeks, but it is from the small proportion of those remaining that hepatocellular carcinomas usually arise (Farber, 1984). As a result, these preneoplastic FAHs are useful indicators of carcinogenic response in the rat and have been considered as end points of carcinogenicity testing by a number of authors (Williams, 1982; Laib *et al.*, 1985a; Laib *et al.*, 1985b). Some forms of GSTs, such as GST-P (Ueno *et al.*, 1992) a member of the pi class of GSTs, have been found to be over-expressed in preneoplastic lesions (Neal *et al.*, 1988) and have been proposed as markers for human and experimental neoplasia, although it possesses a relatively low GSH conjugating activity towards AFB<sub>1</sub> 8,9-epoxide. In addition, Stenius and Hogberg, (1995) demonstrated that GST-P

positive hepatocytes from rats bearing enzyme-altered foci, lack functional p53 protein, suggesting that this thus permits cells with DNA damage to replicate.

## 1.6 Cell Death by Apoptosis and Necrosis

It is important to ascertain by what mechanism hepatocytes are dying after treatment with AFB<sub>1</sub>, since death by apoptosis will eliminate any initiated cells, thereby preventing the onset of cancer, whereas death by necrosis will lead to replacement division which in itself could lead to promotion of any initiated individual cells.

Cell death can be divided into two main categories: necrosis (swelling or accidental cell death) and apoptosis (shrinkage, suicidal or programmed cell death) and a great deal of interest has recently been focused on understanding the workings of the latter which occurs in a variety of physiological settings. Historically, cell death has been viewed using light and electron microscopy and diagnosis made on the morphological appearance of dead cells described as necrosis. There have been several reviews published recently (Hockenbery, 1995; Majno and Joris, 1995) describing the characteristics and differences between apoptosis and necrosis. As described by Majno and Joris (1995) another form of cell death from that of necrosis was recognised in regressing ovarian follicles in mammals by Walther Flemming as early as 1885. He named this spontaneous cell death chromatolysis. The phenomenon received little attention, however, until the critical publication of Kerr *et al.* in 1972 proposing the term apoptosis (used in Greek to describe the 'dropping off' of leaves from trees) to describe a 'mechanism of controlled cell deletion which plays a complementary but opposite role to mitosis in the regulation of animal cell population.'

Apoptosis is identified by a series of morphological changes in dying cells recognisable by electron or light microscopy. Such changes are similar across cell types and species and occur in a fixed sequence; they include cell shrinkage and loss of normal contacts

and dense chromatin condensation with the chromatin becoming pyknotic and packed into smooth masses against the nuclear membrane in a half-moon shape. Cellular budding and fragmentation is also seen when the cell emits processes often containing pyknotic nuclear fragments. These processes may break off and become apoptotic bodies which are rapidly phagocytosed by professional phagocytes such as macrophages or by adjacent cells. Cell shrinkage into a dense rounded mass or a single apoptotic body may also occur. During apoptosis there is little or no swelling of mitochondria or organelles and the cell membrane remains intact. DNA is broken down into fragments that are multiples of approximately 185 base pairs (bp) due to specific cleavage between nucleosomes which leads to the formation of DNA ladders detected on gel electrophoresis analysis (Wyllie *et al.*, 1980). The process is regulated by a number of evolutionarily conserved genes and can be initiated by an internal clock or by extra cellular agents such as hormones, cytokines, killer cells and a variety of chemical, physical and viral agents. In this way, in contrast to necrosis, individual non-clustered dead cells are rapidly removed and any leakage of their noxious contents is avoided. Many apoptotic cells undergo further degeneration with membrane lysis and ultrastructural changes typical of necrosis and this second phase of apoptosis was proposed by Majno and Joris (1995) to be termed apoptotic necrosis as opposed to ischemic, toxic or massive necrosis.

Necrosis is essentially a degenerative process signalled by irreversible changes in the nucleus, namely karyolysis, karyorhexis and pyknosis, and by condensation, intense eosinophilia, loss of structure and fragmentation in the cytoplasm. This is a pathological form of cell death that results from an acute overwhelming cellular injury and cells swell and lyse due to early loss of cell membrane integrity, releasing cytoplasmic material which often triggers an inflammatory response which is absent in the case of apoptotic cell death.

### 1.6.1 Apoptosis in Carcinogenesis

Cancerous cells are generally regarded as those which have evaded cellular growth controls and are proliferating at an increased rate. This ability has been linked with the loss of tumour suppressor genes such as *p53* (Klein, 1987) and with altered expression of proto-oncogenes such as *c-myc* (Dean *et al.*, 1986), *c-fos*, *c-ras* (Porsch-Hallstrom *et al.*, 1989) and *bcl-2* (Mc Donnell *et al.*, 1992; Castle *et al.*, 1993). The *p53* gene product is essential for cells to initiate apoptosis in response to genotoxic damage, and *p53*-deficient cells unable to undergo apoptosis in this situation may be more prone to acquire genetic alterations than normal cells, which could contribute to the high mutation rate observed in many cancers. Wyllie *et al.*, (1987) demonstrated that cancers which showed a low incidence of apoptosis coupled with a high mitotic rate *in vivo* had a distinct advantage for unrestrained cell growth. However, other, *p53*-independent, mechanisms for apoptosis exist: Einspahr *et al.*, (1999) reported an increase in apoptosis correlating with both an increase in cell proliferation, along with an increased frequency of *p53* mutation, in sun-damaged skin when compared to normal skin. They suggested therefore, the presence of a *p53*-independent mechanism for apoptosis, which is a response to increased cellular proliferation.

Impairment of apoptosis resulting in predisposition to oncogenesis has been most clearly shown for the proto-oncogene and intracellular suppressor of apoptosis *bcl-2* (Adams and Corey, 1998) whose oncogenicity was demonstrated by Reed *et al.*, (1988). Consistent with this, *bcl-2* expression has been associated with a poor prognosis in prostatic cancer, colon cancer and neuroblastoma (McDonnell *et al.*, 1992; Castle *et al.*, 1993; Hague *et al.*, 1994). Other apoptosis-suppressing genes such as *bcl-X<sub>L</sub>* and *Mcl-1* show a significant degree of homology with *bcl-2* and form the *bcl-2* family of apoptosis-suppressing genes (Nunez *et al.*, 1990; Boise *et al.*, 1993; Reynolds *et al.*,



1994). The presence of other proteins which exist as heterodimers with *bcl-2* and *bcl-X<sub>L</sub>* and the genes coding for them have been characterised recently. These genes:- *bcl-X<sub>S</sub>*, *bax*, *bak*, and *bad*, act as apoptosis inducers by inhibiting the function of the apoptosis suppressor genes and it now seems that in mammalian cells apoptosis induction is controlled by an equilibrium between suppressor and inducer genes (Oltvai *et al.*, 1993; Yang *et al.*, 1995). The role of a group of proteases, known as caspases, in apoptosis is of importance since they inactivate proteins that protect living cells from apoptosis (e.g. Bcl-2 proteins). It appears that cleavage not only inactivates these proteins, but also produces a fragment that induces apoptosis (for a review see Thornberry and Lazebnik, 1998). Thus, cell viability is maintained either by activating apoptosis-suppressor genes or by inhibiting apoptosis-inducing genes and consequently any mutations which alter the function of any of these genes will have an effect on cell proliferation.

## **1.7 Investigation of AFB<sub>1</sub> Toxicity and Resistance Mechanisms: Strategy**

### **1.7.1 Measurement of Cell Death after Administration of AFB<sub>1</sub>**

The culture of mammalian and bacterial cells offers a useful system for the *in vitro* study of measurements of cytotoxicity as well as the wide range of toxicological mechanisms which play a central role in finding new anti-cancer drugs and in the elucidation of their mechanisms of action. Coles *et al.*, (1985) studied the detoxication of microsomally activated AFB<sub>1</sub> *in vitro* in *Salmonella typhimurium* TA 100 and studies have been carried out using human hepatocytes in toxicological studies (Butterworth *et al.*, 1989; Zhang *et al.*, 1996a; Fernando *et al.*, 1993). Fraser *et al.*, (1986) were successful in culturing primary monolayers of human tumour cells from lung, breast, ovarian and gastrointestinal tumours for subsequent chemosensitivity testing of certain drugs. There are many reports of transformation of cultured rat liver cells by

carcinogens, including AFB<sub>1</sub> (Manson and Green., 1982; Sinha *et al.*, 1987; Zhang *et al.*, 1996a; Blaude *et al.*, 1990; Woo *et al.*, 1992; Lee *et al.*, 1995) and many methods exist for testing the effects of xenobiotics on cells in culture.

Sinha *et al.*, (1987) reported the use of BL8 cells, normal liver epithelial cells, from a male Fischer 344 rat. The majority of cells were in the late S-phase, 16 hours after subculture when they were incubated for 30 minutes with AFB<sub>1</sub> at the desired concentration in HBSS. They reported that the use of a metabolising system of quail microsomes coupled with this partial synchronisation of cells in the late G<sub>1</sub> and S-phase resulted in the transformation of cells after acute treatment with the AFB<sub>1</sub>. Manson and Green (1982) treated both BL8 and JB1 cell lines with AFB<sub>1</sub> in the presence of rat liver microsomes (from animals pre-treated with either phenobarbitone or 3-methyl-cholanthrene) once cell cultures had grown to near confluent monolayers.

A small scale cytotoxicity test for the estimation of drug toxicity against cells in culture of the kind adopted for this thesis was described by Phillips (1973) using Walker tumour cells from the peritoneal cavity of Wistar rats. The method employed in this thesis was adapted from the previously published methods of Manson and Green (1982) and Sinha *et al.* (1987), and either the 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) (MTT) assay (Mosmann, 1983) or trypan blue exclusion were used as a measure of end point. The method has the advantage that any treatments can be carried out on dense cell suspensions, thus allowing many cells to be treated with small amounts of any compound. The use of multi-well microtitre plates in which 96 individual cultures can be set up in one plate gives a considerable saving in cells, medium and space over the use of Petri dishes or flasks. In addition, for each drug treatment a series of eight cultures can be set up at one time which means that counts can be made at intervals leaving the remaining wells undisturbed and allowing for statistical analysis to be carried out on the results. The method can also be adapted for use on cells grown in

flasks or 6-well plates. A disadvantage compared to studies on tissues is that tissues are heterogeneous with respect to their cell population whereas cell cultures are comprised of one cell type only.

## **1.7.2 Investigation of Mechanism of Cell Death after Administration of AFB<sub>1</sub>**

### **1.7.2.1 Detection of Phosphatidylserine Exposure During Apoptosis**

Early on in apoptosis, once the cell is committed to undergoing this process, changes occur at the cell surface, one of which is the translocation of phosphatidylserine (PS) from the inner side of the plasma membrane to the outer layer: thus becoming exposed on the outer surface of the cell. Annexin V, a Ca<sup>2+</sup> dependent phospholipid-binding protein, has a high affinity for PS. Hence this protein can be used as a sensitive probe for PS exposure upon the cell membrane. However, translocation of PS to the external surface of the membrane also occurs in necrosis, the difference being that in the early stages of apoptosis the cell membrane remains intact, only losing its integrity and becoming leaky at the moment of necrosis.

### **1.7.2.2 Measurement of Apoptosis by Annexin V**

The method used in this thesis for measurement of apoptosis was based on that of Koopman *et al.*, (1994): fluorescein isothiocyanate (FITC)-labelled annexin V was used to quantify apoptosis in cell suspensions and monolayer cultures of the rat liver epithelial cell lines RLE and JB1. Koopman *et al.* showed that the phase in apoptosis that is characterised by chromatin condensation coincides with PS exposure. This precedes membrane damage which leads to the release from the cell of enzymes harmful to the surrounding tissues, as is the case in necrosis. By using the FITC-labelled annexin V in conjunction with dye exclusion of propidium iodide (PI) (PI cannot cross

the intact plasma membrane) apoptotic cells could be distinguished from those undergoing necrosis and intact cells using flow cytometry, since apoptotic cells were FITC<sup>+</sup> / PI<sup>-</sup>, necrotic cells were FITC<sup>+</sup> / PI<sup>+</sup>, and those still intact were FITC<sup>-</sup> / PI<sup>-</sup>. Vermes *et al.*, (1995) also developed this method for detection of apoptosis in cultured HSB -2 cells rendered apoptotic by irradiation and on human lymphocytes treated with dexamethasone. This method was developed and applied to adherent cells, the PS detected either by an enzyme linked immunosorbent assay (ELISA) technique or by scanning laser cytometry (LSC).

### **1.7.2.3 Introduction to Enzyme Linked Immunosorbent Assay (ELISA) Technique**

ELISA is a specific and sensitive technique based on a sandwich system in which a primary antibody binds to an antigen (DNA, RNA or protein) immobilised on a multiwell plate. A secondary antigen with an enzyme covalently linked is then bound to the primary antibody and addition of the appropriate substrate in excess results in the formation of a coloured product which can be measured using a spectrophotometer.

The above technique was adapted in this thesis, for use on cells growing in a monolayer in tissue culture-treated 96-well plates. In this case the PS on the cell membrane acted as the antigen, while the annexin V-FITC was the primary antibody. The secondary antibody was anti-fluorescein alkaline phosphatase.

### **1.7.2.4 Introduction to Flow Cytometry**

The applications of flow cytometry have spread through all branches of biological sciences over the last 10 years, the biggest growth being in the clinical field with flow cytometers now being used for routine measurements in immunology, haematology and, to a lesser extent, pathology departments. It is possible to measure a variety of cellular properties such as the DNA content of a nucleus, the expression of a surface antigen, the

activity of an intracellular enzyme or the pH using this technique. The scope of the technique is only really limited by the fluorescent dyes available.

Flow cytometry is the measurement of cells in a flow system which delivers particles in single file past a point of measurement – an instrument which focuses light on to cells and records their fluorescence and the light scattered by them. It has the power to measure several parameters on tens of thousands of individual cells within a few minutes and can therefore be used to define and enumerate accurately sub-populations which, once identified can be sorted physically for further study.

Typically, five parameters may be measured on 20 000 cells. Using blue light for excitation, green, orange and red fluorescence might be measured along with forward scatter of the blue light and it's scatter at right angles to the laser beam. The data is processed by a computer – an essential part of the instrument. The major disadvantage of flow cytometry is that a preparation of single particles (cells, nuclei, chromosomes) is required and hence tissue architecture is destroyed so that information about the relationships of cells to each other is lost (for review see: Ormerod, 1994).

Flow cytometry techniques are widely used for discrimination between live and dead cells, and to distinguish the mode of cell death. Analysis of light scatter, or exclusion of propidium iodide combined with uptake of another fluorochrome such as rhodamine 123 or Hoeschst 33342 are widely used techniques for the detection of cells undergoing apoptotic changes. (for a review see: Cotter and Martin, 1994).

#### **1.7.2.5 Introduction to Laser Scanning Cytometry**

With the laser scanning cytometer (LSC) it is possible to obtain data equivalent to flow cytometry from samples mounted on microscope slides since it applies the properties of flow cytometry and image processing to standard cytological preparations. This

therefore prevents the need for trypsinisation of the monolayer which itself is traumatic to the cells and may interfere with the results when apoptosis and necrosis are under investigation. The LSC scans the slide, measuring fluorescence and forward light scatter, and records the position and time of measurement of each cell so allowing cells of interest to be relocated, visually confirmed, restained, remeasured and their images recorded. For instance, it has recently been employed by Bedner *et al*, (2000) for the identification and phenotypic characterisation of MCF-7 cells growing in microscope slide chambers.

Interaction with the LSC through a personal computer with a Microsoft<sup>TM</sup> Windows operating system: WinCyte<sup>TM</sup>, controls the LSC's operation, data collection and analysis. Data can be displayed as scatter diagrams or histograms to a gating region of any scattergram so allowing the user to identify subpopulations of cells, display, quantitate or relocate them.

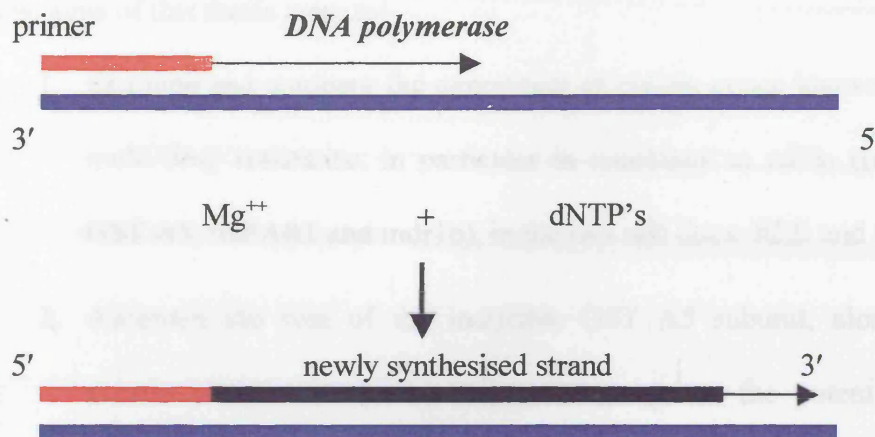
The laser illuminates cells with an intense beam of light that produces fluorescence and as the laser beam crosses a cell, the amount of light intensifies from a background light level which depends upon factors such as the amount of dye in the specimen or on the slide. The intensity of the light signals is translated into an electrical current and once the electrical signal from photomultiplier tube has been amplified, its intensity is analysed and the value is recorded by means of an analog-to-digital converter (ADC). The ADC stores each analog reading of voltage as a digital pixel and a composite of these voltages appear as a laser scan image in shades of grey on a PC screen.

### **1.7.3 Gene Expression by Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)**

Investigation of rAFAR1, *mdr1b*, GST A3, GST A5 mRNA by RT –PCR in the AFB<sub>1</sub>-sensitive RLE cell line and AFB<sub>1</sub>-resistant JB1 cell line, and in ethoxyquin-treated rat liver tissue was carried out in this thesis.

#### **1.7.3.1 The Polymerase Chain Reaction: Basic Principles**

The polymerase chain reaction (PCR) (Mullis and Faloona, 1987) is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA (for a review see: McPherson *et al.*,1991). In the primer extension reaction DNA polymerases carry out the synthesis of a complementary strand of DNA in the 5' to 3' direction using a single-stranded template, but starting from a double-stranded region (figure 1.6). This reaction is the basis for a variety of labelling and sequencing techniques.



**Figure 1.6:** Primer extension: DNA polymerase extends a primer by using a complementary strand as a template

### 1.7.3.2 Reverse Transcriptase PCR (RT – PCR)

With RT – PCR the starting material is RNA so an additional reverse transcriptase (RT) step is required to produce complementary DNA (cDNA) from the target RNA gene sequence before it can undergo the PCR step.

An advantage of RT-PCR is that it is specific for the gene of interest. It is therefore sensitive and is suitable for very low levels of RNA concentration in a sample. Quantification of the level of mRNA is important and several authors have published their findings on reproducibility of the RT-PCR assay and sources of variation which include use of an internal standard (Wang *et al.*, 1989; Zhang *et al.*, 1996b; Hall *et al.*, 1998).



## 1.8 Aims and Objectives

The aims of this thesis were to:

1. Examine and compare the expression of certain genes known to be involved in multi-drug resistance, in particular in resistance to AFB<sub>1</sub> (including GST A3, GST A5, rAFAR1 and *mdr1b*), in the two cell lines: RLE and JB1.
2. Ascertain the role of the inducible GST A5 subunit, along with the aforementioned gene products, in protection against the potent hepatocarcinogen AFB<sub>1</sub>.
3. Investigate the mechanism of cell death induced by the cytotoxic effects of AFB<sub>1</sub>.
4. Produce a model system which can help our understanding of multi-stage models of carcinogenesis, and which could then be applied to other xenobiotics.

This was to be achieved by comparing two rat liver epithelial cell lines, derived from male Fischer 344 rats. One cell line was sensitive to the effects of AFB<sub>1</sub> (RLE); the other cell line used (JB1) was derived from an AFB<sub>1</sub>-induced hepatocellular carcinoma, and was resistant to AFB<sub>1</sub> (Power, 1987; Manson *et al.*, 1981).

Cytotoxic effects of AFB<sub>1</sub> on the cell lines were measured using the MTT assay and the trypan blue dye exclusion assay. An ELISA/MTT technique was developed in this project for detection of PS exposure in adherent cells via annexin V binding to the cell membrane. Results were correlated with those obtained from AFB<sub>1</sub>-treated cells grown as a monolayer on cover slips and on cells that were trypsinised and suspended. Both were stained with the fluorochromes annexin V-FITC and PI and subjected to laser

scanning cytometry and flow cytometry analysis respectively in an attempt to differentiate between apoptosis and necrosis.

The involvement of the A3 and A5 subunits of the GSTs, rAFAR1 and p-glycoprotein (the product of the *mdr1* gene) in protecting against the effects of AFB<sub>1</sub> was investigated by determining their presence, or their absence, in mRNA in RNA samples from both cell lines by RT-PCR, before and after treatment with sublethal doses of AFB<sub>1</sub>. Results were compared with those obtained from RNA samples extracted from livers of rats treated with ethoxyquin in which the A5 subunit, AFAR and p-glycoprotein had been induced.

Chapter 2  
Materials and Methods

## **2 Materials and Methods**

### **2.1 Materials**

The majority of materials were acquired from Sigma Chemical Co., St Louis, USA, and were of analytical grade. Exceptions were: actin primers, obtained from OSWELL DNA Services, Edinburgh; aldehyde reductase primers, obtained from GIBCO BRL Custom Primers; AMV Reverse transcriptase buffer obtained from Promega, USA; AMV Reverse transcriptase enzyme obtained from Promega, USA; annexin V-FITC obtained from Bender Medsystems, Vienna, Austria; anti-Fluorescein alkaline phosphatase obtained from Boehringer Mannheim; Mdr1b primers, kindly donated by Dr. T. Gant MRC University of Leicester (Zhang *et al.*, 1996b); Taq DNA polymerase obtained from GIBCO BRL Life Technologies, Paisley; GST A3 (Hayes, 1994) and A5 subunit (Pulford and Hayes, 1996) primers, kindly donated by Dr. G.E. Neal, MRC, University of Leicester.

### **2.2. Culture and Maintenance of Hepatic Cell Lines**

Two cell lines were used in this study, one of which was sensitive to the carcinogenic compound AFB<sub>1</sub> (RLE) and one of which was resistant (JB1). Both cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS) in the presence of 1% penicillin/streptomycin in 80ml vented tissue culture flasks. Cell cultures were maintained at 37°C in a humid atmosphere containing 5% carbon dioxide. Both cell lines grew as a monolayer and a 1:4 split was made at 90% confluence using a solution of 1x trypsin / ethylene diaminetetracetate (EDTA) to give a concentration of  $1 \times 10^4$  cells/ml. The population mean doubling time was 28 hours for both cell lines and passage numbers of between 8 and 30 were used.

Cell suspensions were seeded out into either 20ml flasks or Falcon 96-well tissue culture treated plates depending on which method was to be employed for detection of cytotoxicity or if cells were to be subjected to RNA extraction. For the trypan blue method as a measure of cytotoxicity and for subsequent RNA extraction 5ml of cell suspension was seeded out into each 20ml flask. For the MTT assay and annexin-V ELISA technique, cells were seeded out onto flat-bottomed Falcon microtest 96-well tissue culture-treated plates (200µl cell suspension/well). For subsequent flow cytometry cells were seeded into 6-well plates (3ml/well), similarly for fluorescence microscopy cells were seeded into 6-well plates containing previously autoclaved 22 x 26 mm coverslips.

### 2.3 Aflatoxin B<sub>1</sub> Assay

The culture of hepatocytes has severe limitations, notably the progressive decrease in cytochrome P450 content possibly due to the loss of the haem moiety (Steward *et al*, 1985). Since mixed function oxidase enzymes play an essential role in activating as well as detoxifying many xenobiotics, including AFB<sub>1</sub>, an extrinsic activating system was added to the reaction medium in the form of quail liver microsomes (prepared by the method of Neal *et al.*, (1986), at a concentration of 6mg protein/ml). An external nicotinamide adenine dinucleotide phosphate (NADPH) generating system was also required (0.5mg NADP, 1.66mg glucose-6-phosphate and 0.08 units glucose-6-phosphate dehydrogenase per 100µl Hanks buffered saline solution (HBSS), preincubated for 5 minutes at 37°C) since the activation of AFB<sub>1</sub> to the epoxide form is an NADPH-dependent reaction. AFB<sub>1</sub> was stored as a stock solution at 2mg/ml in dimethylmethylsulphoxide (DMSO) at 4°C in the dark. The experimental system used was based on previously published methods by Manson and Green (1982) and Sinha *et*

*al.*, (1987). Cells were treated with AFB<sub>1</sub> 48 hours after seeding when the cell population density was around  $3 \times 10^5$  cells/ml, and cells were in the exponential growth phase. Medium was removed by inversion and cells washed with unsupplemented DMEM to remove any traces of FCS. AFB<sub>1</sub> was made up in unsupplemented DMEM using a concentration range of 0.0, 1.0, 2.5, and 5.0 µg AFB<sub>1</sub>/ml. The concentration of DMSO was kept constant throughout at 0.05%. Incubation was with 3ml AFB<sub>1</sub> solution per 20ml flask or per well for 6-well plates, or 200 µl per well for 96-well tissue culture plates in the presence of microsomes (1% total volume) and an external NADPH generating system (1% total volume) for 30 minutes at 37 °C. After incubation, the AFB<sub>1</sub> solution was removed from 96-well plates by inversion. The AFB<sub>1</sub> solution from the flasks was retained and centrifuged (100g, 5 minutes) to pellet non viable cells which became detached from the cell monolayer during this time. The cell pellet was resuspended in 1ml medium and a non viable cell count carried out. The cell monolayer in all cases was washed with unsupplemented DMEM (2 x 100 µl/well for 96-well plates, 2 x 1ml for 20 ml flasks or per well for 6-well plates), medium was replaced (200 µl/well for 96-well plates, 3ml per well for 6-well plates or for 20ml flasks), and cells allowed to recover for either 2 hours for subsequent annexin-V staining or 24 hours for cytotoxicity studies.

NB. In the above protocols all mention of medium refers to DMEM supplemented with FCS and antibiotics as detailed in method 2.2 as opposed to unsupplemented DMEM used solely for AFB<sub>1</sub> assay procedures.

### **2.3.1 Development of AFB<sub>1</sub> Resistant RLE Cell Line.**

JB1 and RLE cells lines were treated according to method 2.3, with the exception that incubation was for 1 hour in 5 µg/ml AFB<sub>1</sub>. Medium was removed, along with any

nonviable cells. Remaining viable cells still attached to the flask were grown up in DMEM until confluent.

### **2.3.1.1 Preparation of Quail Liver Microsomes**

Young (3-4 months old), male Japanese quail were obtained from Norfolk Pheasantries. The liver was rapidly removed immediately after death and placed on ice before being finely chopped. 5g of this tissue was then homogenised in 20ml 150mM KCl, using a Tri-R pestle and glass mortar. The homogenate was then centrifuged (20 minutes, 4°C, 10 000g) and the supernatant subjected to further centrifugation (1hour, 4°C, 100 000g). This super-speed spin yielded a microsomal pellet which was resuspended in 150mM KCl to give 6mg protein/ml.

### **2.3.2 Optimisation of Assay Procedure**

#### **2.3.2.1 Calculation of Cell Loss due to Assay Procedure Alone and Determination of Most Suitable AFB<sub>1</sub> Diluent**

On devising the assay for this thesis several parameters were investigated. The possibility of cell loss due to factors other than the effects of the AFB<sub>1</sub> was taken into account and the effects of three potential AFB<sub>1</sub> diluents on the cell monolayer were compared. Ideally the diluent itself should have little or no detrimental effect on the cells during the incubation period. When working with the small surface areas present in 96-well plates, maximal adherence of the cell monolayer to the plate is desirable and the effects of two different tissue culture plates on cell adherence were compared.

The following procedure was carried out to ascertain where the greatest cell loss, if any, lay during the assay. Cells were seeded out onto 96-well tissue culture-treated plates according to method 2.2. and incubated at 37°C for 48 hours. Medium was then removed by inversion of the plate, and the monolayer washed twice with 100µl

HBSS/well (at 37°C). A viable cell count was calculated using trypan blue (method 2.5), for initial cell number attached to the well surface at the start of the assay. The effects of HBSS, phosphate buffered saline (PBS) and unsupplemented DMEM (200µl/well) respectively were investigated. After 30 minutes incubation, each diluent was removed by pipetting, retained, and centrifuged (100g, 5 minutes). Viable cell counts were carried out on the resuspended pellet for each incubation solution using trypan blue to obtain a value for average cell loss during the 30 minute incubation period for each diluent. The remaining cell monolayer was then washed twice with 100µl of the designated diluent which was again retained for a cell count as above, to give a value for average cell loss at this wash step. Medium was replaced and cells allowed to recover for 2 hours at 37°C, after which medium was removed by inversion. The monolayer was again washed twice with 100µl of designated diluent and a cell count was carried out on remaining cells to give an average cell number per well remaining after the recovery period. In this way an estimate of percentage cell loss at each stage of the procedure (i.e. 30 minute incubation, 1<sup>st</sup> and 2<sup>nd</sup> wash steps ) could be calculated for each diluent investigated. The procedure was repeated using two different types of 96-well plate; the Falcon tissue culture-treated plate and the Nunc 96-well plate to establish whether or not the choice of plate has any effect on cell loss during the assay procedure.

### **2.3.2.2 Determination of Optimum Microsome Concentration**

A titration assay for the quail liver microsome fraction was carried out to ascertain the lowest concentration of microsomes required (v/v total reaction mixture) to activate the AFB<sub>1</sub> to the epoxide, whilst ensuring minimal cytotoxic effect on the cells due to the presence of the microsomal fraction itself.



Cells were seeded out into 96-well plates (method 2.2) in parallel on duplicate plates and incubated for 48 hours, after which medium was removed by inversion of the plate. The monolayer was washed twice with 100µl/well unsupplemented DMEM. A concentration gradient of microsomal fraction (made up in unsupplemented DMEM) from 1% - 9% was tested (200µl per well), along with a 100% solution of the microsomal fraction. The concentration of DMSO was kept constant at 0.05% throughout and an NADPH generating system (1% total volume ) was added to each well. Controls included unsupplemented DMEM only. Plates were incubated at 37 °C for 30 minutes after which solutions were removed by inversion, wells washed twice with 100µl unsupplemented DMEM and medium replaced (200µl/well). Cells were allowed to recover for 2 hours at 37°C after which one plate was subjected to the MTT cytotoxicity assay (method 2.6) while the other plate was subjected to the annexin V ELISA assay (method 2.7.2) to detect any PS exposure occurring due to the effects of the microsomal fraction on the cells.

#### **2.4 HPLC for Detection of Activated AFB<sub>1</sub> in Reaction Medium**

To determine whether or not the AFB<sub>1</sub> was activated to 8,9-epoxide, over a range of 1-5µg AFB<sub>1</sub>/ml with a concentration of 1% microsomes and 1% NADPH generating system in unsupplemented DMEM (as described in section 2.3), the above reaction conditions were set up (incubation period: 10 minutes, 37°C) and any AFB<sub>1</sub> 8,9-epoxide generated was conjugated with GSH (15.3mg/ml) by the BalbC mouse Y<sub>C</sub> subunit (contained in 10µl liver cytosol). The metabolites were extracted in methanol and dried in a vacuum concentrator before reconstitution and analysis by hplc. The presence of this conjugate was looked for on elution from a Chrompack C18 5µm Chromsphep

column (100mm x 3mm glass cartridge). HPLC was kindly carried out by Mr David Judah, MRC, University of Leicester.

## **2.5 Measurement of Cytotoxicity using Trypan Blue Exclusion Method for Cell Viability**

Trypan blue (Congo blue 3B) is a water soluble acid dye with a molecular weight of 960.8. Its uptake by cells is an indication of severe irreversible damage, presumably a consequence of cell membrane damage, and thus represents a late manifestation of cytotoxicity. Nonviable cells therefore stain blue with this dye and so can be distinguished from those that remain viable.

Medium was removed and the cell monolayer was washed with HBSS, cells were trypsinised and centrifuged at 100g for 5 minutes. The cell pellet was resuspended in 1ml medium and 10 $\mu$ l of this suspension was added to 90 $\mu$ l trypan blue. A cell count was carried out using a conventional haemocytometer; four corner squares were counted on each grid and the results were expressed as percentage viable cells per ml medium.

## **2.6 Measurement of Cytotoxicity using MTT (3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide)) Assay**

This method is a simple assay to determine the viability of cells in culture through the formation of a coloured product in a mitochondrial-dependent reaction and was based on the method by Mosmann (1983).

The tetrazolium salt 3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) (MTT) is taken up into cells and reduced in a mitochondrial-dependent reaction to yield a blue coloured formazan product which is insoluble in aqueous medium and hence accumulates in the cell. On solubilisation of the cells the product is dissolved and can be readily detected and quantified using a simple colorimetric method.

### **2.6.1 Optimisation of MTT Concentration**

The MTT assay should be optimised for each cell type used and in this procedure a concentration gradient of MTT was investigated in order to ascertain the optimum concentration of MTT in this case since a high level of MTT will itself prove cytotoxic to the cells.

Cells were seeded out into 96-well plates (method 2.2) and incubated at 37°C for 48 hours after which a concentration gradient of MTT in HBSS over the range of 1 to 20 % was applied to rows 1-10 (20µl per well). Row 11 served as a control and row 12 served as a blank. Plates were incubated for 1 hour at 37°C, after which medium was removed and 50µl DMSO added to dissolve the formazan product accumulated in the cells. Plates were incubated for a further 15 minutes and agitated gently before the amount of formazan product in each well was determined using a spectrophotometer at 495nm. An incubation time greater than 1 hour with the MTT solution led to a considerable amount of cell death which could easily be seen by eye as sheets of nonviable cells detaching from the surface of the well and becoming lost during the removal of the medium.

### **2.6.2 Measurement of Cytotoxicity after Treatment with AFB<sub>1</sub>**

Exponentionally growing cells were seeded into Falcon 96-well tissue culture treated plates according to method 2.2 and after 48 hours incubation were treated with AFB<sub>1</sub> according to method 2.3. After a 24 hour recovery period, 20µl MTT (5mg/ml in HBSS) was added to each well, with the exception of row 12 which served as a blank. After a 1 hour incubation at 37°C the medium was removed, 50µl DMSO was added to each well and plates were incubated at 37°C for 15 minutes. The amount of dissolved

formazan product was then determined for each well using a spectrophotometer at a wavelength of 495nm. The absorbance obtained for test conditions was expressed as a percentage of that obtained in the control situation, and results were expressed as percentage cell survival (i.e. absorbance of treated cells / absorbance of control cells x 100).

## **2.7 Detection of Apoptosis in JB1 and RLE Cells after Treatment with AFB<sub>1</sub>**

### **2.7.1 Detection of Apoptosis in Suspensions of JB1 and RLE Cell Lines by Flow Cytometry**

Cell lines were seeded out onto 6-well plates (method 2.2), incubated for 48 hours and treated with 5µg/ml AFB<sub>1</sub> according to method 2.3. After a two hours recovery at 37°C, medium (containing cells detached due to the effects of AFB<sub>1</sub>) was removed and retained. The monolayer was washed twice with HBSS (200µl/well) and 500µl 1x trypsin EDTA was added prior to 3 minutes incubation at 37°C. Cells were suspended by pipetting to break up the monolayer and added to the original medium for that well. The total cell population was pelleted down (100g, 5 minutes). The pellet was resuspended in 1ml annexin V buffer (10mM HEPES/NaOH pH 7.4, 150mM NaCl, 5mM KCl, 1mM MgCl<sub>2</sub>, 1.8mM CaCl<sub>2</sub>), placed on ice, and 1µl annexin V-FITC was added. Samples were covered and incubated for 8 minutes at room temperature, after which 50µl propidium iodide (50µg/ml PI in PBS) was added.

Further incubation for 2 minutes on ice and in the dark was carried out and the samples were analysed using a Becton Dickinson FACStar Plus flow cytometer with 'Lysis II' software.

### **2.7.2 Enzyme Linked Immunosorbent Assay (ELISA) Technique to Detect PS Exposure in JB1 and RLE Cell Lines**

This method was based on that of Koopman *et al.*, (1994) and was adapted for enzyme linked immunosorbent assay (ELISA). As in Koopman's method the assay should be used in conjunction with a dye exclusion test to establish cell membrane integrity. However in this method it is not possible to do this and so it is possible only to detect annexin V. For this reason the assay is carried out in conjunction with the MTT assay (see method 2.6) on a set of duplicate plates which were seeded out at the same time as and subjected to the same conditions and treatments as the 96-well plates in this procedure.

The ELISA technique was adapted for use on cells growing in a monolayer in tissue culture-treated 96-well plates. In this case the phosphatidylserine on the cell membrane acted as the antigen, while the annexin V-FITC was the primary antibody. The secondary antibody was anti-fluorescein alkaline phosphatase (AP). Cells were seeded out according to method 2.2 and 48 hours later were treated with AFB<sub>1</sub> according to method 2.3. After a 2 hour recovery period medium was removed and cells washed with HBSS (200µl/well), after which 200µl annexin V buffer (see method 2.7.1) containing 1µl/ml annexin V-FITC was added to each well. Cells were incubated for 8 minutes at room temperature after which buffer was removed and cells incubated in a 3% (w/v) BSA/PBS blocking solution for 15 minutes. Removal of the blocking solution was followed by incubation with a 1:1500 solution of anti-fluorescein alkaline phosphatase (in 3% (w/v) BSA/PBS) for 30 minutes at room temperature. Plates were washed three times with wash-buffer (0.05% v/v Tween 20 in PBS) and phosphatase substrate was added in 1% (w/v) DEA (1 tablet/5ml DEA). Plates were incubated at 37 °C for 2 hours after which a yellow colouration was observed and the absorbance at

405nm was read. Controls included wells without annexin V-FITC, anti-fluorescein AP and phosphatase substrate respectively.

$A_{405\text{nm}}$  for the annexin V ELISA technique was calculated in relation to  $A_{495\text{nm}}$  for the MTT assay in the corresponding well of the duplicate plates (i.e.  $A_{405\text{nm}}/A_{495\text{nm}}$ ). This gave a value for annexin V levels expressed in relation to the viable cell number (as indicated by the absorbance value obtained for the MTT assay). Experiments were repeated 8 times.

### **2.7.3 Fluorescence Microscopy and Photography using Confocal Fluorescence Microscope**

The method employed for fluorescence staining was a combination and adaptation of methods 2.7.1 and 2.7.2. Cells were seeded onto 6-well culture plates (each well of which contained a previously autoclaved 22 x 26mm coverslip). according to method 2.2. Cells became attached to the surface of the coverslip, grew into a monolayer and were treated with 5 $\mu\text{g/ml}$  AFB<sub>1</sub> 48 hours later according to method 2.3. After a 2 hour recovery period the coverslips were removed and placed in fresh 6-well plates where the monolayer was washed twice with HBSS (3ml/well) before the addition of 3ml annexin V buffer containing 1 $\mu\text{l/ml}$  annexin V-FITC. Plates were incubated for 8 minutes at room temperature in the dark after which 50 $\mu\text{l}$  propidium iodide (50 $\mu\text{g/ml}$  PI in PBS) was added to the buffer and incubation for a further 2 minutes was carried out. Buffer was removed, cells were washed twice with HBSS (3ml/well) and then fixed in 4 % paraformaldehyde (in PBS) for 10 minutes at 4°C. After fixation, coverslips were removed and mounted onto slides using DABCO before viewing under a Leica TCS4D confocal fluorescent microscope, under oil using either a red or green filter. Cells were kept covered at all stages to avoid quenching of the fluorochromes in the light (for a

review of advanced light microscopes see Ockleford *et al.*, 1997). Experiments were repeated 3 times.

#### **2.7.4 Measurement of Apoptosis in JB1 and RLE Cell Lines using the Laser Scanning Cytometer**

With the laser scanning cytometer (LSC) it is possible to obtain data equivalent to flow cytometry from samples mounted on microscope slides since it applies the properties of flow cytometry and image processing to standard cytological preparations. Interaction with the LSC through a personal computer with a Microsoft<sup>TM</sup> Windows operating system, WinCyte<sup>TM</sup>, controls the LSC's operation, data collection and analysis.

JB1 and RLE cells were seeded out into 6-well plates containing an autoclaved coverslip at a density of  $1 \times 10^4$ /ml as in method 2.7.2. Two day old cultures were treated with a concentration of AFB<sub>1</sub> 5µg/ml in the presence and absence of microsomes according to method 2.3. After a two hour recovery period, cells growing on the coverslips were stained with annexin V-FITC and PI prior to fixation in 4% paraformaldehyde and mounting in DABCO (method 2.7.2). Counts were made using the laser scanning cytometer, and the number of cells staining individually for annexin V-FITC and PI were measured using green and red parameters respectively. X and Y values for the scan area were as follows; X; 56479 to 22095, Y; 69181 to 36317 for all slides. To obtain a total cell count for each slide, PI was added to the permeabilised (due to fixation in paraformaldehyde) cells after this initial count was made, as every cell should now take up the PI giving a total cell count per slide. Each slide was rescanned covering the same points, and the number of cell nuclei staining red were recorded. From this, the proportion of cells staining green only (apoptotic) and those staining red and green (necrotic) was calculated for each dose of AFB<sub>1</sub> used.

## 2.8 RNA Analysis

### 2.8.1 Isolation of RNA from Cell Lines

The single step method of RNA isolation was that of Chomczynski *et al.*, (1986) and produced total RNA from both RLE and JB1 cells. The following procedure was carried out on a 20 cm<sup>2</sup> flask of a two day old culture (approx. 3 x10<sup>5</sup> cells) of each cell line. Medium was removed and the monolayer washed with HBSS before the addition of 1ml Solution D (4M guanidinium thiocyanate (GTC), 25mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1M  $\beta$ -mercaptoethanol). GTC and  $\beta$ -mercaptoethanol are both powerful RNase inhibitors and the GTC acts with the sarcosyl to disrupt cell membranes and nucleoprotein complexes, thus allowing RNA to be released into a solution free of proteins. The flasks were gently agitated until the solution became viscous and the cells had lysed. The homogenate was transferred to an eppendorf for extraction of RNA and the following added sequentially on ice with thorough mixing after each addition; 0.1ml of 2M sodium acetate pH 4.0, 1ml water saturated phenol and 0.2ml chloroform isoamyl alcohol mixture (24:1). The solution was placed on ice for 15 minutes. Samples were centrifuged (20 minutes, 100g, 4°C), the low pH causes the RNA to remain in the aqueous phase while DNA and proteins are partitioned in the interphase and phenol phase. RNA was precipitated from the aqueous phase by the addition of 1ml of isopropanol and standing for 1 hour at -20°C. A further centrifugation step (20 minutes, 100g, 4°C) was carried out and the pellet was resuspended in 0.3ml Solution D and precipitated with 0.3ml isopropanol (-20°C, 1 hour) after which the RNA was pelleted down (20 minutes, 100g, 4°C) and resuspended in 75% ethanol. A further precipitation step was carried out (1 hour, -20°C), the sample was centrifuged (20 minutes, 100g, 4°C) and the RNA redissolved in 50 $\mu$ l DEPC-treated water. Samples were stored at



-20°C prior to use.

### 2.8.2 RNA Preparation from Tissue Samples

Liver tissue from an ethoxyquin-fed (5% v/v feed, *ad libitum*, 6 weeks) male Fischer rat was kindly donated by Dr G.E. Neal, MRC University of Leicester.

It was snap frozen and stored in liquid nitrogen. When required a small sample from around the edge of the tissue, roughly around 100mg, was removed and placed in 1ml of solution D containing 1µl antifoam C. The tissue was then homogenised and RNA extraction carried out according to method 2.8.1.

### 2.8.3 Assessment of RNA Quality, Concentrations and Yields.

RNA concentrations were determined using spectroscopy; 5µl of RNA sample was added to 995µl DEPC-treated water in a cuvette and the absorbance at 260nm and 280nm was recorded. An optical density (OD)<sub>260nm</sub> of 1 corresponds to 40µg/ml RNA, 50µg/ml DNA.. The  $A_{260}/A_{280}$  ratio was calculated to give an estimate of RNA/DNA present in the sample. A value of 1.8 denotes pure DNA while a value of 1.6 denotes pure RNA.

The quality of the RNA samples was assessed by electrophoresis of 10-20µg samples of total RNA in 1% agarose gels. 0.5g Seakem agarose was melted in 50ml 1 x TAE in a microwave oven, and 2.5µl ethidium bromide added, the gel was cast and left to set at 4°C. Electrophoresis was carried out on 10-20µg RNA (in 5µl) with the gel submerged in 1 x TAE gel running buffer containing 2.5µl ethidium bromide (10mg in 1ml UP water)/50ml buffer. Under ultra violet light two bands corresponding to the 18s and 28s subunits of RNA for each sample could be seen. Any DNA contamination in the sample

showed up as a bright band which remained in the well owing to its comparatively much larger size.

All equipment used was treated in 1% (v/v) H<sub>2</sub>O<sub>2</sub> for 30 minutes before use to avoid contamination with RNase.

## **2.9 Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) Analysis of Gene Expression**

### **2.9.1 Detection of rAFAR1, mdrlb, GST A3, GST A5 and Actin Genes by RT – PCR**

In a typical analysis, 100ng RNA was mixed with 1.5µl reverse primer (10pm/µl) (Figure 1.6) and the final reaction volume made up to 10µl with diethylpyrocarbonate (DEPC) treated water. An RNA- negative control was included in this step. Reverse primers were annealed at 70°C for 5minutes (mdrlb primers were annealed at 23°C for 10 minutes) after which 5µl 5x AMV buffer (10mM dithiothreitol (DTT), 1mM each deoxynucleotide triphosphate (dNTP), 20U ribonuclease (Rnase) inhibitor, 50U Expand<sup>TM</sup> reverse transcriptase /20µL) was added along with 2.5µl 10mM dNTP's, 0.62µl RNasin (an RNase inhibitor) (33U/µl) and 0.62µl AMV RT enzyme (10U/µL). An RT enzyme-negative control for each sample was included at this step, and the final reaction volume was made up to 25µl with DEPC treated water. Products were extended at 42 °C for 1 hour. The PCR stage was carried out by the addition of 1µl each of forward and reverse primer (10pm /µl) to 5µl of the RT reaction mix along with 5µl 10 x reaction buffer (0.9mM tris (hydroxymethyl)amino methane (TRIS), pH 8.8, 0.22mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.9mM MgCl<sub>2</sub>, 4pM each dNTP, 0.1mM β-mercaptoethanol, 10mM ethylene diaminetetracetate (EDTA), pH 8, 2.2mg/ml bovine serum albumin (BSA) in a total volume of 50µl made up with ultra pure water. Samples were covered

with 50µl paraffin oil and PCR was carried out using a Hybaid omnigene thermal cycler as follows with 1µl *Thermus aquaticus* (Taq) DNA polymerase (1U/µL) added after the first denaturation step: **mdr1b gene**; 28 cycles at an annealing temperature of 56°C (2 minutes each cycle). Denaturation was at 95°C for 1 minute each cycle except the first where it was extended to 5 minutes. The extension time was 2 minutes (72°C) in each cycle except the last, in which it was extended to 5 minutes. **GST A3 and A5, rAFAR1, actin genes**; 35 cycles at an annealing temperature of 60 °C (30 seconds each cycle). Denaturation was at 95°C for 30 seconds each cycle except the first where it was increased to 3 minutes. The extension time was for 30 seconds (72°C).

### 2.9.2 Detection of RT–PCR Products by Gel Electrophoresis

After amplification by PCR, 5µl samples were electrophoresed in 3% agarose gels. 3g Nusieve and 1.5g Seakem agarose were melted in 150ml 1 x TAE by microwaving and 2.5µl ethidium bromide /50ml 1 x TAE was added. The gel was cast and set at 4°C. Electrophoresis was carried out with the gel submerged in 1 x TAE gel running buffer containing 2.5µl ethidium bromide (10mg/ml UP water)/50 ml buffer. A DNA molecular weight marker PHI X174 (1µg DNA/well) was run alongside the samples to give an indication of the size of PCR products formed, which were separated according to their size (table 2.1). Bands formed by PCR products were visualised by the incorporation of the ethidium bromide which allowed for their detection under ultra violet light.

Target Gene	PRIMERS, Forward and Reverse	Region Spanned	PCR Product (bp)
<b>GST A3</b>	5' AAGGAGTGGCGGATCTGGAG <sup>3'</sup>	439-460	247
	3' GGGAAAGTTGGCCAAAGCGCTG <sup>5'</sup> (ref: Hayes,1994)	685-706	
<b>GST A5</b>	5' GGTGTGGCCGATCTGGAGTTG <sup>3'</sup>	520-541	245
	3' GGGAAAGTTGTCCACAATGCCT <sup>5'</sup> (ref: Pulford and Hayes, 1996)	764-85	
<b>rAFAR1</b>	5' GGCTGAGATTTGTACCCTC <sup>3'</sup>	505-524	205
	3' ATTCTTCCCATCCTTATCC <sup>5'</sup> (ref: Ellis <i>et al</i> , 1993)	709-728	
<b>mdr1b</b>	5' GAAATAATGCTTATGAATCCCAAAG <sup>3'</sup>	1910-1934	325
	3' GGTTTCATGGTCGTCGTCTCTTGA <sup>5'</sup> (ref: Zhang <i>et al.</i> , 1996b)	2212-2235	
<b>Actin</b>	5' TCATCACCATTGGCAATGAGCG <sup>3'</sup>	750-772	389
	3' CTAGAAGCATTGCGGTGGACG <sup>5'</sup> (ref: Ponte <i>et al.</i> , 1984)	1138-1160	

**Table 2.1:** Oligonucleotide primer sequences for GST A3 and A5, rAFAR1, mdr1b and actin genes.

## **2.10 Safety Precautions**

### **2.10.1 AFB<sub>1</sub>**

Due to the ability of DMSO to rapidly penetrate the skin care was taken to avoid any contact with the AFB<sub>1</sub>/DMSO solution. When handling AFB<sub>1</sub>/DMSO gloves were always worn as well as a laboratory coat. Safety goggles were worn if not working in a fume hood or laminar flow cabinet. All work areas were swabbed with 0.5% sodium hypochlorite after use. Aflatoxin solutions and contaminated glassware and plasticware were decontaminated by soaking in 0.5% sodium hypochlorite over night.

### **2.10.2 Trypan blue**

Trypan blue is a known teratogen and gloves and a laboratory coat were always worn when handling this chemical.

### **2.10.3 MTT**

MTT is a known teratogen and gloves and laboratory coat were always worn when using this chemical.

## **2.11 Statistics**

Statistics were carried out on typically 8 sets of normally distributed data using the students t-test (assuming equal variances) for a 95% confidence interval.

## Chapter 3

### Optimisation of Toxicity Assay Procedure

### 3 Optimisation of AFB<sub>1</sub> Toxicity Assay

#### 3.1 Introduction

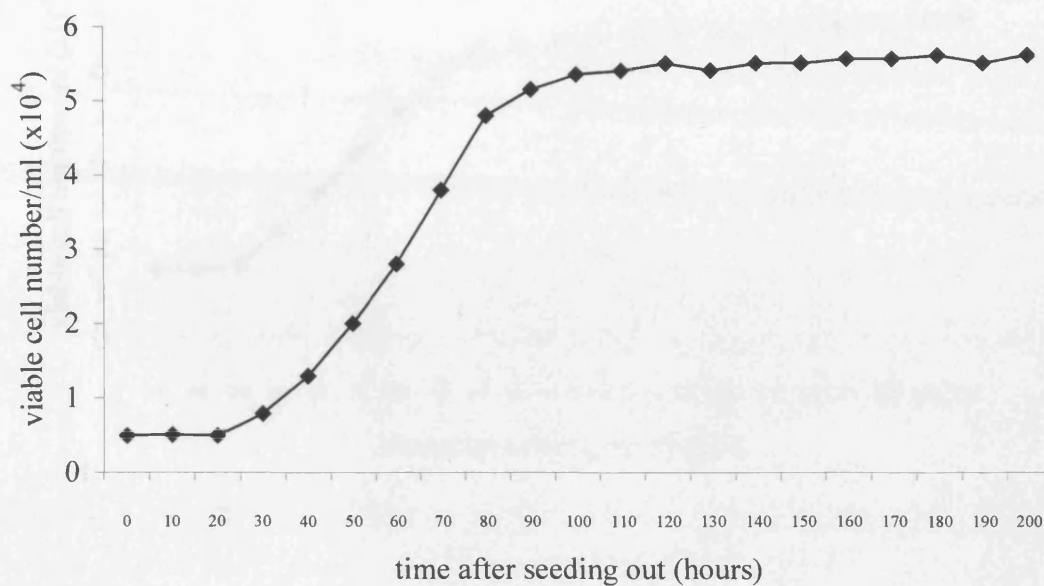
The experimental system used to study the effects of AFB<sub>1</sub> on both JB1 and RLE cell lines was based upon the previously published methods of Manson and Green (1982) and Sinha *et al.*, (1987). The aim of the work described in this chapter was to adapt these two methods and devise a single assay which would allow for the detection of cytotoxicity by various simple techniques. The assay was therefore required to be suitable for use on a range of cell culture vessels depending on the method to be employed for detection of cell death or for subsequent RNA extraction. Preliminary experiments highlighted a number of problems with the assay, which was subsequently redesigned. The important parameters for investigation were, choice of diluent for the AFB<sub>1</sub>, choice of tissue culture plate, concentration of microsomes (v/v), and concentration of, and incubation time with, MTT.

Firstly, the assay procedure was carried on cultures of JB1 and RLE cell lines in the absence of AFB<sub>1</sub>, as controls. The effects of three possible diluents for the AFB<sub>1</sub> solution on the cells was compared in order to determine where, if at all, any cell loss occurred which was attributable solely to the procedure or the effects of the diluent used. In addition the optimum concentration for the microsome fraction was investigated since initial studies in this project showed the microsome fraction alone to have a toxic effect on the cells. Once the assay was developed it was carried out on the two cell lines and cytotoxicity was measured using either the trypan blue exclusion method or the MTT assay (after optimisation).

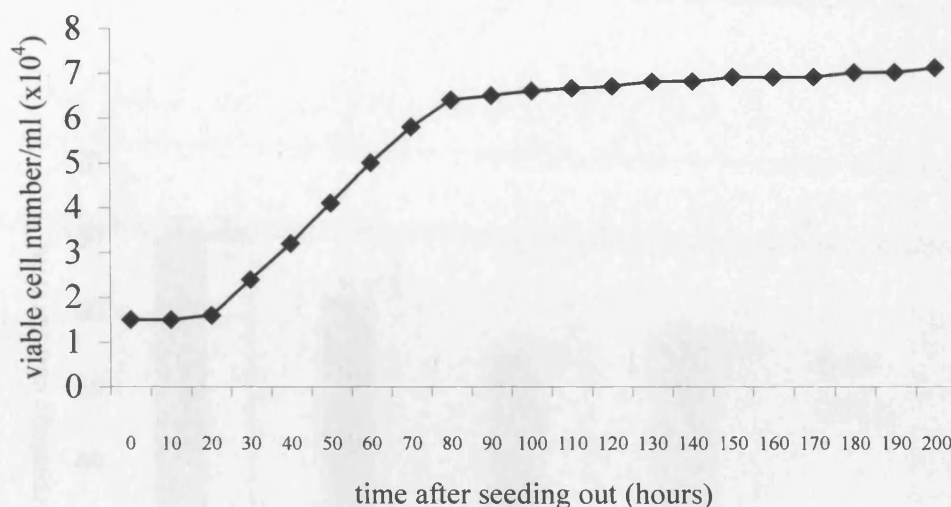
### **3.2 Growth curve for JB1 and RLE cell lines**

It was necessary to establish growth curves for both cell lines to determine when they were undergoing cell division. Suspensions of JB1 and RLE cell lines were seeded out into 96-well plates at a density of  $1 \times 10^4$  cells/ml, and plates incubated at 37°C (method 2.2). Viable cell counts were taken manually at regular time intervals from 10 hours after seeding out onwards from 8 wells at a time using the trypan blue exclusion method for cell viability (method 2.5). The results given are an average of three sets of data. A growth curve for both cell lines was established which showed an initial lag phase for both cell lines of around 24 hours after which followed exponential growth over the next 50 hours before growth levelled off reaching a plateau 80 hours after seeding out. An average cell doubling time for both cell lines was taken to be 28 hours (figures 3.1 and 3.2).





**Figure 3.1:** Growth curve obtained for JB1 cell line over 200 hours in DMEM supplemented with 10% FCS and 2% PS at 37°C in an atmosphere of 5% CO<sub>2</sub> showing an average cell doubling time of 28 hours over the exponential growth phase between 24 and 80 hours after seeding out of cell suspensions.

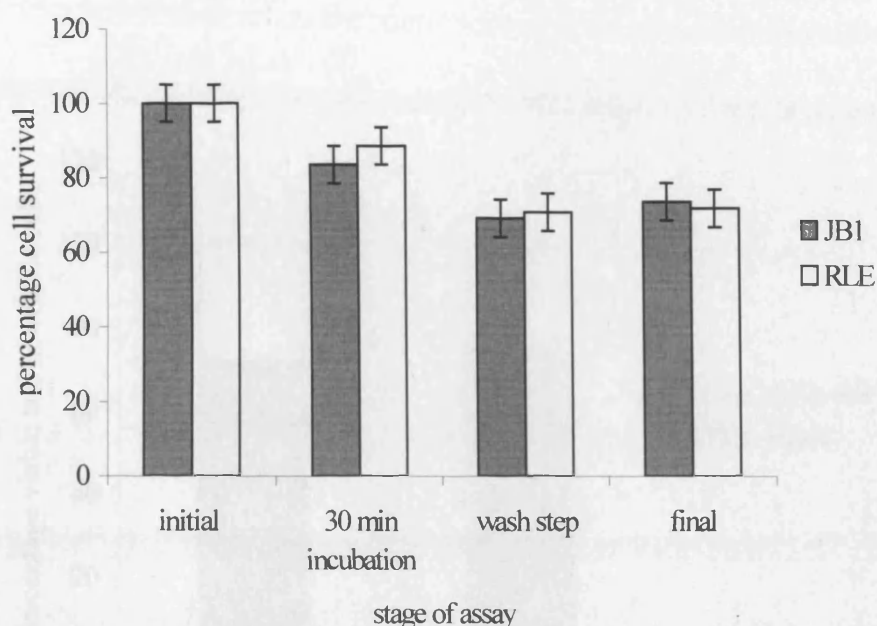


**Figure 3.2:** Growth curve obtained for RLE cell line over 200 hours DMEM supplemented with 10% FCS and 2% PS at 37°C and 5% CO<sub>2</sub> showing an average cell doubling time of 28 hours over the exponential growth phase between 24 and 80 hours after seeding out.

### 3.3 Determination of most suitable diluent for AFB<sub>1</sub> solutions.

Sinha *et al.*, (1987) treated cells with AFB<sub>1</sub> made up to the required concentration in HBSS. However initial studies in this project showed a great deal of cell loss when this method was repeated. The assay was carried out in the absence of AFB<sub>1</sub> and cell number was measured using trypan blue and a haemocytometer to give an estimate of original cell number per well in the 96 – well plates. In this way cell loss at each stage of the assay was calculated. Similar estimates of cell number remaining per well at the end of the assay allowed for the calculation of overall percentage cell loss for the assay and for percentage cell loss at each individual step. Results showed that the procedure led to an overall loss of around 30% of the initial cell number for both the JB1 and RLE

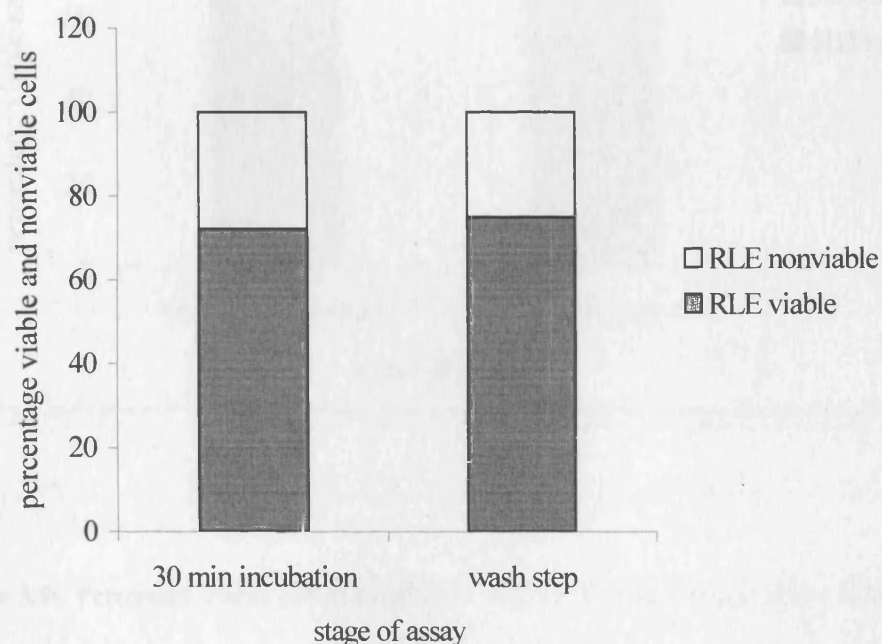
cell lines with HBSS, the majority of which occurred during the 30 minute incubation period and in the subsequent wash step (figure 3.3).



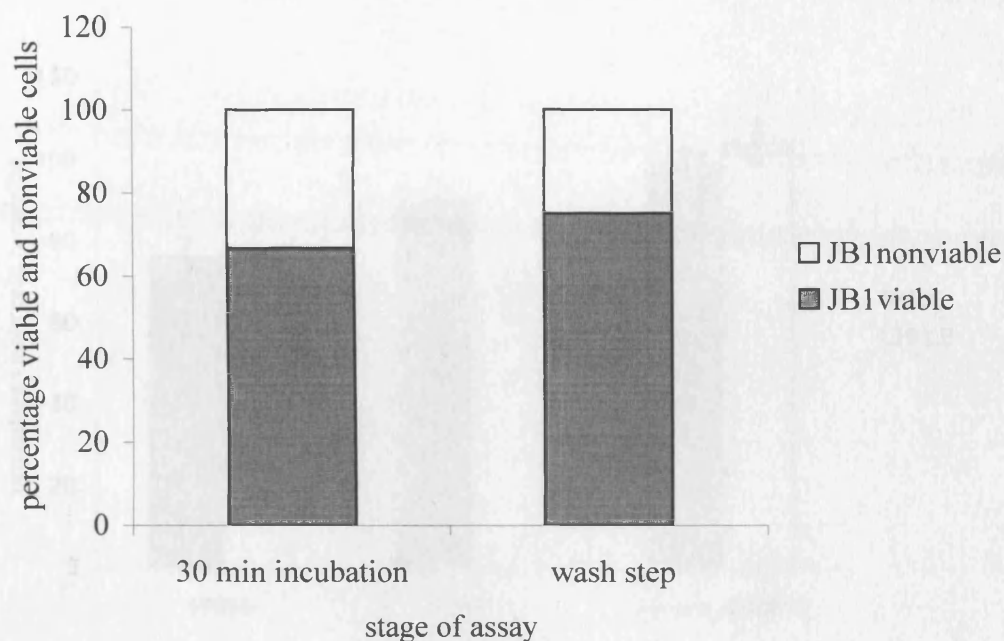
**Figure 3.3:** Percentage cell survival (i.e. cell number remaining per well expressed as a percentage of initial cell number) for JB1 and RLE cell lines at each stage of the cytotoxicity assay using HBSS alone. Note that after the 30 minute incubation step cell loss was around 20% which increased to around 35% after the 2<sup>nd</sup> wash step. This was the case for both cell lines. The final cell count was carried out 2 hours after the 2<sup>nd</sup> wash step.

Total cell number at the initial stage of the assay comprised 5% non viable cells compared with 5-7% non viable in the final cell count when calculated after trypsinisation and using the trypan blue method for cell viability. The cell number that had become detached from the monolayer during the 30 minute incubation step and the subsequent wash step were expressed as percentage viable and nonviable cells (figures 3.4 and 3.5) and showed that 66% of the JB1 cells that had detached stained negative for trypan blue and were therefore still viable and 72% of the RLE cells that had detached were still viable. On visual inspection using a phase contrast microscope at a

magnification of  $\times 20$  many of the remaining RLE cells appeared rounded up but still attached to the monolayer, this was seen to a lesser extent in the JB1 cell line. Cells washed off in the subsequent wash step were found to comprise 75% viable cells for the JB1 cell line and 69% viable cells for the RLE cell line.

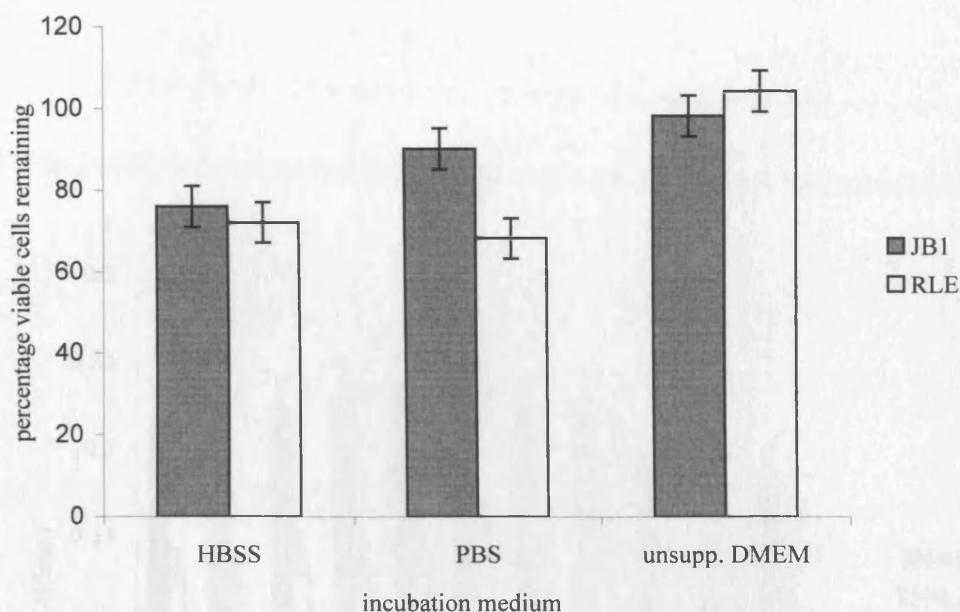


**Figure 3.4:** Percentage of viable and nonviable RLE cells in the total cell loss at the 30 minute incubation and wash stage of the assay procedure when the effects of HBSS on cell number were investigated.



**Figure 3.5:** Percentage viable and nonviable JB1 cells in the total cell loss at the 30 minute incubation and wash stage of the assay procedure when the effects of HBSS on cell number was investigated.

Similar results were found when PBS was investigated as a possible diluent in that around 30% of the initial cell number was lost during the assay procedure. However when unsupplemented DMEM was used this resulted in virtually no cell loss for either cell line at any stage during the procedure (figure 3.6). There was no difference found in cell loss during the procedure when the two different plate types were compared.



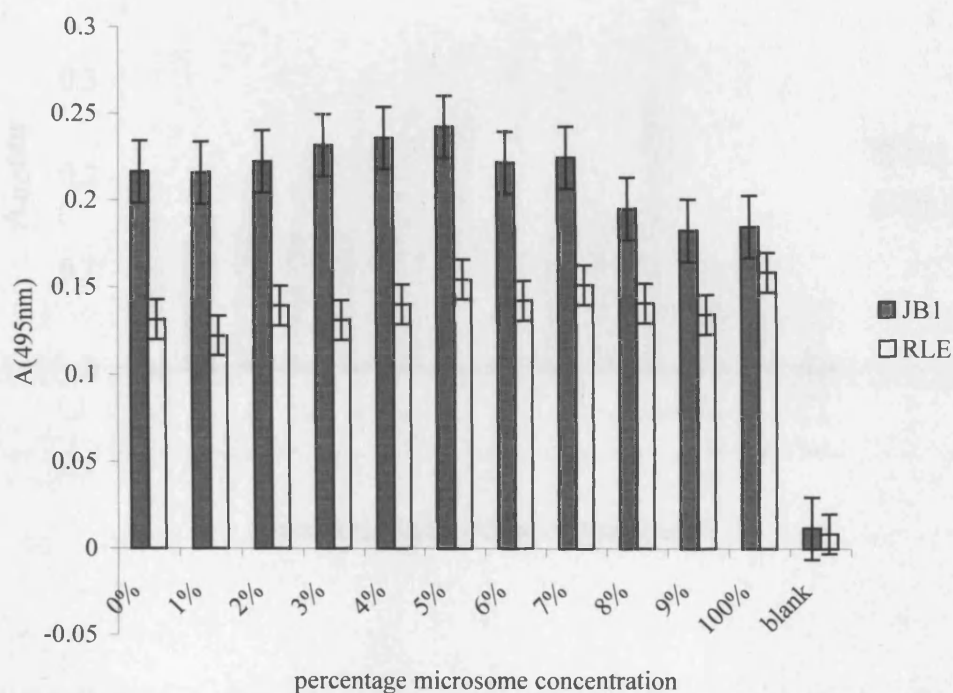
**Figure 3.6:** Comparison of overall percentage cell loss for JB1 and RLE cell lines for the assay procedure when carried out using HBSS, PBS and unsupplemented DMEM.

### 3.4 Determination of optimum microsome concentration.

The effects of male quail liver microsomes (6mg protein/ml), extracted via the method of Neal *et al.*, (1986), on JB1 and RLE cell lines over a concentration range of 0% to 100% dilutions in DMEM (method 2.3.2.2) were analyzed. Cell viability was examined using the MTT assay (method 2.6) and the annexin V ELISA technique (method 2.7.2) was also carried out on a set of duplicate plates to assess whether or not the cells were induced to undergo apoptosis or necrosis. The procedure was repeated three times and an average result is shown.



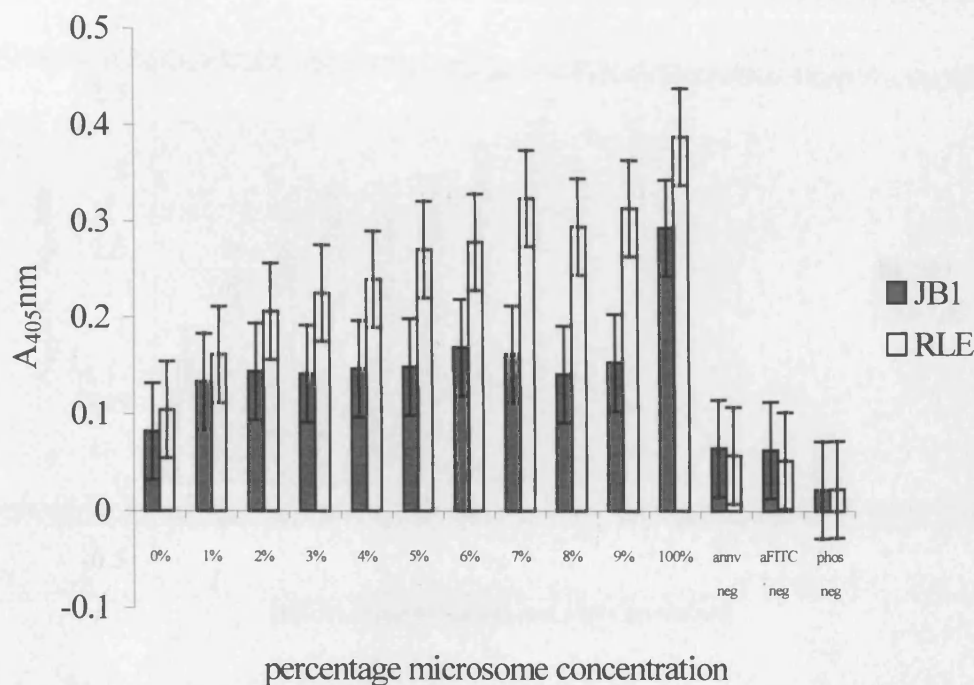
No significant difference in absorbance was seen for either cell line over the concentration range of microsomes applied. (figure 3.7) when results were subjected to the two sample t-test (95% confidence interval).



**Figure 3.7:** Absorbance values at 495<sub>nm</sub> obtained for MTT assay, with JB1 and RLE cell lines over a concentration range of 0% to 100% dilutions of stock suspension of quail liver microsomes (6mg protein/ml).

Results of the annexin V ELISA assay for the RLE cell line showed a steady increase in the absorbance at 405<sub>nm</sub> over the concentration range of 1% to 100% microsomes applied. A value of over three times that of the control (media only) was recorded for a 100% microsome concentration. This was found to be highly significant ( $P < 0.001$ ). Results for the JB1 cell line showed no significant difference in absorbance at 405<sub>nm</sub>

over a concentration range 1% to 9%. However, again, for a concentration of 100% microsomes, an absorbance of three times that of the control situation was recorded (figure 3.8). This increase in absorbance was statistically significant ( $P < 0.001$ ).

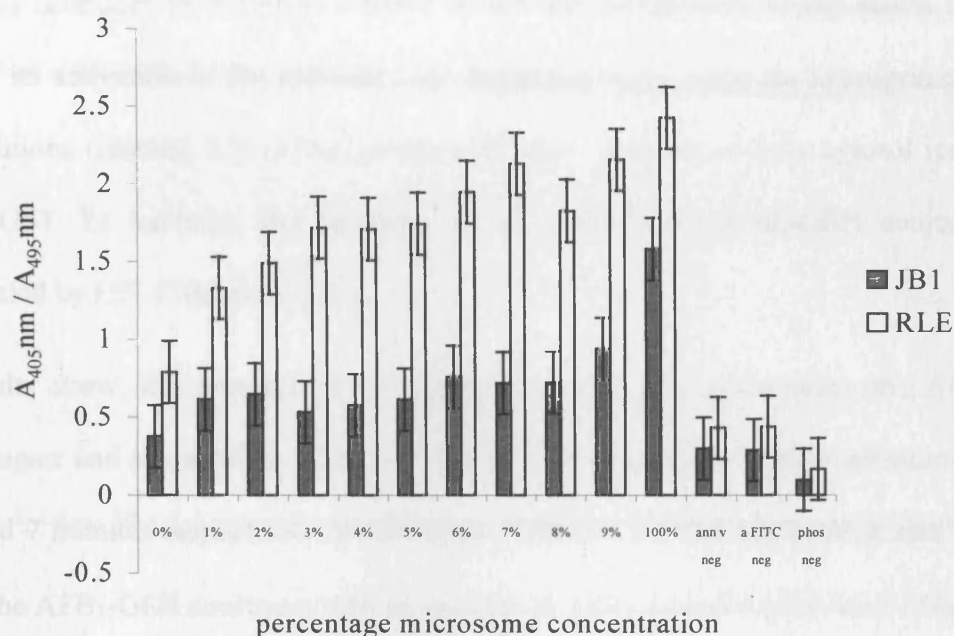


**Figure 3.8:** Comparison of PS exposure, measured by ELISA, with the control situation (0% quail liver microsomes) over a concentration range of 1% to 100% microsomes for JB1 and RLE cell lines.

The average recorded  $A_{405nm}$  was calculated in relation to the average recorded  $A_{495nm}$  for each concentration of microsomes (i.e. in relation to viable cell number) (figure 3.9) to give a value for 'PS index'. Results obtained showed no significant increase in the value of PS index for the JB1 cell line over the concentration range of 1-9% microsomes. However, a reading of over three times the value for the control was recorded at 100% microsome concentration, this was highly significant ( $p < 0.001$ ) For the RLE cell line, absorbance readings gradually increased over the concentration range of microsomes applied until a value of over three times that of the control situation was



recorded for a 100% microsome concentration. Again, this was highly significant. For concentrations of 5-9% and 100% microsomes, differences in absorbance readings between both cell lines were highly significant.

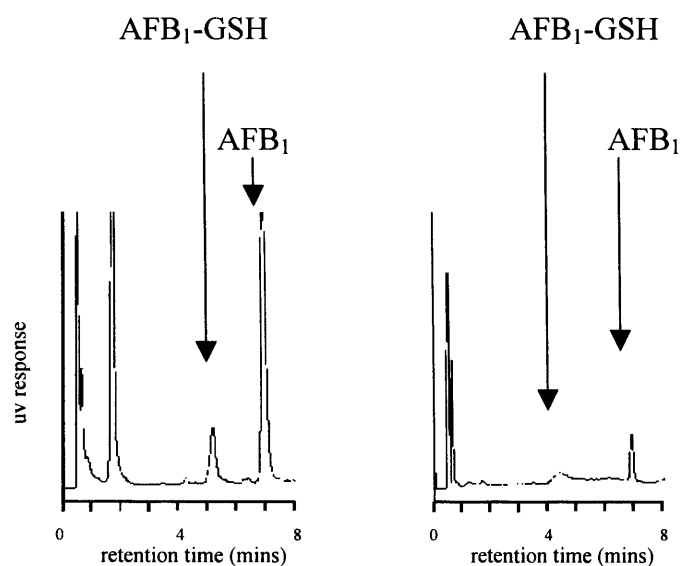


**Figure 3.9:** JB1 and RLE cell lines were treated with a concentration range of 1-100% (v/v) microsome stock solution (6mg protein.ml) in DMEM for 30 minutes at 37°C. Levels of PS exposure (A<sub>405nm</sub>) were determined by ELISA after annexin V-FITC labelling, and were expressed in relation to viable cell count as determined by the MTT assay (A<sub>495nm</sub>). A PS index (A<sub>405nm</sub>/A<sub>495nm</sub>) was then calculated for each set of experimental conditions.

### **3.5 Demonstration of activation of AFB<sub>1</sub> to the epoxide using a 1% solution of quail liver microsomes in unsupplemented DMEM by HPLC.**

Once the choice of unsupplemented DMEM was established as a diluent for the AFB<sub>1</sub>, it was necessary to ascertain whether or not any components of the media interfered with its activation to the epoxide. By incubating AFB<sub>1</sub> with the appropriate reaction conditions (method 2.3) in the presence of GSH and mouse liver cytosol (containing the GST Yc subunit), the presence of the AFB<sub>1</sub> 8,9-epoxide-GSH conjugate was detected by HPLC (method 2.4).

Results show, the presence of two peaks (figure 3.10), representing the AFB<sub>1</sub>-GSH conjugate and any residual AFB<sub>1</sub>, which were distinguishable by their retention times of 5 and 7 minutes respectively (Hayes *et al.*, 1991c). An increase in peak size was seen for the AFB<sub>1</sub>-GSH conjugate with an increase in AFB<sub>1</sub> concentration from 1-5µg/ml.



**Figure 3.10:** Example of results of hplc analysis on incubation of a solution of AFB<sub>1</sub> (1 µg/ml) with 1% (v/v) quail liver microsomes (method 2.3), in the presence of GSH and the BALB-C mouse liver cytosol (containing GST Yc subunit) (10 minutes incubation time). Two peaks can be seen, corresponding the AFB<sub>1</sub>-GSH conjugate and residual AFB<sub>1</sub>, which are differentiated by their retention time on a Chrompack C18 5 µm Chromsphep column (5 and 7 minutes respectively) (Hayes *et al.*, 1991c).

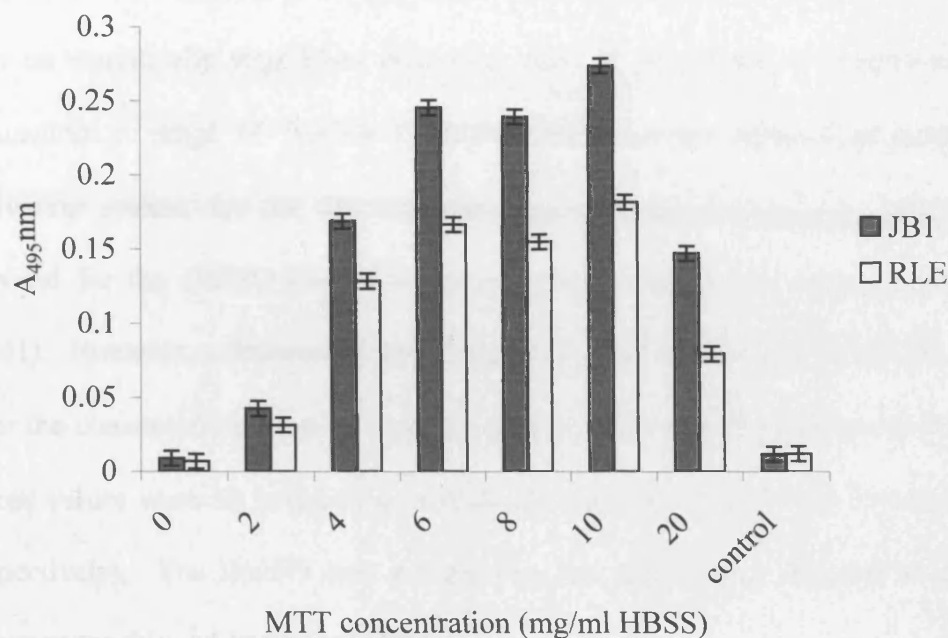
### **3.6 Cell Viability Studies on JB1 and RLE Cell lines After Treatment with AFB<sub>1</sub>**

Once a suitable assay technique had been established for use on a cell monolayer the effects of AFB<sub>1</sub> on the two cell lines JB1 and RLE were investigated. Cell viability after treatment with AFB<sub>1</sub> over a range of 0 to 5µg/ml in the presence and absence of an external microsomal activating system was measured via the trypan blue exclusion method (2.5) and compared with results obtained using the MTT assay (2.6) for cell viability.

#### **3.6.1 Optimisation of MTT concentration**

It was necessary to optimise the MTT assay for use on the cell lines used and the effects of a concentration gradient of MTT/HBSS solution ranging from 0 to 20mg/ml were studied. Two day old cultures of JB1 and RLE cell lines which had been seeded out into 96-well plates at a concentration of  $1 \times 10^4$  cells/ml were used. A concentration gradient of 0-20mg/ml MTT/HBSS was investigated in order to ascertain the optimum MTT concentration required to give a high enough reading on the spectrophotometer to be detectable but above which it did not itself cause cytotoxicity.

Absorbance at 495nm was seen to increase with an increase in concentration of MTT from 0 to 10 mg MTT per ml HBSS after which it decreased at a concentration of 20 mg/ml MTT (figure 3.11).



**Figure 3.11** Absorbance readings at 495nm after 1 hour incubation with MTT over a concentration range of 0 to 20 mg/ml MTT in HBSS for JB1 and RLE cell lines.

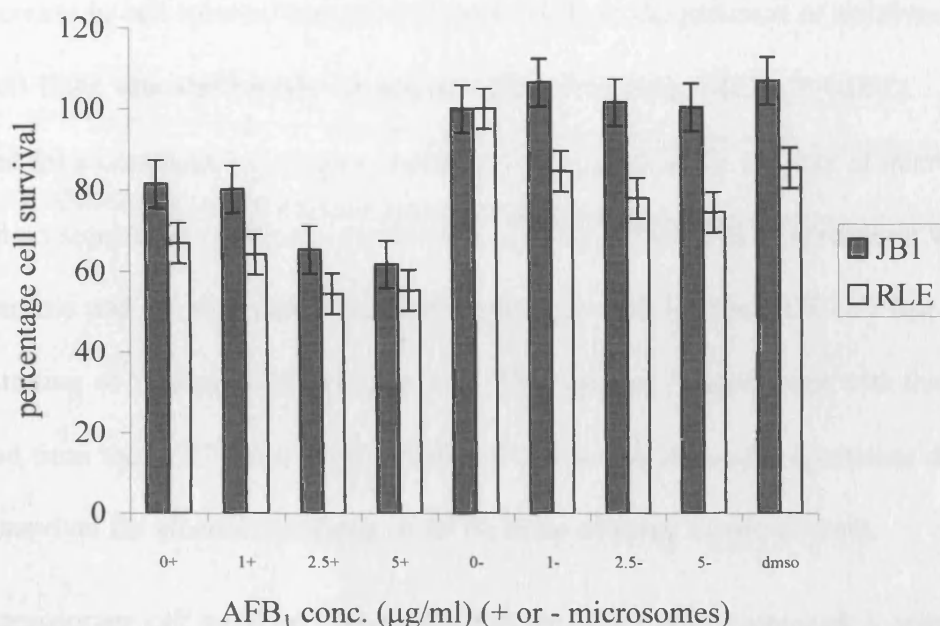
The optimum concentration of MTT adopted for use in this project was 5mg /ml MTT in HBSS. This gave readings at 495nm of between 0.1 and 0.25, which was sensitive enough to be detected but not off the scale of the spectrophotometer but at the same time caused the minimal amount of toxicity to the cells themselves.

### 3.6.2 Toxicity of AFB<sub>1</sub> on JB1 and RLE cell lines measured by MTT assay.

Percentage cell survival for both JB1 and RLE cell lines was measured 24 hours after treatment with AFB<sub>1</sub> in the presence and absence of microsomes using the MTT assay (figure 3.12). Results obtained for percentage cell survival after 24 hours showed a dose response with an increase in cytotoxicity for both cell lines when compared to the

control (0µg/ml AFB<sub>1</sub> minus microsomes), over the concentration range of activated AFB<sub>1</sub> applied (These results were all found to be highly statistically significant when subjected to the two sample t-test with a 95% confidence interval ( $P < 0.001$ ). There was no statistically significant difference seen in percentage cell survival over the concentration range of 1µg/ml to 5µg/ml AFB<sub>1</sub> in the absence of a microsomal activating system, for the JB1 cell line, nor was the difference in percentage cell survival for the DMSO control statistically significant to the control situation ( $P = 0.161$ ). However, a decrease in percentage cell survival was seen for the RLE cell line over the concentration range of 1µg/ml AFB<sub>1</sub> to 5µg/ml in the absence of microsomes. These values were all found to be statistically significant ( $P=0.022$ ,  $P<0.001$ ,  $P<0.001$  respectively). The DMSO only control was not significantly different to the control situation for this cell line ( $P = 0.065$ ).

Lower values for percentage cell survival were obtained for the RLE cell line compared with the JB1 cell line with unactivated AFB<sub>1</sub> over the range 1µg-5µg/ml. This was found to be statistically significant for all concentrations applied ( $P = 0.015$ ,  $P = 0.001$ ,  $P<0.001$  respectively). However in the presence of activated AFB<sub>1</sub>, over the concentration range applied, a highly significant difference in percentage survival rates was seen between the two cell lines ( $P<0.001$ ) with microsomes alone and for a concentration of 1µg/ml, and for a concentration of 2.5µg/ml AFB<sub>1</sub> ( $P = 0.024$ )(figure 3.11), with the JB1 cell line exhibiting a higher level of resistance to the AFB<sub>1</sub>.



**Figure 3.12:** Percentage cell survival for JB1 and RLE cell lines 24 hours after a 30 minute incubation with AFB<sub>1</sub>, over a concentration range of 0 to 5 µg/ml in the presence (+) and absence (-) of an external microsomal activating system, showing a dose response effect for both cell lines. Cell viability was measured using the MTT assay and cell survival was calculated as a percentage of the absorbance reading at 495nm obtained for the control situation.

### 3.6.3 Cytotoxicity of AFB<sub>1</sub> measured by trypan blue exclusion method

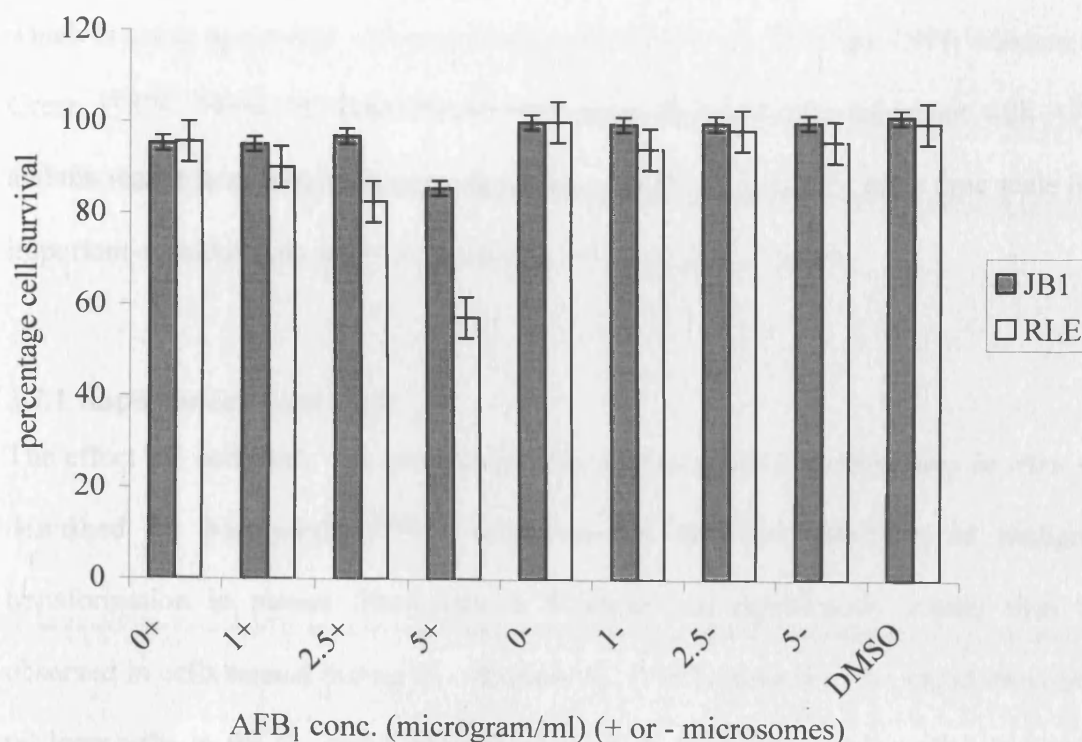
Cytotoxicity of AFB<sub>1</sub> on JB1 and RLE cell lines was measured using the trypan blue exclusion method for cell viability 24 hours after treatment and results were expressed as percentage cell survival when compared with the control situation (0µg/ml AFB<sub>1</sub> minus microsomes) (figure 3.13). Similar trends in cell survival rates were obtained as for the MTT assay except that higher values were recorded over the concentration range of 0 to 2.5µg/ml AFB<sub>1</sub> in the presence of microsomes. The only significant difference in percentage cell survival in either cell line for a 95% confidence interval using the two

tailed t-test, was seen in the RLE cell line for 1 and 2.5µg/ml AFB<sub>1</sub> in the presence of 1% microsomes ( $P = 0.015$ ,  $P < 0.001$  respectively).

The decrease in cell survival seen with 5µg/ml AFB<sub>1</sub> in the presence of microsomes for both cell lines was statistically significant (JB1:  $P < 0.001$ ), (RLE:  $P = 0.001$ ). Results obtained for a concentration range of AFB<sub>1</sub> of 0 to 5µg/ml in the absence of microsomes showed no significant difference for the JB1 cell line, which was in agreement with the MTT results and no significant decrease in cell survival for the RLE cell line until a concentration of 5µg/ml AFB<sub>1</sub> was reached. This was not in agreement with the results obtained from the MTT assay for the RLE cell line which showed a significant decrease in cell survival for all concentrations of AFB<sub>1</sub> in the absence of microsomes.

When percentage cell survival values for both cell lines were compared, a statistically significant difference was found for concentrations of 2.5 and 5µg/ml AFB<sub>1</sub> in the presence of microsomes. This is not in agreement with the results of the MTT assay in which the percentage cell survival for the JB1 cell line was significantly higher than the RLE cell line for all concentrations of AFB<sub>1</sub> in the absence of microsomes. However the significant difference found between percentage cell survival for both cell lines at 2.5µg/ml AFB<sub>1</sub> in the presence of microsomes seen with the MTT assay was in accordance with the results obtained for the trypan blue assay.





**Figure 3.13:** Percentage cell survival for JB1 and RLE cell lines 24 hours after 30 minutes incubation with AFB<sub>1</sub> over a concentration range of 0 to 5 µg/ml in the presence (+) and absence (-) of an external microsomal activating system, showing a dose response effect. Cell viability was measured by the trypan blue exclusion method and results expressed as a percentage of the value obtained for the control situation.

### 3.7 Discussion

The toxicity of AFB<sub>1</sub> is well documented and results of this thesis are in agreement with that of other authors (Manson *et al.*, 1981), in that JB1 exhibits resistance to the toxic effects of this mycotoxin, when compared with the more sensitive RLE cell line. A

dose response is seen with both cell lines over the concentration range of AFB<sub>1</sub> applied, and the RLE cell line appears to be sensitive to the effects of unactivated AFB<sub>1</sub>, indicating that it may possess the ability to intrinsically activate the mycotoxin itself, which is not in agreement with previously published work (Phillips, 1974; Manson and Green 1982). However, these results were taken 24 hours after treatment with AFB<sub>1</sub>, and the reader is referred to further discussion on this in chapter 5, since time scale is an important consideration when investigating the mode of cell death.

### 3.7.1 Importance of cell cycle

The effect of cell cycle on chemically induced malignant transformation *in vitro* was described by Marquardt (1974) who reported that the induction of malignant transformation in mouse fibroblasts in S phase was significantly greater than that observed in cells treated during G<sub>1</sub>. Sinha *et al.*, (1987), described the transformation of rat liver cells in the G<sub>1</sub> and S phase with AFB<sub>1</sub> in the presence of quail microsomes. However other authors do not report the stage of cell cycle at which cells were treated; Manson and Green., (1982) state that BL8 and JB1 cell lines were 80% confluent when treated with AFB<sub>1</sub> while Fernando *et al.*, (1993) treated human hepatoma cells with AFB<sub>1</sub> when they had reached 60% confluence in minimal essential medium.

Since the principal way that AFB<sub>1</sub> causes mutagenesis is by formation of DNA and protein adducts, in the method developed for this thesis it was necessary to introduce the AFB<sub>1</sub> epoxide when the cells were undergoing DNA synthesis during the exponential growth phase. In addition cell growth was required to be in the exponential stage for subsequent MTT assays for cell cytotoxicity which were measured 24 hours after treatment with AFB<sub>1</sub>. Results showed that after a 24 hour lag phase both JB1 and RLE cell lines used in this study entered an exponential growth phase for the next 50 hours reaching a stationary phase 80 hours after seeding out. For the purpose of the toxicity assay both cell lines were treated with AFB<sub>1</sub> 48 hours after seeding out when

they were in the exponential growth phase with a cell doubling time of 28 hours. At this point they had reached 80% confluence and they were still in the exponential phase 24 hours later when the MTT assay was carried out.

### 3.7.2 Choice of AFB<sub>1</sub> diluent

Unsupplemented DMEM was found to be the most suitable diluent for the AFB<sub>1</sub> compared with both HBSS and PBS. Cell numbers remained at around 100% of the initial cell number for both cell lines at the end of the assay after 30 minutes incubation in this diluent. The 2% cell loss obtained for the JB1 cell line can be attributed to the effects of trypsinisation. This was also apparent in both cell lines when the assay was carried out in the presence of HBSS alone, when a total initial cell count was shown to consist of 5% nonviable cells. This was in accordance with 5-7% nonviable cells in the final cell count. Since the initial cell count was taken prior to any treatment and after washing of the monolayer to remove any cell debris the nonviable cell count in this instance can be taken to be that amount of cell death which occurs due to the effects of trypsinisation of the cell monolayer.

This is in accordance with the cell count taken after the 1<sup>st</sup> wash step with nonviable cells amounting to between 1% and 5% of the total number and the final cell count taken after a 2 hour recovery period which was comprised of between 5% and 7% nonviable cells, although the slight increase of 2% could be attributed to cells dying and detaching from the monolayer during this time. Since any nonviable cells present at the above mentioned stages of the assay are likely to be due to trypsinisation of the monolayer it can be concluded that there was little or no death of cells at any of these stages.

The greatest cell loss with HBSS, was seen after the 30 minute incubation step when around 20% of the total cell number was lost with a further 15% detaching from the

monolayer during the subsequent wash step leading to an overall loss of 35%; 30% of which was taken to be due to the effects of the incubation with HBSS and 5% due to trypsinisation. The cell number increased slightly over the two hour recovery stage by 6% of the initial cell number which could mask the effects of any further cell loss during this time. This was not in accordance with the results of Sinha *et al.*, (1987) who did not report any cell loss due to the effects of the HBSS diluent. However other authors did in fact use unsupplemented medium as a diluent for the carcinogen under investigation (Marquardt *et al.*, 1974; Manson and Green, 1982; Woo *et al.*, 1992).

### 3.7.3 Optimisation of microsome concentration

The results of the effects of quail liver microsomes on the RLE cell line ( $A_{405\text{nm}}/A_{495\text{nm}}$ ) showed an increase in the amount of annexin V binding and therefore phosphatidyl serine (PS) exposed on the cell surface of the cells over the concentration range 0% to 100% until three times more PS was exposed at 100% microsome concentration than in the control situation. There was twice the amount detected at the lowest dose of 1% microsome concentration than at 0%.

The concentration of microsomes did not appear to have as great an effect on the JB1 cell line however, there only being an increase in phosphatidyl serine detection of 1.5 times that of the control situation for a dose of 1% microsomes. This value remained constant until a concentration range of 8% microsomes was reached when annexin V detection increased until a value of three times the control situation was recorded at 100% microsome concentration. The effect of 100% microsome concentration therefore was to increase PS exposure on the outer surface of the cell membrane of both cell lines by three times that of the control (0% microsomes) although the JB1 cell line appeared to exhibit a greater resistance to the effects of the microsomes at the lower concentration range.

A comparison of the readings obtained for duplicate sets of plates with the MTT assay and the annexin V ELISA, showed that annexin V - FITC binding increased over the concentration range applied whilst there was no significant difference in viable cell number. This would seem to suggest that the effect of the microsomes is to increase the amount of PS exposed on the outer cell membrane with an increasing concentration of microsomes therefore indicating the induction of apoptosis in these cells.

A major disadvantage of the annexin V ELISA technique developed in this project for the detection of PS on the outer cell membrane of adherent cells is that it is difficult to differentiate between the occurrence of apoptosis or necrosis since the flipping of PS to the outer surface of the cell membrane occurs in both instances and therefore both types of cell death would show positive for staining with annexin V – FITC (for further discussion see chapter 5).

#### **3.7.3.1 Extrinsic Activation of AFB<sub>1</sub>**

The presence of an external microsomal activating system is essential in studies concerned with the effects of AFB<sub>1</sub> on established cell lines such as the ones used in this study since on culturing they lose the ability to metabolise the AFB<sub>1</sub> to the reactive epoxide form due to the progressive decrease in cytochrome P450 content which occurs during culturing. Manson and Green (1982) reported no metabolism of AFB<sub>1</sub> (as shown by HPLC) when the compound alone was incubated with JB1 or BL8L cell lines. However, they demonstrated an increase in GGT activity in the hepatic epithelial cell lines when treated with AFB<sub>1</sub> in the presence of microsomes obtained from the livers of male Fischer 344 rats pretreated with either 0.1% phenobarbitone or 3-methylcholanthrene.

Phillips (1974) also demonstrated the requirement for an extrinsic activating system for cyclophosphamide, reporting that the compound had no effect on Walker tumour cells

from the peritoneal cavity of Wistar rats unless rat liver microsomes were added.

Furthermore, they reported that the microsomes had no effect upon cell viability. Sinha *et al.*, (1987) used quail liver microsomes in the activation of AFB<sub>1</sub> when studying its effects on BL8 cells and did not report any effects of the microsomes on cell viability. The microsomes used in this study were prepared by the method of Neal *et al.*, (1986) who reported that the rate of microsomal metabolism of AFB<sub>1</sub> by male quail hepatic microsomal P450 in vitro was 8 times greater than that catalysed by male rat cytochrome. Also of note is that in the quail almost all of the metabolism proceeded via 8,9-AFB<sub>1</sub> epoxidation, whereas in the rat only 36% of the soluble metabolites were formed by this pathway. The 3-methyl-cholanthrene induced cytochromes had very different properties in the two species; in particular a species of P450 was present in quail microsomes with a high efficiency for metabolism of AFB<sub>1</sub>.

Judah and Davies, (1994) reported that primary rat hepatocytes cultured on matrigel, a laminin – rich extracellular matrix, plus phenobarbital for 5 days led to cells retaining 74% of the initial cytochrome P450 content compared with <10% for cells grown on plastic alone. However this method was not suitable for this project since established cell lines were used rather than primary cultures and therefore loss of the P450 content had already occurred.

Other studies which have been carried out attempting to slow or prevent this loss of P450 on culturing cells have involved manipulating the composition of the culture medium either by the addition of various compounds, including haem (Englemann *et al.*, 1985) and hormones (Paine *et al.*, 1980) or the removal of other constituents such as cysteine (Paine and Hockin, 1980). In general however this has resulted in only marginal differences in the rate of loss of xenobiotic metabolising capacity. Although the nature of the substratum has been examined, for instance collagen (Bissell and Guzelian, 1980) or fibronectin (Johannson and Hook, 1984), as it could influence the

adaptation of the hepatocytes to culture conditions, a general finding has been that these substrata do not significantly influence the rate of alteration of hepatic gene expression in cultured cells.

HPLC analysis demonstrated the presence of the AFB<sub>1</sub> dihydrodiol when AFB<sub>1</sub> was incubated at 37°C with a 1% microsome concentration (6mg protein/ml), which proved that the AFB<sub>1</sub> was in fact activated at this level of microsome concentration. A lower concentration of microsomes was decided against due to the impracticalities of measuring out very small volumes of the microsome fraction when 96-well plates were used.

#### **3.7.4 Considerations for the MTT Assay**

The use of the MTT assay for cell viability performed in microtitre trays in conjunction with an automatic scanning spectrophotometer offers major advantages in speed, simplicity, cost and safety over other assays such as [<sup>3</sup>H] thymidine uptake assay. The method originally described by Mosmann (1983) involves cleavage of MTT by living metabolically active cells, but not by dead or metabolically inactive cells; the amount of formazan product generated by dehydrogenase activity being directly proportional to cell number and this has been confirmed by other authors (Green *et al.*, 1984; Denizot and Lang, 1986; Twentyman and Luscombe, 1987; Carmichael and DeGraff, 1987; Hansen *et al.*, 1989).

A good correlation between the MTT assay and [<sup>3</sup>H] thymidine incorporation has been reported (Mosmann, 1983; Heeg *et al.*, 1985; Tada *et al.*, 1986) and Twentyman and Luscombe (1987) reported a good agreement for comparative chemosensitivity data results between the MTT assay and total cell counts. Carmichael and DeGraff, (1987) reported a good correlation between results obtained for the clonogenic assay and MTT. However Hansen *et al.*, (1989), reported that the original MTT method described

sometimes gave rise to results which differed from those obtained with dye-uptake methods while Dotsika and Sanderson, (1987) reported that Mosmann's assay was relatively less sensitive than uptake of [ $^3\text{H}$ ] thymidine as did Denizot and Lang (1986).

#### 3.7.4.1 Parameters Affecting MTT Absorbance Spectrum

The presence of phenol red in the media was thought by Mosmann (1983) to interfere with the absorbance spectrum of the formazan product but Twentyman and Luscombe (1987) reported that this did not present a major problem as it was turned yellow by any acid in the solvent. Denizot and Lang (1986) added HCl to isopropanol, when used as a solvent of the formazan, which converted the indicator to a yellow noninterfering colour. Hansen *et al.*, (1989) however, stated that phenol red did not interfere with measurements for a pH of  $<5.5$ . This was important in that it allowed for the elimination of any wash steps during the assay which could lead to cell loss.

Other factors such as the effects of the amount of serum in the media were considered by Twentyman and Luscombe (1987) who recommended the use of serum-free media immediately before MTT addition to circumvent the problem of protein precipitation and Green *et al.*, (1984) recommended addition of a detergent solution (3% w/v) sodium dodecyl sulphate in  $\text{H}_2\text{O}$  to alleviate the problem of alcohol-induced protein precipitation when high serum concentrations of  $> 15\%$  were used. However, Hansen *et al.*, (1989) reported that serum proteins at a concentration of up to 25% have no influence on the results of the MTT assay, but that the pH of the extraction buffer used was of the utmost importance, since a pH of  $>5$  gave rise to false signals.

The presence of traces of media in the solvent was reported to greatly impede solubilisation of formazan in either mineral oil or acidified isopropanol by Twentyman and Luscombe (1987). However they also demonstrated that solubilisation was rapid and complete with  $< 40\mu\text{l}$  medium left over per well if DMSO was used as a solvent



although a chemical reaction took place between the medium and DMSO which was only considered to cause a significant variation in optical density when  $> 20\mu\text{l}$  medium was present in the well.

In the adaptation of the original assay used in this project however these factors were not considered to be problematic since the amount of residual medium in wells after inversion was minimal ( $< 10\mu\text{l}$ ) and the concentration of FCS in the media was only 10%. The chosen solvent for the MTT assay in this instance was DMSO which was in accordance with several other authors; Carmichael *et al.* (1987) compared it with acid isopropyl alcohol as a solvent of the formazan crystals and reported that total solubilisation of the formazan was incomplete using the latter but total solubilisation was achieved rapidly ( $< 5$  minutes) in DMSO (Twentyman and Luscombe, 1987). Hansen *et al.*, (1989) developed an extraction buffer (20% w/v SDS in 50% solution of DMF and demineralised water, pH 4.7) which required overnight incubation which has obvious disadvantages over the use of the more rapid solubilisation in DMSO. Ethanol and propanol have also been used as solvents (Denizot and Lang, 1986) but it was thought that these could pose problems due to evaporation of the solvent and the possibility of protein precipitation.

Although MTT formazan is readily soluble in many organic solvents of very different polarities absorption curves obtained for the different solvents vary with the major peak occurring at different wavelengths. For instance the major peak is seen at 505nm for DMF and 560nm for n hexane (Altman, 1974), 570nm for acid isopropanol (Mosmann, 1983), 560nm for propanol (Denizot and Lang, 1986), 570nm for mineral oil and 540nm for DMSO (Carmichael *et al.*, 1987) while Twentyman and Luscombe (1987) reported a major peak at 550nm for DMSO. A wavelength of 495nm was used in this project since it was the nearest filter we had in this laboratory to the maximum absorbance

wavelength for formazan in DMSO. 630nm was chosen as the reference wavelength since the formazan does not absorb at this wavelength.

#### 3.7.4.2 Adaptations to the MTT Assay

Many adaptations to Mosmann's method have been made and the need for optimisation of assay conditions for each individual cell line has been stressed. One other factor which requires optimisation is MTT concentration. Mosmann (1983) used 10µl of 5mg/ml MTT in PBS per 100µl medium as did Green *et al.* (1984), Dotsika and Sanderson (1987) and Hansen *et al.* (1989). This is in accordance with the results found in this project i.e. that a concentration of 5mg/ml MTT is the optimum and at concentrations greater than 10mg/ml MTT in HBSS absorbance decreases due to the cytotoxic effects of the MTT on the cells possibly due to the production of a free radical intermediate in the cleavage of the MTT to the formazan (Altman, 1974). This is also in accordance with the results of Tada *et al.*, (1986) who reported an increase in absorbance at 540nm up to a concentration of 8mg/ml MTT after which at higher concentrations absorbance decreased.

Incubation time with the MTT is an important factor to be taken into account. Results of this project showed that for the two hepatocyte cell lines used; JB1 and RLE, incubation times of longer than 1 hour caused cell death visible as cells detaching from the surface of the culture vessel and subsequently becoming lost during the inversion of the plate.

This was again possibly due to the formation of a free radical intermediate or interaction of a non H bonded structure of the formazan with relatively polar cellular components (Altman 1974). Other authors report times of between 2 to 6 hours incubation with MTT depending on the cell lines used; 4-6 hours for a mouse tumour cell line (Twentyman and Luscombe, 1987); 4 hours for human lung cancer cell lines

(Carmichael, 1987) and 3 hours for T lymphoma cell lines (Denizot and Lang, 1986).

Mosmann (1983) noted a linear increase in absorbance from  $\frac{1}{2}$  to 2 hours incubation with MTT and a lesser rate of increase from 2 to 4 hours while Green *et al.*, (1984) and Tada *et al.*, (1986) reported an increase in absorbance with time over 4 hours.

Cell number must also be taken into account and optimised for each cell line. Hansen *et al.*, (1989) demonstrated a linear correlation between the number of seeded cells and OD readings within the range of 300 – 52 000 cells per well stating that below 300 cells the signal was not reproducible and above 50 000 cells the curve reached a plateau.

Tada *et al.*, (1986) reported that  $A_{590}$  was directly proportional to the number of viable cells over a range of 1000 to 80 000 cells per well. Green *et al.*, (1984) stated that the detection limit was between 500 to 50 000 cells per well after which the relationship became nonlinear due to the complete reduction of the MTT to the formazan. In the original method of Mosmann (1983) it was stated that the assay was suitable for detecting from 200 to 50000 cells per well.

Reproducibility of the assay was reported by Green *et al.* (1984) to be within 2-5% of the mean for triplicate samples which was in accordance with Twentyman and Luscombe (1987) who reported a similar reproducibility of within 2% of the mean for triplicate samples. Assay temperature was recommended to be 37° C by Hansen *et al.* (1989) who noted that OD at room temperature and therefore MTT conversion was half that at 37°C. The MTT assay conditions adopted for this project therefore were based on recommendations by other authors and on optimisation studies carried out as part of the project.

### 3.7.4.3 Advantages of MTT Assay

MTT is cleaved by all living metabolically active cells but not by dead cells and the amount of formazan is directly proportional to cell number over a wide range. An advantage of the method is the speed with which samples can be processed and in conditions where the components of the medium do not interfere the assay can be read with no wash steps which increases the speed of the assay and minimises variability between samples leading to good reproducibility. The MTT assay measures the number and activity of living cells at the end of the assay whereas [ $^3\text{H}$ ] thymidine incorporation measures the number of cells synthesising DNA during the last few hours of the assay so the MTT assay correlates well with visual examination of cells at the end of the assay. Also the MTT assay is preferable due to there being no radioisotopes used. Multiple replicates of microcultures can be processed rapidly so leading to the generation of statistically reliable data and the MTT assay is compatible with computer analysis programs.

However optimal conditions must be elucidated for each cell line and it must be taken into account that the assay depends upon both the number of cells present and mitochondrial activity per cell. MTT is only cleaved by active mitochondria and certain compounds may selectively affect the mitochondria of the cells resulting in a greatly overestimated level of activity. The assay is therefore not suitable for use with cells with low mitochondrial activity. It is also essential that sufficient time is allowed for cell death and loss of dehydrogenase activity to occur and that cells are in the exponential growth phase at the time of the assay.

### 3.7.5 Trypan blue exclusion as an index of cell viability

The ability of cells to exclude trypan blue and other dyes is frequently used as an index of cell viability and several authors have used dye exclusion techniques to estimate cell kill following exposure to cytotoxic drugs (Durkin *et al.*, 1979). However certain factors must be taken into account such as that sufficient time must elapse before lethally damaged cells lose their membrane integrity following a cytotoxic insult and this will differ between cell types. During this time surviving cells may proliferate and this along with the fact that some lethally damaged cells may undergo an early disintegration and therefore are not present to be stained at the end of the recovery period may lead to an underestimate of cell kill if the results of the assay are based upon percent viability as in this project.

Weisenthal *et al.*, (1983) reported that using a constant number of duck red blood cells as an internal standard allowed for direct comparison of the number of surviving cells present in control and drug-treated cultures so increasing the sensitivity of the dye exclusion method in detecting cell kill.

Another potential problem with monolayer systems is that nonviable cells which detach from the culture flask will be washed off and therefore lost. This was compensated for to some extent in this project with the trypan blue assay as the incubation medium containing the AFB<sub>1</sub> was retained along with any HBSS used in the wash steps and cells pelleted down and taken into account when total cell number was calculated for each well. This however was not possible for the MTT assay as nonviable cells which had detached were inevitably washed away prior to addition of medium and the subsequent recovery period.

### 3.7.6 Conclusion

The results of the above analyses showed that the most suitable diluent for the AFB<sub>1</sub> was unsupplemented DMEM since this had little or no detrimental effect on cell viability during the assay, compared to HBSS or PBS which led to an overall loss of 30% of initial cell number, most of which occurred during the 30 minute incubation period. Cell loss due to trypsinisation of the monolayer was between 1% and 7% although this was not relevant for cytotoxicity assays where trypsinisation of the monolayer was not required, such as for the MTT assay or annexin V ELISA technique. A microsome concentration of 1% (v/v) of the total assay volume was adopted as the optimum concentration for the microsomes, since at higher concentrations cells showed an increase in PS exposure, which is an indication of the occurrence of either apoptosis or necrosis. An optimum incubation period of 1 hour, with a concentration of 5mg/ml MTT in PBS is recommended for use with the JB1 and RLE cell lines, for measuring percentage cell survival rates.

The toxic effects of AFB<sub>1</sub> on the JB1 and RLE cell lines have been demonstrated, along with the relative resistance of the JB1 cell line compared to the more sensitive RLEs. The RLE cell line appears to activate the AFB<sub>1</sub> to the epoxide in the absence of an extrinsic microsomal activating system, albeit at much lower levels than when quail liver microsomes are present, and is also more sensitive to the toxic effects of quail liver microsomes.

Chapter 4

Analysis of Gene Expression

## 4 Analysis of Gene Expression

### 4.1 Introduction

The differences between the JB1 and RLE cell lines, with respect to AFB<sub>1</sub> cytotoxicity have been demonstrated in the previous chapter. The JB1 cell line shows a resistance to the toxic effects of AFB<sub>1</sub>, whereas the RLE cell line is much more sensitive over the same dose range. The aim of this chapter was to investigate and compare expression of certain genes involved in resistance to AFB<sub>1</sub>, in particular the GST A3 and A5 subunits, the AFB<sub>1</sub>-aldehyde reductase (rAFAR1), and *mdr1b* (which codes for the p-glycoprotein pump). In this way the genotype of both cell lines could be elucidated with respect to the afore mentioned genes, and their importance in protecting against the toxic effects of AFB<sub>1</sub> could be ascertained.

RNA was extracted from JB1 and RLE cell lines before and after treatment with AFB<sub>1</sub> (method 2.3), and from tissue obtained from ethoxyquin-treated rats (method 2.8.2). In addition, RNA from AFB<sub>1</sub>-resistant cells (obtained from clonal growth of surviving JB1 and RLE cells after 1 hour incubation with 5µg/ml AFB<sub>1</sub> plus 1% microsomes) (method 2.3.1) was obtained.

All RNA samples were subjected to RT-PCR analysis (method 2.9) to look for the presence of GST A3 and A5 subunit, aldehyde reductase and *mdr1b* (p-glycoprotein) mRNA. Actin, a house-keeping gene, was used as a positive control in all cases.

### 4.2 Results

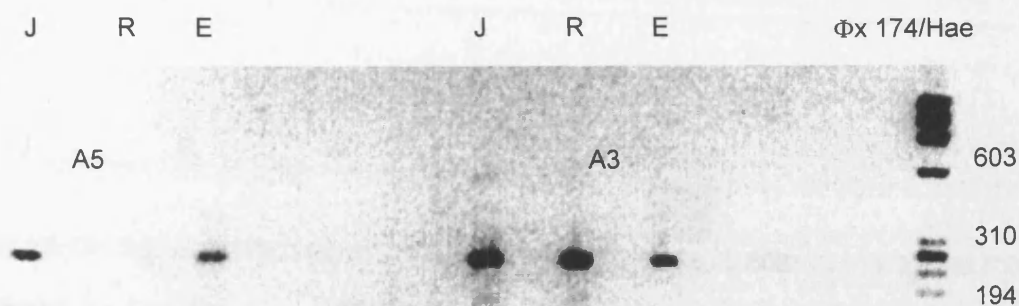
Results showed that the AFB<sub>1</sub>-resistant JB1 cell line, was positive for both GST A3 and A5 subunit mRNA (figure 4.1), as well as for rAFAR1 (figure 4.2) and *mdr1b* (figure 4.3), i.e these genes are constitutively expressed in this cell line.



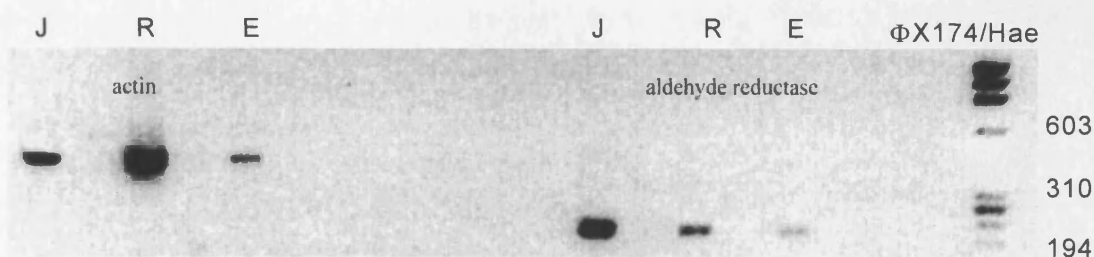
The AFB<sub>1</sub>- sensitive RLE cell line however, was positive for the GST A3 (figure 4.1), rAFAR1 (figure 4.2) and *mdr1b* mRNA (figure 4.3), but did not constitutively express the A5 subunit (figure 4.1) prior to AFB<sub>1</sub> treatment..

The GST A5 subunit mRNA was present in the AFB<sub>1</sub>-sensitive cells however, after treatment with activated AFB<sub>1</sub> (figure 4.4).

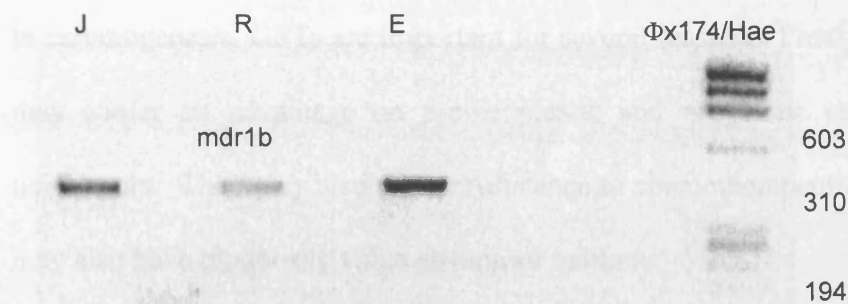
The RNA from ethoxyquin treated rats was also found to possess mRNA for all of the genes investigated, including the GST A5 subunit (figures 4.1, 4.2, 4.3). The reader is referred to table 2.1 for information on primers used and length of PCR product. All samples were positive for actin (figure 4.2). The rAFAR1 and *mdr1b*, gave a stronger band on gel electrophoresis for the JB1 cell line compared with the RLE cell line, which may indicate a greater expression of these genes in the AFB<sub>1</sub>-resistant cell line.



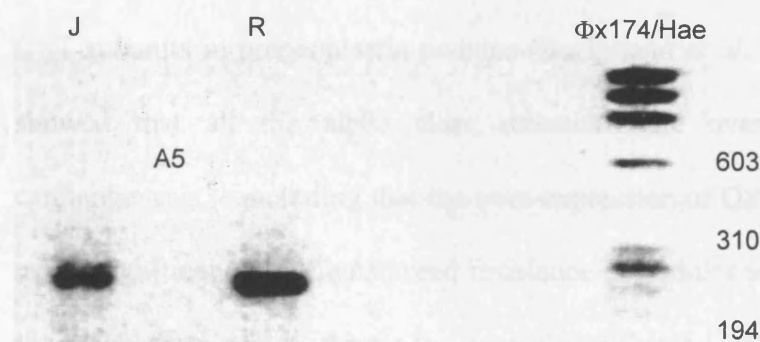
**Figure 4.1:** RT-PCR products using GST A3 (247bp) and A5 (245) primers on mRNA samples from JB1 (J) and RLE (R) cell lines, and ethoxyquin-treated rat liver tissue (E). JB1 is positive for both genes, as is ethoxyquin-treated rat liver. The RLE cell line is positive for GST A3 and negative for GST A5.



**Figure 4.2:** RT-PCR products using actin (389bp) and rAFAR1 (aldehyde reductase) (205bp) primers on mRNA samples from JB1 and RLE cell lines, and ethoxyquin-treated rat liver. All samples are positive for both genes. The rAFAR1 band for the JB1 cell line is stronger compared with the RLE cell line, which may indicate a greater expression of this gene in the AFB<sub>1</sub>-resistant cell line.



**Figure 4.3:** RT-PCR products using *mdr1b* (325bp) primers on mRNA samples from JB1 and RLE cell lines, and ethoxyquin-treated rat liver tissue. All samples are positive for this gene. The *mdr1b* band for the JB1 cell line is stronger compared with the RLE cell line, which may indicate a greater expression of this gene in the AFB<sub>1</sub>-resistant cell line.



**Figure 4.4:** RT-PCR products using GST A5 (245bp) primers on mRNA samples from JB1 and RLE cell lines after treatment with AFB<sub>1</sub>. Both samples are positive for this gene i.e. it is induced in the RLE cell line by treatment with AFB<sub>1</sub>.

### 4.3 Discussion

#### 4.3.1 Protective Effect of the GST A5 subunit

In carcinogenesis, GSTs are important for several reasons. Firstly, it appears likely that they confer an advantage on pre-neoplastic and neoplastic cells over their normal neighbours. They may also confer resistance to chemotherapeutic drugs and in addition may also have diagnostic value as tumour markers.

The GSTs represent a highly complex supergene family of proteins that until recently were known to only comprise six subclasses:- alpha, mu, pi, theta, sigma and zeta (Hayes and Pulford, 1995). However, a seventh subclass, omega has now been identified (Board *et al.*, 2000). Coles *et al.*, (1985) reported that among GSTs only the class alpha (Ya, Yc and Yk) are responsible for the metabolism of activated AFB<sub>1</sub> and its conjugation with GSH. It appears likely that the expression of class alpha GST within AFB<sub>1</sub>-resistant nodules is of critical importance in their ability to resist the toxic effects of AFB<sub>1</sub>, and previous work has shown an elevated expression of alpha class GST subunits in preneoplastic nodules (Buchmann *et al.*, 1985). Hayes *et al.*, (1990c) showed that all the alpha class subunits were overexpressed in AFB<sub>1</sub>-induced carcinogenesis, concluding that the over-expression of GST A1 and A2 subunits was of major significance in the acquired resistance of nodules to AFB<sub>1</sub>. In addition, hamster liver, which has been shown to contain significant levels of AFB<sub>1</sub>-GSH conjugating activity (Lotlikar *et al.*, 1984; Raj *et al.*, 1984), appears to contain substantial amounts of a Yc-type subunit (Hayes and Mantle, 1986) which represents the major alpha class GST polypeptide in this tissue (Hayes, 1988).

The expression of the GST A5 subunit gene, a member of the alpha class Yc subfamily, in the AFB<sub>1</sub>-resistant JB1 cell line and not in the AFB<sub>1</sub>-sensitive RLE cell line, indicates that it confers a protective effect on rat liver hepatocytes. The inducibility of the GST

A5 subunit is also shown by its expression in the RLE cell line after treatment with AFB<sub>1</sub>. This is in accordance with the results of Hayes *et al.*, (1991a) who reported that the inducibility of the rat GST A5 subunit was responsible for high activity towards AFB<sub>1</sub> 8,9-epoxide. Further evidence for the protective role of the GST A5 subunit in preventing AFB<sub>1</sub>-induced carcinogenesis in the rat comes from comparisons of male and female livers. Female rats display higher constitutive levels of A5 protein with a corresponding higher rate of AFB<sub>1</sub>-epoxide detoxification (Meyer *et al.*, 1993).

The potential for xenobiotic compounds to protect animals from carcinogenesis has been explored and developed over recent years. Pearson *et al.*, (1983) reported increased GST, rate of synthesis and mRNA for GST proteins in mouse hepatic cytosols in response to the anticarcinogenic antioxidants 2(3)-tert-butyl-4-hydroxyanisole (BHA), ethoxyquin and disulfiram. Cabral and Neal (1983) also reported the inhibitory effects of ethoxyquin on the carcinogenic action of AFB<sub>1</sub> in rats, which coincided with an increase in hepatic enzyme activities. The means by which such chemoprotectors reduce the incidence of chemical carcinogenesis is diverse and multifactorial, but it is likely that the levels of xenobiotic metabolising enzymes are altered by many of these chemicals, thus increasing the capacity for carcinogen detoxification and excretion.

#### **4.3.1.1 Induction of GST A5 and AFAR in Protection Against AFB<sub>1</sub> Toxicity**

The list of chemicals able to induce hepatic GST activity is large and includes GST substrates, chemical carcinogens, tumour promoters and synthetic phenolic antioxidants. Manson *et al.*, (1997) examined a range of potential chemoprotective agents for their ability to elevate both phase I and phase II enzymes, including A5 and (rAFAR1), and reported that butylated hydroxytoluene, indole-3-carbinol, phenethyl isothiocyanate and ethoxyquin were potent bifunctional inducing agents. Oltipraz was found to be a weak inducer of phase I enzymes but was a potent inducer of phase II enzymes. Their finding

that GST A5 was important in the detoxification of AFB<sub>1</sub> in rodents and was inducible by ethoxyquin was also in accordance with the results of this thesis. In a similar study, McLellan *et al.*, (1994) showed that increased levels of A5 subunit in rat liver coincided with a substantial rise in GST activity towards AFB<sub>1</sub> 8,9-epoxide and that ethoxyquin was an effective inducing agent for the GST A5 subunit.

In view of this apparent importance of the rat A5 subunit in providing protection against AFB<sub>1</sub>, identification of factors controlling both basal and inducible expression of this subunit are important. The spectrum of compounds that elevate the levels of specific GST isoenzymes suggests the existence of several distinct induction mechanisms. Rushmore *et al.*, (1990a) identified a region in the 5' flanking sequence of the GST Ya subunit that contains a xenobiotic responsive element (XRE) which is responsible in part for the basal and inducible expression of the Ya subunit gene by planar aromatic compounds such as  $\beta$ -naphthoflavone and 3-methylcholanthrene (3-MC). In addition Rushmore *et al.*, (1991) identified the antioxidant responsive element (ARE) in the 5'-flanking region of the rat GST Ya subunit gene suggesting that the ARE is responsive to reactive oxygen species and thus may represent part of a signal transduction pathway that allows eukaryotic cells to sense and respond to oxidative stress. Paulson *et al.*, (1990) by means of *in situ* hybridisation, showed that all hepatocytes in uninduced mice express GST Ya mRNA, but that 3-methylcholanthrene can induce 15- to 20-fold the expression of this subunit in pericentral cells. By analysing the upstream regions of the GST Ya gene, four nuclear protein-binding sites were identified in this enhancer region; one site required for inducible expression of the Ya subunit being bound by a 3-MC-induced protein.

Pulford and Hayes (1996) have putatively identified an ARE in the 5'-flanking region of GST A5 which may mediate protection against AFB<sub>1</sub>. More recently, Hayes *et al.*, (1998) have reported on the regulation of GST A5 by chemopreventive agents. They

report that since GST A5 possesses peroxidase activity and has the ability to conjugate GSH with various epoxides as well as 4-hydroxynonenal (a major product of lipid peroxidation) this suggests that A5 serves an important role as an antioxidant enzyme *in vivo*. In accordance with the results of Manson *et al.*, (1997) they report that oltipraz and ethoxyquin are potent inducing agents of A5 and also suggest that coumarin is a particularly potent A5 inducer. Furthermore, Kelly *et al.*, (2000b) report that, in rats, coumarin is a major inducer of AFAR and GST A5 subunit, and hence a coumarin containing diet can provide protection against the initiation of AFB<sub>1</sub> hepatocarcinogenesis. However, it must also be noted that a marked species variation in chemoprotection by coumarin has been demonstrated by Goeger *et al.*, (1998).

#### 4.3.1.2 Chemoprotective Value of Oltipraz

Oltipraz has recently been considered for human use to protect against AFB<sub>1</sub>-induced hepatocarcinogenesis based on its proven protective effect in rats, as pretreatment with oltipraz has been shown to protect rats from developing liver cancer (Kensler *et al.*, 1985; Meyer *et al.*, 1993). More recently, Li *et al.*, (2000) reported that oltipraz reduces AFB<sub>1</sub> risk biomarkers in the tree shrew in a manner similar to that observed in rats and humans.

Studies have been undertaken to use oltipraz as a chemoprotective agent in Phase II clinical trials in AFB<sub>1</sub>-exposed humans in the Qidong region in China (Kensler, 1994; Zhang *et al.*, 1997; Jacobson *et al.*, 1997; Kensler *et al.*, 1998b; Wang *et al.*, 1999), the outcome of which has highlighted the feasibility of induction of various phase II detoxification enzymes in humans as a chemopreventative strategy.

Buetler *et al.*, (1996) showed that the GST A5 steady state mRNA level was induced 5-fold by oltipraz treatment and that this corresponded with the level of the A5 protein and AFB<sub>1</sub>-epoxide conjugating activity in rat liver. They concluded that the A5 subunit may

contribute significantly to protection against AFB<sub>1</sub>-induced carcinogenesis in the rat, but that there was no evidence from their investigations, or others to date, of A5 -like GST isozyme with high AFB<sub>1</sub>-epoxide conjugating activity in human liver.

However the value of oltipraz treatment in the prevention of AFB<sub>1</sub>-induced hepatocarcinogenesis in humans is questionable since as well as induction of an A5 subunit the CYP 1A2 steady-state mRNA level has been shown to be increased 3-fold in oltipraz treated rats. Although CYP 1A2 does not appear to play a part in AFB<sub>1</sub> activation in rat liver (CYP 3A2 being the major rat P450 capable of activating AFB<sub>1</sub> in the rat) it has been identified as the major human P450 capable of activating AFB<sub>1</sub> at low concentrations (Buetler *et al.*,1996). Therefore, oltipraz treatment could possibly lead to an increase in activation of AFB<sub>1</sub> in humans by induction of CYP 1A2, whilst at the same time not affording any protection against the AFB<sub>1</sub>-epoxide by increased GST-mediated detoxification. Further studies are required to be carried out on the safety of oltipraz treatment in humans before the benefits of its role as a chemoprotective agent can be confirmed.

Alternatively, oltipraz may provide a different mechanism of protection in human liver which does not involve GST isozymes. Such a mechanism may be provided by rAFAR1, which metabolises the dialdehyde form of AFB<sub>1</sub>-dihydrodiol formed via hydrolysis of the epoxide. The study carried out by Manson *et al.*, (1997) identified both A5 and rAFAR1 as uniquely important in detoxification of AFB<sub>1</sub> in rodents. However, this was not in accordance with the results found in this study as the expression of rAFAR1 was found in both the AFB<sub>1</sub>-resistant JB1 cell line and the AFB<sub>1</sub>-sensitive RLE cell line when RNA extracts were subjected to RT-PCR analysis, although to differing degrees. However, RT-PCR analysis in this thesis was qualitative and not quantitative, and it remains to be seen whether levels of rAFAR1 in the AFB<sub>1</sub>-



sensitive RLE cell line are comparatively much lower than in the AFB<sub>1</sub>-resistant JB1 cell line.

McLellan *et al.*, (1994) reported that the induction of rAFAR1 by ethoxyquin is likely to make a major contribution to the anticarcinogenic effects of ethoxyquin in rats. It should also be noted that ethoxyquin has been shown to enhance the ability of liver microsomes to detoxify AFB<sub>1</sub> to AFQ<sub>1</sub> and AFM<sub>1</sub> (Mandel *et al.*, 1987).

However, Hayes *et al.*, (1993) demonstrated that there are notable differences in the control of A5 and rAFAR1, reporting that foetal rat liver contains high levels of A5 but very little rAFAR1. The findings of McLellan *et al.*, (1994) showed tissue-specific regulation to be a further example of differences in regulation of A5 and rAFAR1 since high levels of A5 found in the epididymis did not correspond with the levels of rAFAR1 in this tissue. In addition, substantial levels of rAFAR1 were found in the kidney whereas A5 was undetectable. This raises the question of whether the mechanisms governing drug induction of these AFB<sub>1</sub>-detoxifying enzymes are similar for both A5 and rAFAR1.

#### **4.3.2 Chemoprotective Function of Efflux pumps**

Once formed, elimination of GSH S-conjugates from cells is important because their accumulation can lead to inhibition of GSTs and GSH reductase, which may have deleterious consequences with respect to cellular function and viability. The AFB<sub>1</sub>-epoxide-GSH conjugates are actively pumped from the cell by an unidentified ATP-dependent export pump or pumps, but the protein or proteins responsible for this have not yet been physically or chemically identified. One possible candidate is the 170 kDa P-glycoprotein (Pgp) coded for by the rat *mdr1b* gene. The results of this thesis showed that it was present in both JB1 and RLE cell lines though to a lesser extent in the RLE cell line. It also appeared to be more strongly present in the ethoxyquin-treated rat liver

cell line. This is in accordance with work published by other authors such as Lee *et al.*, (1995), who reported that class II Pgp, expressed at a very low level in normal liver, was overexpressed in several models of rat liver carcinogenesis, concluding that an association between Pgp mRNA and cytoskeletal elements may underlie the mechanism that regulates class II stability.

Pgp has been shown to mediate multidrug resistance in many tumour cell lines. Bradley *et al.*, (1992) showed that distinct changes occurred in the expression of Pgp glycoprotein during stepwise liver cancer in rats, and that in contrast to  $\gamma$ -glutamyl transpeptidase and GST P, whose expression appeared to correlate with early steps of liver carcinogenesis, Pgp was associated with a more progressed malignant phenotype in liver carcinogenesis.

Silverman and Hill, (1995) reported that the expression of the rat *mdr1b* gene was increased in primary rat hepatocytes by exposure to carcinogens including AFB<sub>1</sub>, and that this was due to an increase in transcription. However, their data suggested that the carcinogen induction of *mdr1b* expression in rats and human MDR1 is mediated through sequences that overlap or are identical to the basal promoter element concluding that it is likely that several factors interact to regulate this gene.

Loe *et al.*, (1997) have investigated the role of another multidrug resistance protein, the 190 kDa MRP protein which belongs to the same family of ABC transporter genes as the MDR1 Pgp. Their results showed that using membrane vesicles from MRP-transfected cells, GSH conjugates of both the *endo*- and *exo*- isomers of AFB<sub>1</sub> 8,9-epoxide were transported in an ATP-dependent manner. In contrast, membrane vesicles from Pgp-overexpressing cells showed very low levels of transport and they concluded that Pgp does not play a significant role in either unmodified or conjugated GSH-conjugated AFB<sub>1</sub>, suggesting that a chemoprotective function may also exist for MRP.

### 4.3.3 Improving the Quality of RNA Starting Material

The sensitivity of the RT-PCR technique can be improved by the quality of RNA used as starting material. The method of RNA extraction employed in this project, that of Chomczynski and Sacchi (1986), involved disruption of the cell membrane by GTC and sarcosyl to allow for the release of RNA which was separated from any DNA or proteins by centrifugation with aqueous phenol. This has the disadvantage that it is fairly time consuming and that the GTC and  $\beta$ -mercaptoethanol involved are highly toxic. An alternative method for extraction of RNA from cell or tissue samples is that of the dynabeads<sup>®</sup> mRNA direct kit available from Dynal. This allows isolation of mRNA directly from crude extracts of animal tissues and cells using magnetic dynabeads. The advantages of this technique are that it is quick and efficient and the beads are reusable therefore making the procedure cost effective. Another RNA extraction kit is available from Genosys Biotechnologies Ltd, which allows for the additional isolation of DNA and proteins. The isolation is achieved through the improvement of the method of Chomczynski and Sacchi (1986) but has the advantage that the entire procedure can be performed in one hour. Again DNA and proteins can be isolated by this procedure. An advantage is that the determination of DNA content in samples used in studies of gene expression creates the possibility of normalising the results of gene expression studies based on DNA content instead of on the more variable total RNA content.

### 4.3.4 Alternative Methods for Identification of GSTs

There are many other methods for demonstrating the expression of the particular genes investigated in this thesis. For instance, where antibodies against the gene products are available, immunohistochemical techniques can demonstrate the presence of these proteins in tissue sections (Lauro *et al.*, 1989; Cairns *et al.*, 1992). This has the

advantage that localised distribution of the gene products can be detected which is particularly important in the liver since metabolic zonalisation means that enzyme concentrations vary across the acinus and with different cell types. This is also significant in heterogeneous tumours composed of a variety of cell types, and this is obviously a disadvantage with using established hepatocyte cell lines, as in this project, which are composed of the same cell type. Harrison *et al.*, (1989) demonstrated a heterogeneity of GST isoenzyme distribution in renal carcinomas which suggests that different parts of the same tumour may have varying responses to cytotoxic chemotherapy. However, the problems of cross-reactivity both between and within classes of GST should be taken into account and monoclonal antibodies should be employed where they are available.

Additional methods for demonstration of GSTs include *in situ* hybridisation studies (Evarts *et al.*, 1987) using single stranded mRNA probes to detect mRNA levels for the protein of interest within single cells in tissue sections. Northern blotting techniques carried out on RNA extractions from tissue samples and using labelled cDNA probes have also been used to demonstrate the expression of GST genes (Moscow *et al.*, 1989), and Coates *et al.*, (1991) published a method for detecting low copy mRNAs using PCR methods applied to paraffin embedded fixed tissue sections.

The characterisation of GSTs is also made possible by the availability of affinity chromatography gels to which these enzymes bind, in particular glutathione-agarose matrices (Hayes *et al.*, 1990c). GSTs have also been defined by their elution behaviour from 2-(diethylamino)ethyl (DEAE)-cellulose and carboxymethyl (CM)-cellulose (Hayes *et al.*, 1992) and by reverse phase high pressure liquid chromatography and sodium dodecyl-sulphate (SDS)-PAGE (Hayes *et al.*, 1990c).

#### **4.3.5 Conclusion**

The protective effect of the GST A5 subunit towards AFB<sub>1</sub> has been demonstrated in this chapter, by its constitutive expression in the AFB<sub>1</sub>-resistant JB1 cell line and its absence in the AFB<sub>1</sub>-sensitive RLE cell line. However, it has been shown to be inducible by treatment with AFB<sub>1</sub> in the RLE cell line, and by ethoxyquin treatment. All of the other genes investigated: GST A3, rAFAR1 and mdr1b, were found to be constitutively expressed in both cell lines. The rAFAR1 and mdr1b appeared to be more strongly expressed in the AFB<sub>1</sub>-resistant JB1, although it is necessary to further quantitate expression of these genes.

Chapter 5

Induction of Cell Death in JB1 and RLE Cell Lines by AFB<sub>1</sub>

## **5 Induction of Cell Death in JB1 and RLE Cell Lines by AFB<sub>1</sub>**

### **5.1 Introduction**

In chapter 3, the cytotoxic effect of AFB<sub>1</sub> on the JB1 and RLE cell lines was demonstrated and compared. The aim of this chapter was to establish whether cell death seen after treatment with an acute dose of AFB<sub>1</sub> (method 2.3) was attributable to either necrosis or apoptosis (the reader is referred section 1.6). This was attempted in several ways using annexin V conjugated with FITC as marker for PS exposure; firstly by way of the ELISA technique developed in this thesis in conjunction with the MTT assay (method 2.7.2); secondly by carrying out flow cytometry on the cell suspension after trypsinisation (method 2.7.1); and, thirdly by subjecting the cell monolayer to analysis using the laser scanning cytometer (LSC) (method 2.7.4). Cells were visualised after staining with annexin V-FITC and propidium iodide using the confocal fluorescence microscope and photographed (method 2.7.3).

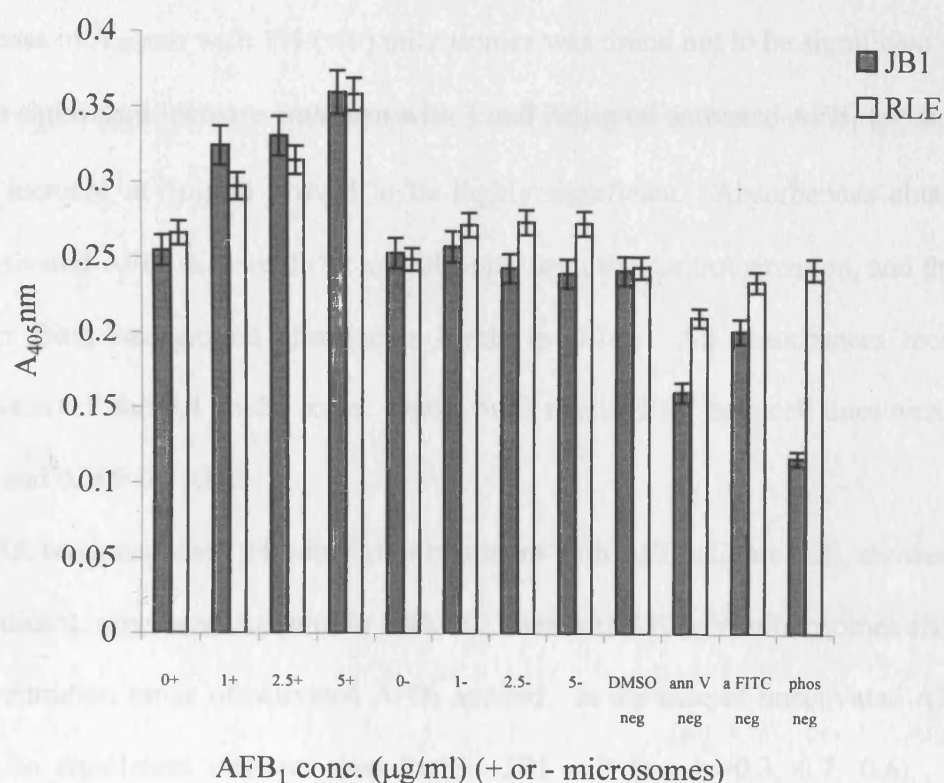
### **5.2 Detection of PS exposure by the annexin V ELISA technique**

Cells were treated with a concentration range of 0, 1, 2.5 and 5µg AFB<sub>1</sub>/ml, in the presence and absence of an external microsomal activating system (1% v/v) on a set of duplicate 96-well plates, 48 hours after seeding out. After either 2 or 24 hours recovery, the annexin V ELISA technique (method 2.7.2) was carried out on one plate of each set and the MTT assay carried out on the other (method 2.6.2). With both methods, a dose response effect was seen for both cell lines corresponding to an increase in activated AFB<sub>1</sub> over the range applied. Results given here were an average of 8 sets of data. For percentage cell survival values see sections 3.6.2 (24 hours) and 5.2.2 (2 hours).

### 5.2.1 Analysis of ELISA data

No significant difference in  $A_{405\text{nm}}$  (for a 95% confidence interval using the two sample t-test,  $n = 8$ ) was seen in the 'medium only' control when compared with the '0.05% (v/v) DMSO only' ( $0\mu\text{g}$  AFB<sub>1</sub> minus microsomes) control ( $p = 0.2$ ) in any of the assays carried out, and further mention of the control will refer to the 'medium only' control.

Results of the annexin V ELISA carried out 2 hours after treatment (figure 5.1) showed



**Figure 5.1:** Absorbance readings ( $A_{405\text{nm}}$ ) for the annexin V-ELISA technique carried out on JB1 and RLE cell lines. Results were obtained 2 hours after treatment with a concentration gradient of AFB<sub>1</sub> (0–5  $\mu\text{g}/\text{ml}$ ) and a 1% (v/v) microsomal activating system (AFB<sub>1</sub><sup>+</sup>). An increase in annexin V-FITC binding was seen for both cell lines with activated AFB<sub>1</sub>. Values obtained with unactivated AFB<sub>1</sub> (AFB<sub>1</sub><sup>-</sup>) for the JB1 cell line were significantly different from background levels (annexin V neg) indicating a low level of PS exposure in these cells.



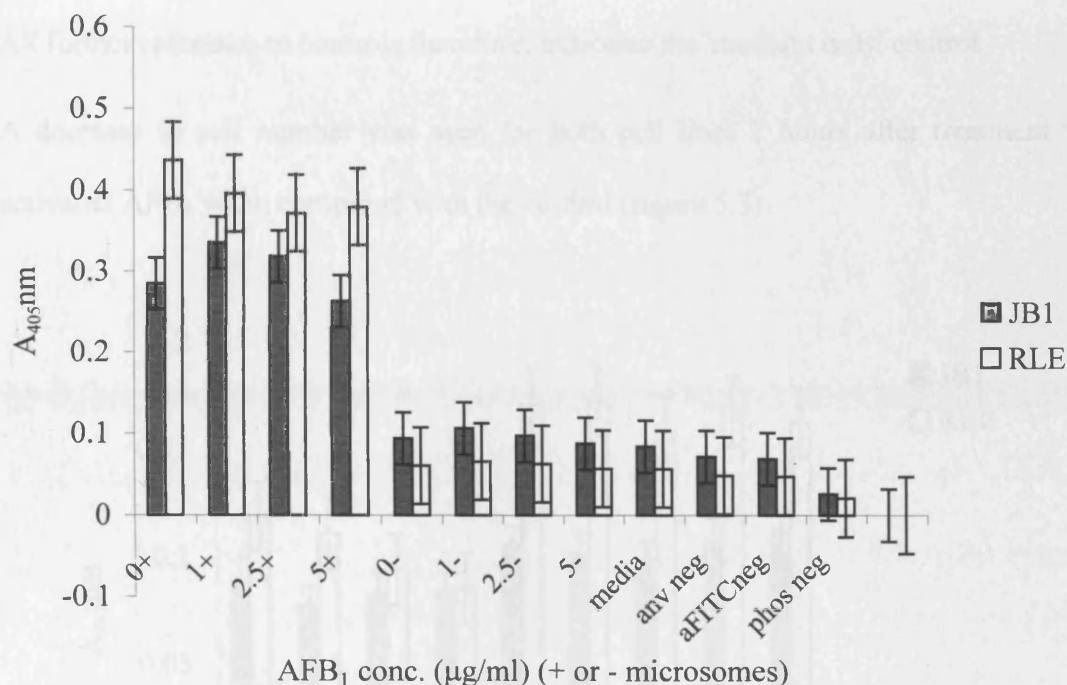
an increase in A<sub>405nm</sub> in a dose responsive manner for both cell lines on addition of activated AFB<sub>1</sub>, ( i.e. in the presence of 1% (v/v) microsomes), when compared with the control situation. In the JB1 cell line, the increase in A<sub>405nm</sub> was found to be statistically significant over the range of 1-5µg/ml of activated AFB<sub>1</sub> (p=0.01, 0.007, 0.003 respectively), but no significant difference was seen with 1% (v/v) microsomes alone. No significant increase in A<sub>405nm</sub> was seen, when compared with the control, for unactivated AFB<sub>1</sub>, but the absorbance obtained for the control differed significantly from background levels (p= 0.01).

A similar trend was seen with the RLE cell line 2 hours after treatment with AFB<sub>1</sub>. The increase in A<sub>405nm</sub> with 1% (v/v) microsomes was found not to be significant (p= 0.06), but a significant increase was seen with 1 and 2.5µg/ml activated AFB<sub>1</sub> (p= 0.01, 0.03). The increase at 5µg/ml proved to be highly significant. Absorbances obtained with unactivated AFB<sub>1</sub> did not differ significantly from the control situation, and this did not differ from background absorbance levels (p=0.14). All absorbances recorded lay between 0.1 and 0.4 on the scale. Background readings for both cell lines were: 0.16 for JB1 and 0.209 for RLE.

ELISA readings taken 24 hours after treatment with AFB<sub>1</sub> (figure 5.2), showed a highly significant increase in A<sub>405nm</sub> for both cell lines with 1% (v/v) microsomes and over the concentration range of activated AFB<sub>1</sub> applied. In the case of unactivated AFB<sub>1</sub>, there was no significant increase seen for the JB1 cell line (p=0.3, 0.7, 0.6). Readings obtained though, were significantly different from background readings (p= 0.007 for control), indicating a low level of PS exposure in these cells. For the RLE cell line, again, no significant difference was seen in absorbance with unactivated AFB<sub>1</sub> compared to the control (p=0.3, 0.7, 0.1), but the difference in absorbance between the control and background levels was highly significant (p=0.00005). Background absorbance readings of 0.069 and 0.047 were obtained for JB1 and RLE cell lines

respectively. Lower readings were obtained overall when compared with the results

obtained 2 hours after treatment; ranging between 0 and 0.5.

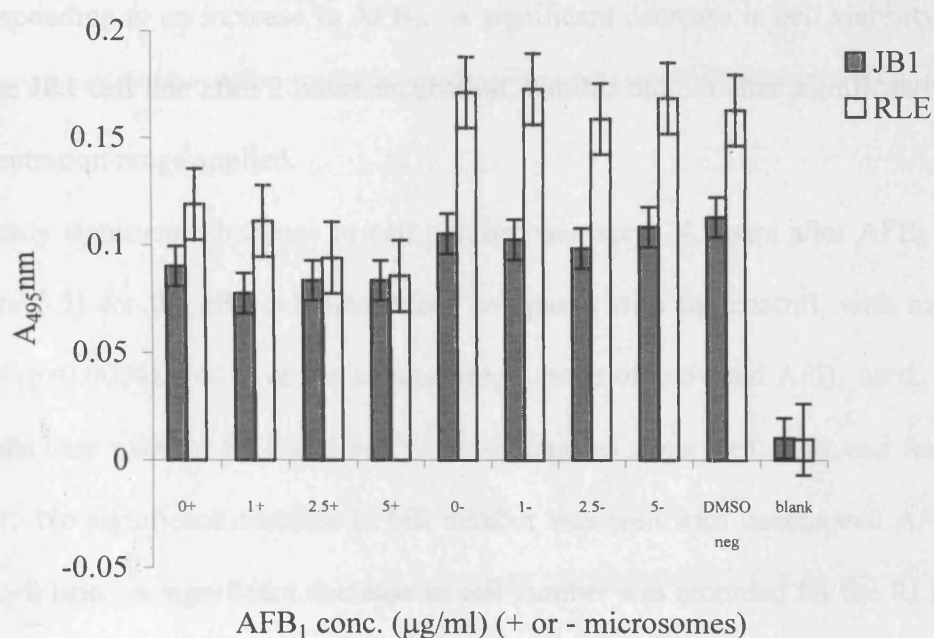


**Figure 5.2:** Absorbance readings ( $A_{405nm}$ ) for the annexin V-ELISA technique carried out on JB1 and RLE cell lines. Results were obtained 24 hours after treatment with a concentration gradient of AFB<sub>1</sub> (0-5 µg/ml) and a 1% (v/v) microsomal activating system (AFB<sub>1</sub><sup>+</sup>). An increase in annexin V-FITC binding was seen for both cell lines with activated AFB<sub>1</sub> and in the presence of 1% (v/v) microsomes alone. Values obtained with unactivated AFB<sub>1</sub> (AFB<sub>1</sub><sup>-</sup>) also differed significantly from background levels (annexin V neg).

### 5.2.2 Analysis of MTT assay results

No statistically significant difference was evident (for a 95% confidence interval using the two sample t-test,  $n = 8$ ) between the '0.05% DMSO only' and 'medium only' controls for either cell line ( $p = 0.98$ , JB1;  $0.68$ , RLE) in any of the assays carried out. All further reference to controls therefore, indicates the 'medium only' control.

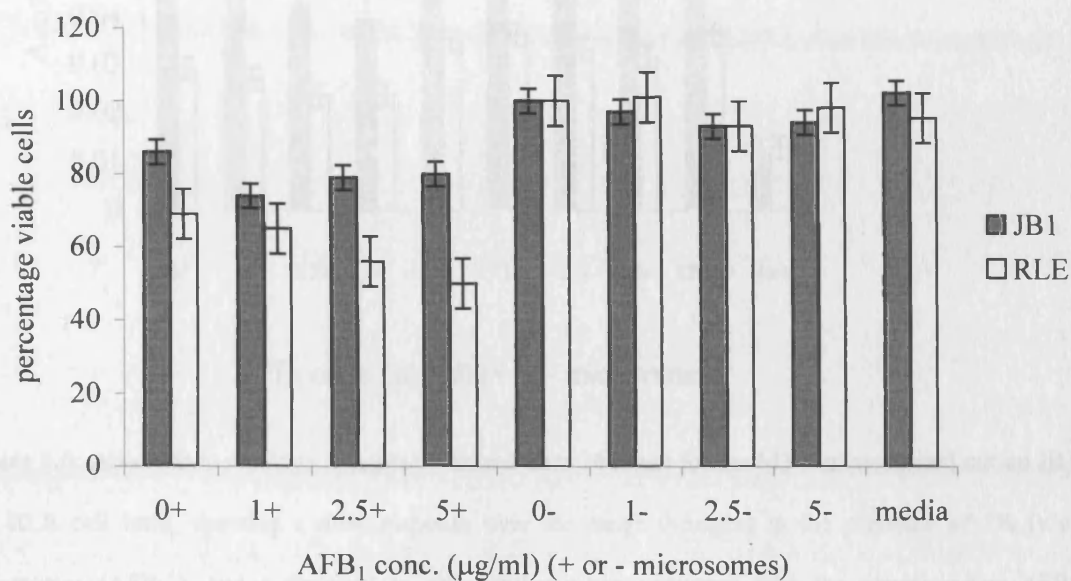
A decrease in cell number was seen for both cell lines 2 hours after treatment with activated AFB<sub>1</sub> when compared with the control (figure 5.3).



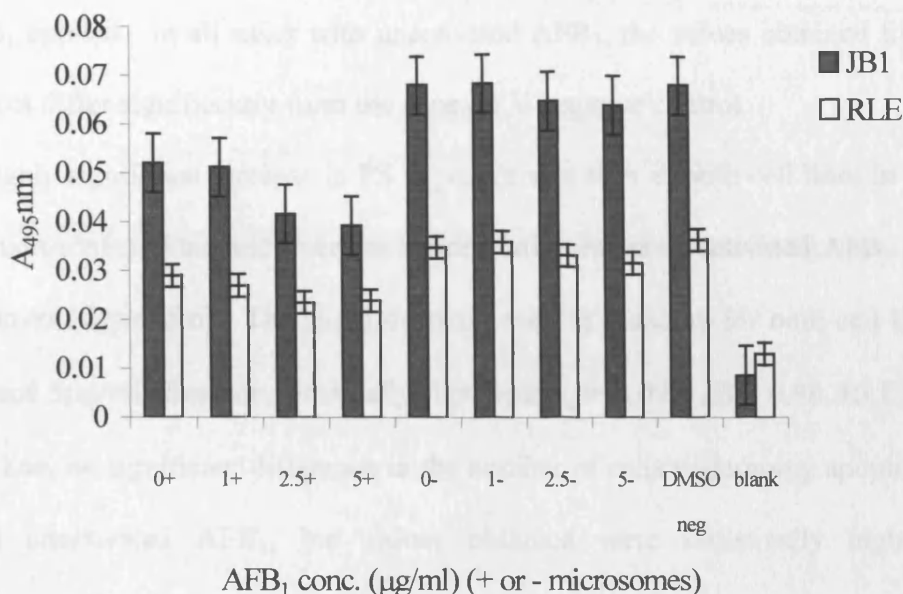
**Figure 5.3:** Absorbance readings ( $A_{495nm}$ ) obtained after 2 hours for the MTT assay carried out on JB1 and RLE cell lines. A statistically significant decrease in viable cell number over the range of 1-5 µg/ml can be seen for the JB1 cell line compared with the control (0 µg/ml AFB<sub>1</sub><sup>-</sup>), whilst the RLE cell line exhibits a dose response over the range 0 - 5 µg/ml in the presence of 1% (v/v) microsomes (AFB<sub>1</sub><sup>+</sup>). There is a decrease in cell number when compared with the corresponding AFB<sub>1</sub> concentration in the absence of a microsomal activating system (AFB<sub>1</sub><sup>-</sup>) for both cell lines. Note the decrease in viable cell number with 1% (v/v) microsomes (0 µg/ml AFB<sub>1</sub><sup>+</sup>) when compared to the control situation (0 µg/ml AFB<sub>1</sub><sup>-</sup>).

In the JB1 cell line the decrease was statistically significant over the range of 1-5µg/ml AFB<sub>1</sub> (p=0.008, 0.047, 0.041 respectively) compared with the control, but there was no significant difference in cell number seen with microsomes alone (p=0.17) or with unactivated AFB<sub>1</sub>. In the RLE cell line however, the decrease in cell number in the presence of a 1% microsome solution was highly significant as were the differences seen over the concentration range of 1-5µg/ml activated AFB<sub>1</sub>. Control readings of 0.105 and 0.171 were obtained for JB1 and RLE cell lines respectively. Percentage cell survival values (figure 5.4) showed a dose response for the RLE cell line after incubation with activated AFB<sub>1</sub>, with a significant increase in cell death (p< 0.001) corresponding to an increase in AFB<sub>1</sub>. A significant decrease in cell viability was seen for the JB1 cell line after 2 hours incubation, but this did not alter significantly over the concentration range applied.

A highly significant decrease in cell number was seen 24 hours after AFB<sub>1</sub> treatment (figure 5.5) for the JB1 cell line, when compared with the control, with microsomes alone (p=0.0004), and over the concentration range of activated AFB<sub>1</sub> used. This was also the case with the RLE cell line with microsomes alone (p=0.009), and for activated AFB<sub>1</sub>. No significant decrease in cell number was seen with unactivated AFB<sub>1</sub> for the JB1 cell line. A significant decrease in cell number was recorded for the RLE cell line for a dose of 5µg/ml unactivated AFB<sub>1</sub> (p=0.038).



**Figure 5.4:** Percentage cell survival values for JB1 and RLE cell lines 2 hours after 30 minutes incubation with AFB<sub>1</sub> over a concentration range of 0-5 µg/ml in the presence (+) and absence (-) of an external microsomal activating system. A statistically significant decrease in percentage viable cell number over the range of 1-5 µg/ml can be seen for the JB1 cell line compared with the control (0 µg/ml AFB<sub>1</sub>), whilst the RLE cell line exhibits a dose response over the range 0 - 5 µg/ml in the presence of 1% (v/v) microsomes (AFB<sub>1</sub><sup>+</sup>). Cell viability was measured using the MTT assay and cell survival was calculated as a percentage of the absorbance reading at 495nm obtained for the control situation.



**Figure 5.5:** Absorbance readings ( $A_{495nm}$ ) obtained after 24 hours for the MTT assay carried out on JB1 and RLE cell lines, showing a dose response over the range 0-5 $\mu$ g/ml in the presence of 1% (v/v) microsomes (AFB<sub>1</sub><sup>+</sup>), and a decrease in cell number when compared with the corresponding AFB<sub>1</sub> concentration in the absence of a microsomal activating system (AFB<sub>1</sub><sup>-</sup>) for both cell lines. Note the decrease in viable cell number with 1% (v/v) microsomes (0 $\mu$ g/ml AFB<sub>1</sub><sup>+</sup>) when compared to the control situation (0 $\mu$ g/ml AFB<sub>1</sub><sup>-</sup>).

### 5.2.3 Analysis of data generated for PS indices

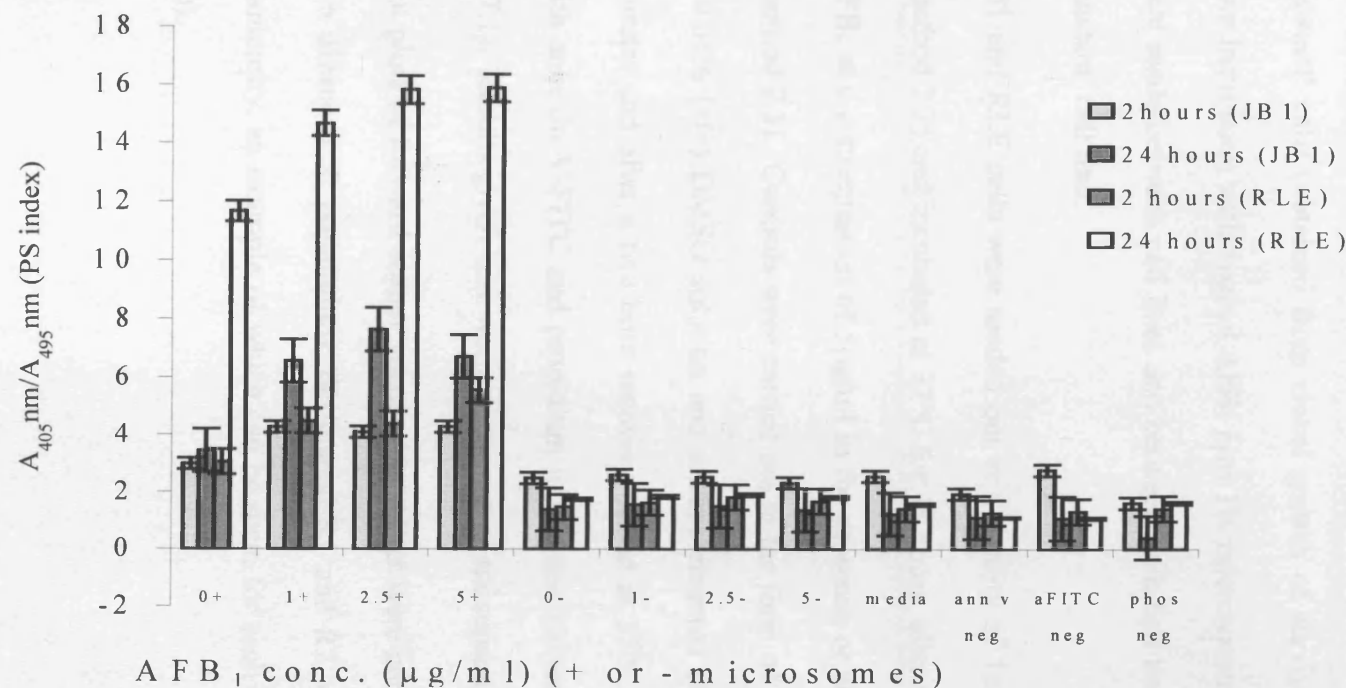
In both cell lines, when the density of viable cells per well was taken into account, the level of annexin V-FITC binding per well was expressed in proportion to the number of cells present ( $A_{405nm}/A_{495nm}$ ), giving a 'PS index' for each set of results.

An increase in PS exposure, corresponding to an increase in dose of activated AFB<sub>1</sub> was apparent after 2 hours (figure 5.6). In the JB1 cell line, differences in apoptotic indices were statistically significant (for a 95% confidence interval using the two sample t-test,  $n = 8$ ) for doses of 1, 2.5 and 5 $\mu$ g/ml AFB<sub>1</sub> ( $p=0.001$ , 0.002, 0.001 respectively), but there was no significant difference with microsomes alone ( $p=0.33$ ). For the RLE cell line however, an increase in PS exposure was evident with microsomes alone ( $p=0.006$ ),

and a highly significant increase was seen over the concentration range of activated AFB<sub>1</sub> applied. In all cases with unactivated AFB<sub>1</sub>, the values obtained for PS indices did not differ significantly from the annexin V negative control.

A highly significant increase in PS exposure was seen in both cell lines in the presence of microsomes alone and over the concentration range of activated AFB<sub>1</sub> applied after 24 hours (figure 5.6). The slight decrease seen in numbers for both cell lines between 2.5 and 5 µg/ml was not statistically significant ( $p=0.099$ , JB1: 0.98, RLE). In the JB1 cell line, no significant difference in the number of cells undergoing apoptosis was seen with unactivated AFB<sub>1</sub>, but values obtained were statistically higher than the background readings. For the RLE cell line however, the absorbance readings obtained with unactivated AFB<sub>1</sub> showed an increase in PS exposure for concentrations of 1 and 2.5 µg/ml compared with the control. This proved to be statistically significant, ( $p=0.006$ , 0.009 respectively). The slight decrease in PS exposure seen at 5 µg/ml AFB<sub>1</sub> was also statistically significant ( $p=0.0008$ ).

The level of PS exposure after treatment with activated AFB<sub>1</sub> in the RLE cell line, was more than twice that of the JB1 cell line. PS indices obtained for the JB1 cell line were around 50% of the values recorded 2 hours after treatment with activated AFB<sub>1</sub>. For the RLE cell line however, values obtained at this time point were similar to those obtained 2 hours after treatment. For both cell lines, values obtained for PS indices after treatment with unactivated AFB<sub>1</sub> dropped significantly after 24 hours.



**Figure 5.6:** PS indices ( $A_{405\text{nm}}/A_{495\text{nm}}$ ) obtained 2 and 24 hours after AFB<sub>1</sub> treatment for the JB1 and RLE cell line. An increase in PS exposure is seen for both cell lines in a dose responsive manner, after 2 and 24 hours, over a concentration range of 0-2.5 $\mu\text{g/ml}$  AFB<sub>1</sub> in the presence of microsomes (AFB<sub>1</sub><sup>+</sup>). A slight decrease in value of PS index was seen with 5 $\mu\text{g}$  AFB<sub>1</sub>/ml. It can also be seen that the RLE cell line is more sensitive to the effects of activated AFB<sub>1</sub> than the JB1 cell line. Of note is the relatively high reading obtained with 1% microsome (v/v) solution (0 $\mu\text{g/ml}$  AFB<sub>1</sub><sup>+</sup>) when compared with the control (0 $\mu\text{g/ml}$  AFB<sub>1</sub><sup>-</sup>).

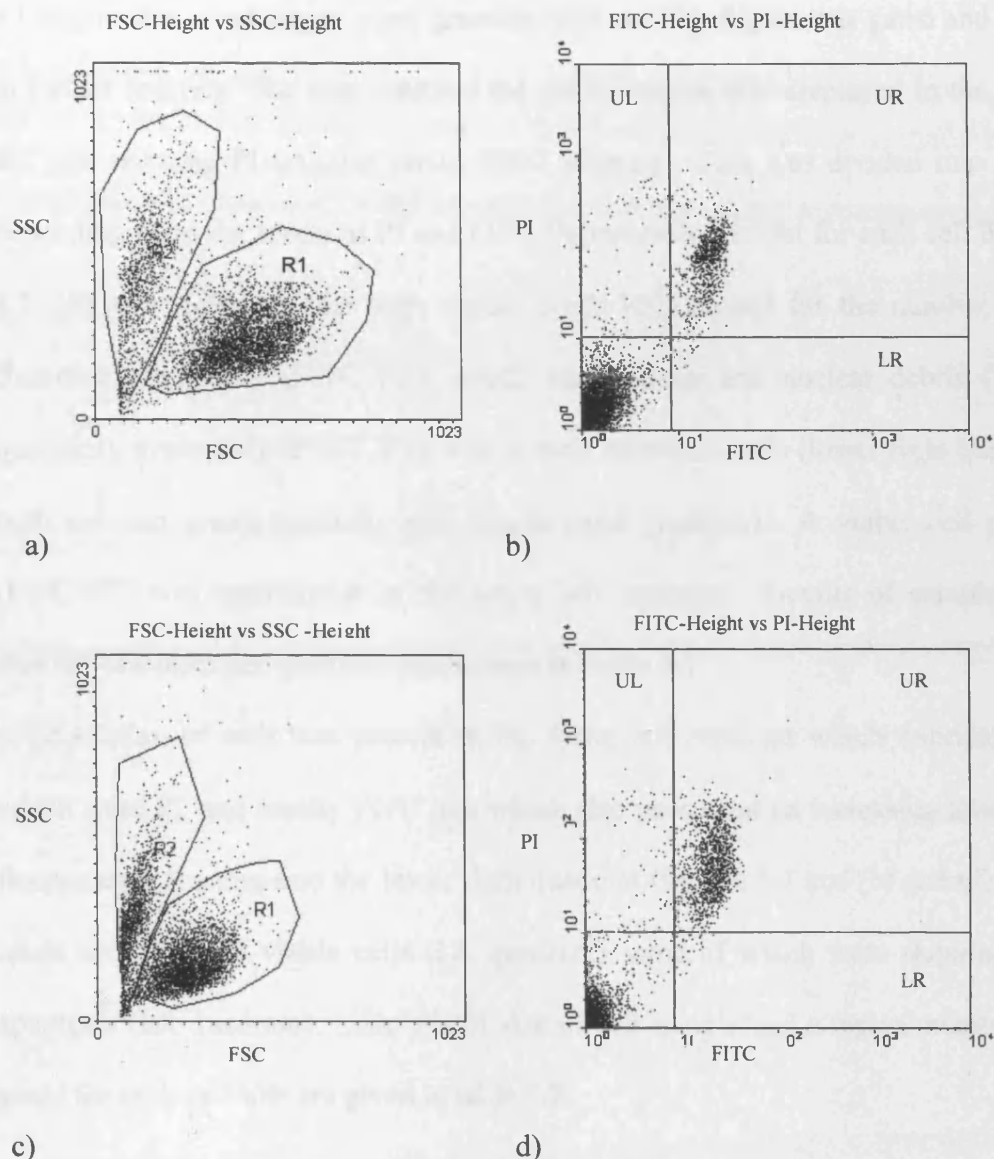


### 5.3 Detection of Apoptosis in Suspensions of RLE and JB1 Cell Lines by Flow Cytometry

This procedure was carried out on JB1 and RLE cell lines, and in addition on 'AFB<sub>1</sub>-resistant' cells (obtained from clonal growth of surviving JB1 and RLE cells after 1 hour incubation with 5µg/ml AFB<sub>1</sub> plus 1% microsomes) (method 2.3.1). Comparisons were made between cell lines and between each cell line and its corresponding 'AFB<sub>1</sub>-resistant' cell line.

JB1 and RLE cells were seeded out at a density of  $1 \times 10^4$  cells/ml into 6-well plates (method 2.2) and incubated at 37°C for 48 hours, after which they were incubated with AFB<sub>1</sub> at a concentration of 5µg/ml in the presence or absence of 1% microsomes (v/v) (method 2.3). Controls were carried out in the form of a 1% microsome (v/v) solution, a 0.05% (v/v) DMSO solution and unsupplemented DMEM. Incubation was for 30 minutes and after a two hour recovery period at 37°C, cells were trypsinised, stained with annexin V-FITC and propidium iodide and subjected to flow cytometry (method 2.7.1). Results given here were an average of six separate sets of data.

Dot plots of forward scatter versus side scatter were generated showing the presence of two distinct cell populations, designated R1 and R2, differentiated by their size and granularity, an example of which can be seen, for each cell line, in figures 5.8 ((a) and (c)).



**Figure 5.7:** Scatter plots for AFB<sub>1</sub>-treated JB1 ((a) and (b)) and RLE ((c) and (d)) cell lines obtained by flow cytometric analysis after treatment with annexin V-FITC and PI. Two separate populations (R1 and R2) were evident in the plot of forward scatter (FSC) vs side scatter (SSC) ((a) and (c)). Region R1 consisted of large cells with a high granularity while R2 was made up of small particles of cellular and nuclear debris. Further analysis of R1 in the form of a second dot plot showing levels of FITC fluorescence (FL1-Height) vs PI fluorescence (FL2-Height) ((b) and (d)), showed that this region consisted mainly of a PI<sup>+</sup>/FITC<sup>+</sup> (necrotic) population (UR quadrant) and a FITC<sup>-</sup>/PI<sup>-</sup> (normal cell) population (LL quadrant). These two populations overlapped slightly to give a FITC<sup>+</sup>/PI<sup>-</sup> (apoptotic) population (LR quadrant). A fourth population of PI<sup>+</sup>/FITC<sup>-</sup> cells (UL quadrant) which consisted of cellular and nuclear debris was also present.

Events contained in R2 represented small particles of cellular and nuclear debris. The R1 region contained larger, more granular cells and this region was gated and subjected to further analysis. The data obtained for the R1 region was displayed in the form of a dot plot showing PI staining versus FITC staining. This was divided into quadrants depending upon the levels of PI and FITC fluorescence present for each cell line (figure 5.7 ((b) and (d))). In this way, values could be obtained for the number of events fluorescing red only (FITC<sup>-</sup>/PI<sup>+</sup>), which was cellular and nuclear debris (upper left quadrant), green only (FITC<sup>+</sup>/PI<sup>-</sup>), which were apoptotic cells (lower right quadrant), or both red and green; necrotic cells (upper right quadrant). A viable cell population (FITC<sup>-</sup>/PI<sup>-</sup>) was represented in the lower left quadrant. Details of sample size and number of events per quadrant can be seen in table 5.1.

A population of cells was present in the lower left quadrant which consisted of cells which were PI<sup>-</sup> and mostly FITC<sup>-</sup> but which also possessed an increasing level of green fluorescence running into the lower right quadrant (figures 5.7 and (b) and (d)). This was taken to be largely viable cells (LL quadrant) some of which were showing signs of apoptosis (LR quadrant). Details of size of cell sample and a typical number of cells gated for each cell line are given in table 5.2.

		<i>AFB<sub>1</sub> plus micros.</i>	<i>AFB<sub>1</sub> without micros.</i>	<i>1% micros.</i>	<i>0.05% DMSO</i>	<i>control</i>
JB1 cell line						
number of events per quadrant	total	3752	3853	4100	3855	3757
	UL	28	36	41	46	31
	UR	917	423	659	354	431
	LL	2723	3308	3368	3425	3253
	LR	84	86	32	30	42
RLE cell line						
number of events per quadrant	total	3650	3802	3856	3752	4059
	UL	39	27	21	41	36
	UR	1407	540	792	431	572
	LL	2198	3205	3038	3197	3429
	LR	6	30	5	83	22

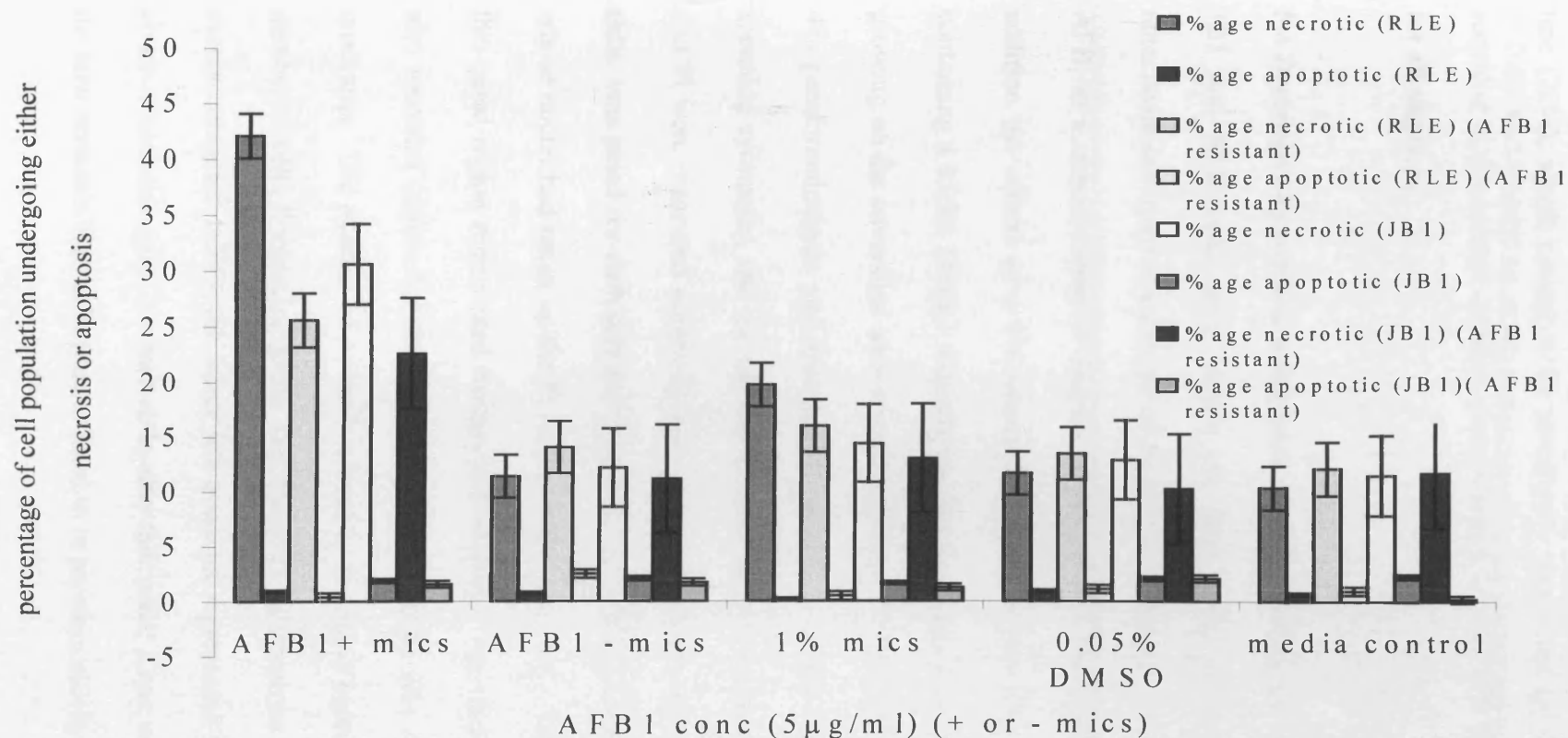
**Table 5.1:** RLE and JB1 cells were subjected to 30 minutes incubation in solutions containing 5µg/ml AFB<sub>1</sub> in the presence and absence of a 1% (v/v) microsomal activating system. Controls were carried out in the form of a 1% (v/v) microsomes solution, 0.05% (v/v) DMSO solution and unsupplemented DMEM. Cell counts were obtained for both cell lines by flow cytometric analysis after staining with annexin V-FITC and PI as markers for apoptosis and necrosis. Total cell number investigated (region R1, figure 5.8 ((a) and (c)) is given along with the number of cells undergoing apoptosis (LR quadrant), necrosis (UR quadrant), and viable cells (LL quadrant). Events contained within the UL quadrant represent cellular and nuclear debris ( figure 5.8 (b) and (d)).

		<i>AFB<sub>1</sub> plus</i>	<i>AFB<sub>1</sub></i>	<i>1%</i>	<i>0.05%</i>	<i>control</i>
Cell line		<i>micros.</i>	<i>without</i> <i>micros.</i>	<i>micros.</i>	<i>DMSO</i>	
JB1	total events	5000	5000	5000	5000	5000
	gated events (%age)	3752 75.04%	3853 77.06%	4100 82.0%	3855 77.1%	3757 75.14%
RLE	total events	5000	5000	5000	5000	5000
	gated events (%age)	3650 73.0%	3802 76.02%	3856 77.12%	3752 75.04%	4059 81.18%

**Table 5.2;** RLE and JB1 cells were subjected to 30 minutes incubation in solutions containing 5µg/ml AFB<sub>1</sub> in the presence and absence of a 1% (v/v) microsomal activating system. Controls were carried out in the form of a 1% (v/v) microsomes solution, 0.05% DMSO solution and unsupplemented DMEM. After a 2 hour recovery period, cells were stained with annexin V-FITC and PI as markers for apoptosis and necrosis. Cell counts were obtained for both cell lines by flow cytometric analysis. The total number of cells counted for each cell line is given, and the number of cells in gated region (R1; figures 5.8 (a) and (c)) is expressed as a percentage of total cell count.

The proportion of cells undergoing necrosis and apoptosis was expressed as a percentage of total cell number (figure 5.8). In all flow cytometry samples for both cell lines the intensity of green fluorescence in the apoptotic population was lower than that seen in the necrotic cells (figures 5.7 (b) and (d)).

Results showed that no significant difference was seen (for a 95% confidence interval using the two sample t-test,  $n = 6$ ) between the medium control and either the 0.05% DMSO control, or when cells were incubated with AFB<sub>1</sub> in the absence of microsomes, for any sample. Any further mention of control therefore will refer to the 'medium only' control. Within two hours of treatment with AFB<sub>1</sub>, cell death in all samples was seen to occur largely by necrosis. The levels of apoptosis did not differ significantly from the control for any of the samples investigated (0-3% of total population). With regard to the percentage of cell population undergoing necrosis, a statistically significant increase was seen for both the JB1 cell line (31% necrosis) ( $P < 0.001$ ) and the 'AFB<sub>1</sub>-resistant JB1' cell line (22% necrosis) ( $p=0.028$ ) compared with the control, after treatment with AFB<sub>1</sub> in the presence of 1% (v/v) microsomes. The difference in the values obtained between these two cell lines was not, however, significantly different ( $p= 0.16$ ). For the RLE cell line, a highly significant increase was seen in the percentage of cell population undergoing necrosis after incubation with AFB<sub>1</sub> in the presence of 1% (v/v) microsomes, (38% necrosis) ( $p<0.001$ ). The increase in necrosis seen in the 'AFB<sub>1</sub>-resistant RLE' cell line (26% necrosis) compared with the control was also statistically significant ( $p=0.004$ ). In addition, the difference in values obtained (for percentage of cell population undergoing necrosis) between the RLE and 'AFB<sub>1</sub>-resistant RLE' cell lines was found to be statistically significant ( $p=0.006$ ). The difference seen between the JB1 and RLE cell lines, for percentage of cell population undergoing necrosis was also found to be statistically significant ( $p=0.04$ ). No increase



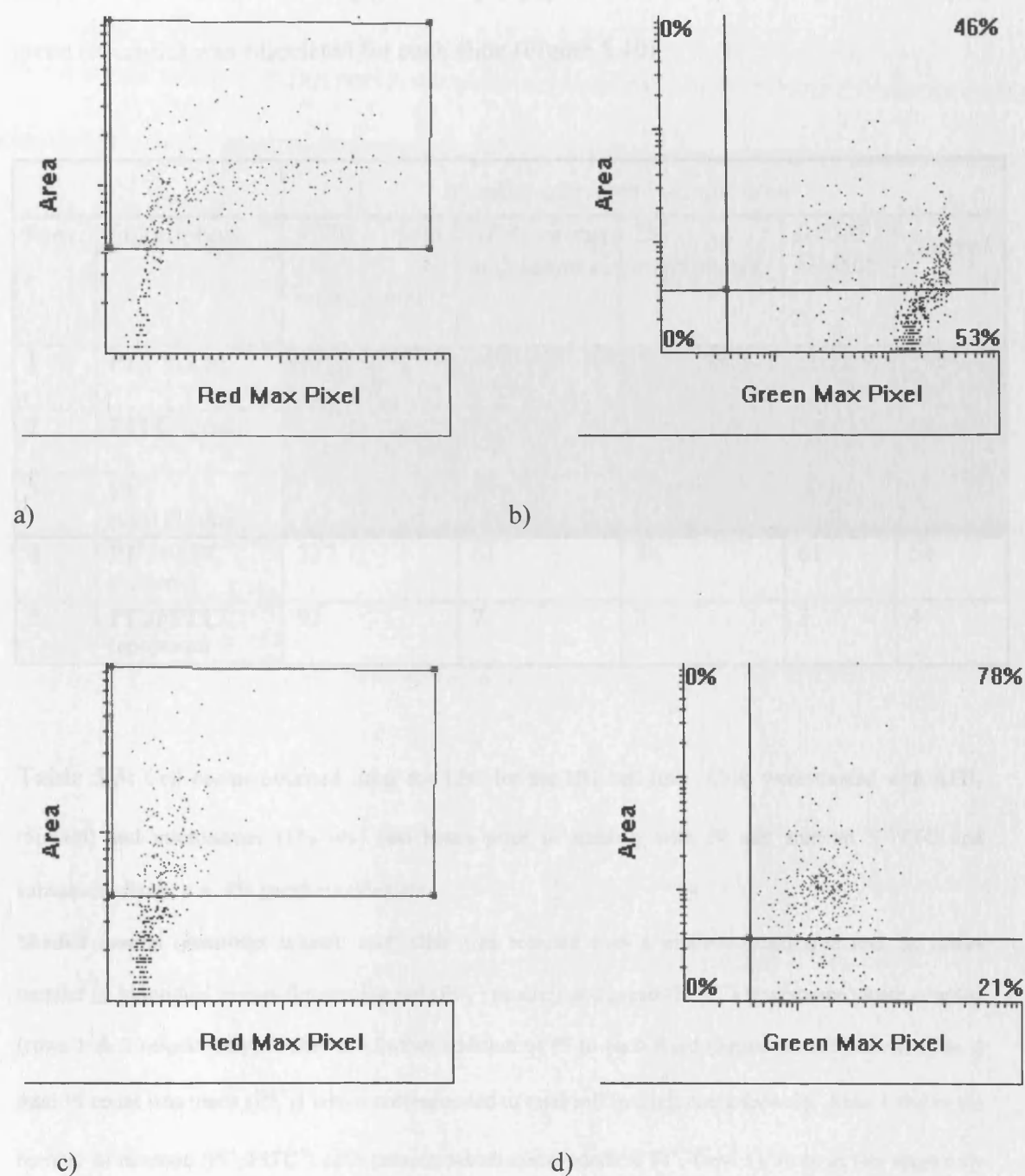
**Figure 5.8:** Percentage of total cell population showing necrosis (PI<sup>+</sup>/FITC<sup>+</sup>) (UR quadrant figure 5.8 (b) and (d)), and apoptosis (PI<sup>-</sup>/FITC<sup>+</sup>) (LR quadrant figure 5.8 (b) and (d)) for JB1 and RLE cell line after acute treatment with 5  $\mu$ g/ml AFB<sub>1</sub> in the presence and absence of a 1% (v/v) microsomal activating system. Analysis was by flow cytometry after staining with annexin V-FITC and PI as markers for necrosis and apoptosis. Results shown are an average of 6 separate sets of data.

in the level of necrosis was evident for either cell line in the presence of AFB<sub>1</sub> without microsomes. When cells were incubated with 1% microsomes alone, necrosis was not induced in the JB1 cell line. However, an increase in necrosis was seen for the RLE cell line (20%), which proved to be statistically significant ( $p=0.03$ ). Levels of necrosis recorded in the control situation were between 10 and 15% of the total cell population for all samples.

#### **5.4 Detection of apoptosis using laser scanning cytometer**

JB1 and RLE cells were seeded out into 6-well plates containing a previously autoclaved coverslip at a density of  $1 \times 10^4$ /ml. Two day old cultures were treated with AFB<sub>1</sub> at a concentration of 5 µg/ml in the presence and absence of microsomes. In addition the effects of a 1% microsome solution were also examined and a control containing a 0.05% DMSO solution was set up. After a two hour recovery period cells growing on the coverslips were stained with annexin V-FITC and PI prior to fixation in 4% paraformaldehyde and mounting in DABCO. Counts were made using the laser scanning cytometer, and the number of cells staining individually for annexin V-FITC and PI were measured separately and scatter plots were obtained. A population of PI<sup>+</sup> cells was gated for each cell line (figure 5.9 (a) and (c)), which represented those cells whose nuclei had taken up the PI (i.e. necrotic cells only). The events recorded outside this gated region represented background staining. The number of FITC<sup>+</sup> events was also recorded (figure 5.9 (b) and (d)), and the scatter plot obtained was divided into quadrants. The number of events recorded in the upper right quadrant represented the number of cells fluorescing green (i.e necrotic and apoptotic cells), and the number of events contained within the lower left quadrant represented background staining. To obtain a total cell count for each slide after this initial count was made, PI was added to the now permeabilised cells (due to fixation in paraformaldehyde). This was taken up





**Figure 5.9:** Scatter Plot obtained using laser scanning cytometric analysis of AFB<sub>1</sub> –treated JB1 ((a) and (b)) and RLE ((c) and (d)) Cell lines (5 $\mu$ g/ml + 1% microsomes) after staining with annexin V-FITC and PI as markers for necrosis and apoptosis. The gated region in a) and c) represents the number of cells whose nuclei have taken up the PI (i.e. necrotic cells). The upper right quadrant of b) and d) represents the number of cells fluorescing green (i.e. total of necrotic and apoptotic cells).

by every cell nucleus to give a total cell count per slide (table 5.3 and 5.4). From this, the proportion of cells staining green only (apoptotic) and those staining both red and green (necrotic) was calculated for each slide (Figure 5.10).

Row	Fluorophore	Number of events / sample area				
		<i>AFB<sub>1</sub> plus 1% microsomes</i>	<i>AFB<sub>1</sub> minus microsomes</i>	<i>1% microsomes</i>	<i>0.05% DMSO</i>	<i>Control</i>
1	PI <sup>+</sup> <sub>i</sub> count	332	61	84	61	54
2	FITC <sup>+</sup> count	424	68	89	64	58
3	PI <sup>+</sup> <sub>f</sub> (total PI count)	511	467	504	425	453
4	PI <sup>+</sup> <sub>i</sub> /FITC <sup>+</sup> (necrotic)	332	61	84	61	54
5	PI <sup>-</sup> <sub>i</sub> /FITC <sup>+</sup> (apoptotic)	92	7	5	3	4

**Table 5.3:** Cell counts obtained using the LSC for the JB1 cell line. Cells were treated with AFB<sub>1</sub> (5µg/ml) and microsomes (1% v/v) two hours prior to staining with PI and annexin V-FITC and subsequent fixation in 4% paraformaldehyde.

**Shaded results (readings taken);** each slide was scanned over a predetermined area and the initial number of individual events fluorescing red (PI<sup>+</sup><sub>i</sub>) (nuclei) and green (FITC<sup>+</sup>) (membrane) were counted (rows 1 & 2 respectively). After the further addition of PI to each fixed (hence permeabilised) slide, a total PI count was made (PI<sup>+</sup><sub>f</sub>) which corresponded to total cell (nuclei) count (row 3). Row 4 shows the number of necrotic (PI<sup>+</sup><sub>i</sub>/FITC<sup>+</sup>) cells present, which corresponds to PI<sup>+</sup><sub>i</sub> (row 1), since at this stage only cells whose membranes are no longer intact (a feature of necrotic cells) can take up the PI into their nuclei. We can assume, therefore, that the membranes of these cells will also stain positive for annexin V-FITC, due to exposure of PS. From these results, the number of apoptotic cells (PI<sup>-</sup><sub>i</sub>/FITC<sup>+</sup>) (row 5), could be calculated (row 2 value – row 1 value), i.e. those which fluoresced green but not red during the initial PI (row 1) and FITC (row 2) counts.

Row	Fluorophore	Number of events / sample area				
		<i>AFB<sub>1</sub> plus 1% microsomes</i>	<i>AFB<sub>1</sub> minus microsomes</i>	<i>1% microsomes</i>	<i>0.05% DMSO</i>	<i>Control</i>
1	<b>PI<sup>+</sup><sub>i</sub> count</b>	355	114	66	41	56
1	<b>FITC<sup>+</sup> count</b>	403	116	72	44	61
3	<b>PI<sup>+</sup><sub>f</sub> (total PI count)</b>	554	238	238	337	376
4	<b>PI<sup>+</sup><sub>i</sub>/FITC<sup>+</sup> (necrotic)</b>	355	114	66	41	56
5	<b>PI<sup>-</sup><sub>i</sub>/FITC<sup>+</sup> (apoptotic)</b>	48	2	6	3	5

**Table 5.4:** Cell counts obtained using the LSC for the RLE cell line. Cells were treated with AFB<sub>1</sub> (5µg/ml) and microsomes (1% v/v) two hours prior to staining with PI and annexin V-FITC and subsequent fixation in 4% paraformaldehyde.

**Shaded results (readings taken);** each slide was scanned over a predetermined area and the initial number of individual events fluorescing red (PI<sup>+</sup><sub>i</sub>) (nuclei) and green (FITC<sup>+</sup>) (membrane) were counted (rows 1 & 2 respectively). After the further addition of PI to each fixed (hence permeabilised) slide, a total PI count was made (PI<sup>+</sup><sub>f</sub>) which corresponded to total cell (nuclei) count (row 3). Row 4 shows the number of necrotic (PI<sup>+</sup><sub>i</sub>/FITC<sup>+</sup>) cells present, which corresponds to PI<sup>+</sup><sub>i</sub> (row 1), since at this stage only cells whose membranes are no longer intact (a feature of necrotic cells) can take up the PI into their nuclei. We can assume, therefore, that the membranes of these cells will also stain positive for annexin V-FITC, due to exposure of PS. From these results, the number of apoptotic cells (PI<sup>-</sup><sub>i</sub>/FITC<sup>+</sup>) (row 5), could be calculated (row 2 value – row 1 value), i.e. those which fluoresced green but not red during the initial PI (row 1) and FITC (row 2) counts.

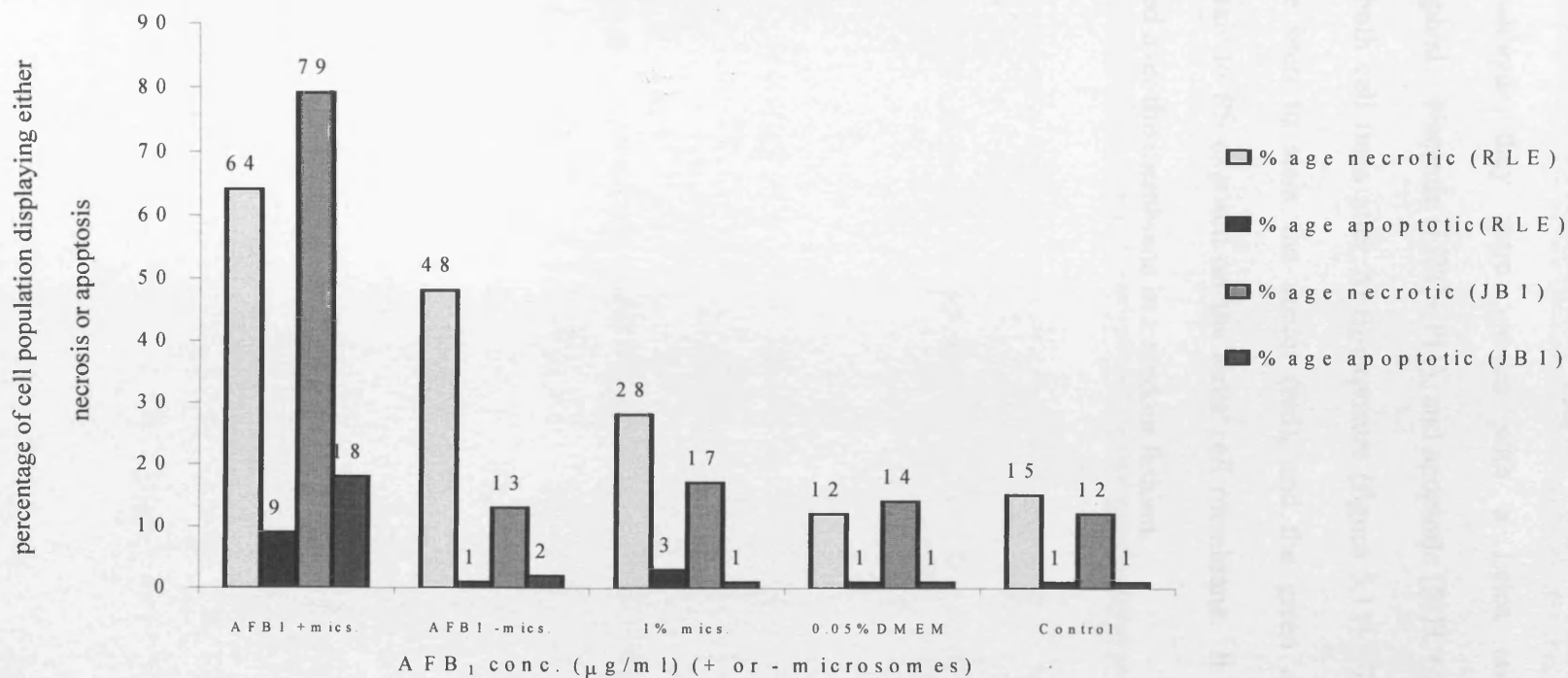
Results showed that for both cell lines the method of cell death was largely by necrosis; 65% of total cell number for the JB1 cell line and 64% for the RLE cell line compared with 19% and 8.6% respectively undergoing apoptosis, when cells were subjected to 5µg/ml AFB<sub>1</sub> in the presence of 1% quail liver microsomes. Also of note is that 48% of cells were seen to be undergoing necrosis in the RLE cell line with AFB<sub>1</sub> alone (i.e. unactivated) compared with just 13% of the JB1 cells. This value of 13% did not differ greatly from that seen for the DMSO only control (14% necrotic cells for JB1 and 12% for RLE). In both cell lines there were very low levels of apoptosis seen with unactivated AFB<sub>1</sub>; 1.5% of JB1 cells and 0.8% of RLE cells. It can also be seen that the RLE cell line was more sensitive to effects of a 1% microsome concentration than the JB1 cell line, with 28% of RLE cells undergoing apoptosis compared with 17% of JB1 cells. Again the values obtained for apoptosis in both cell lines were similar to results obtained with the DMSO only control; 1% of cells undergoing apoptosis for the JB1 cell line compared with 0.7% for the control; 2.5% for RLE compared with 1% for the control.

From these results it can be seen that in all of the control situations, the RLE cell line was more sensitive to the effects of the AFB<sub>1</sub> and the microsomes than the JB1 cell line. However, when the AFB<sub>1</sub> was activated in the presence of a 1% (v/v) microsome solution, the JB1 cell line was seen to be as sensitive as the RLE line at this concentration, which was not in agreement with previous results.

The scattergrams obtained for the RLE cell line after two hours incubation with activated AFB<sub>1</sub> (figure 5.9 (c) and (d)) showed that as well as there being an increase in the number of the PI<sup>+</sup> cells, they were of a similar size to those in the control situation but fluoresced more brightly, i.e. had taken up more of the DNA stain. In the case of the JB1 cell line however (figure 5.9 (a) and (b)), although there was a greater number

of PI<sup>+</sup> cells after treatment with activated AFB<sub>1</sub>, these were of a similar size and showed a similar level of fluorescence when compared with the control situation.

When taking green staining into account, the scattergrams show that for the RLE cell line after treatment with activated AFB<sub>1</sub>, FITC<sup>+</sup> cells were similar in size and level of fluorescence to the control situation, although they were greater in number. For the JB1 cell line there appears to be the presence of a more strongly staining cell population with a varying cell size when compared to the control.

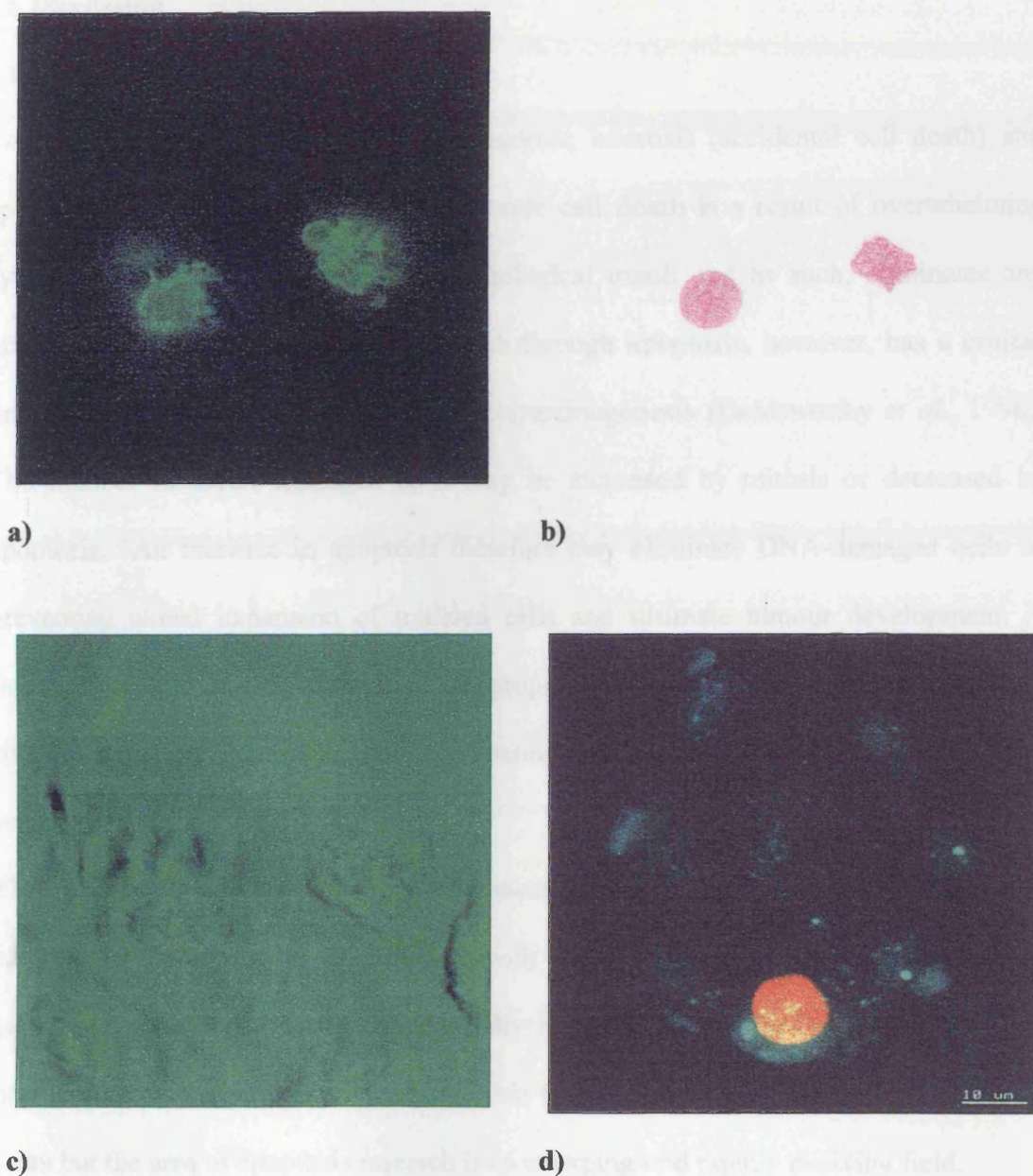


**Figure 5.10:** Individual cell counts were obtained for JB1 and RLE cell line (PI<sup>+</sup> and FITC<sup>+</sup> cells) using the LCS. A total cell count was then carried out for each slide and the results expressed as the proportion of the total cell number undergoing necrosis (PI+/FITC+) and apoptosis (PI/FITC<sup>+</sup>). Results obtained were an average of 3 sets of data.

### **5.5 Confocal Fluorescence Microscopy and Photography**

JB1 cells were grown on coverslips and stained with annexin V-FITC and PI 2 hours after 30 minutes incubation with 5µg/ml AFB<sub>1</sub>. After fixation in 4% (w/v) paraformaldehyde they were viewed with a Leica confocal microscope and photographed. Necrotic (FITC+/PI+), and apoptotic (FITC+/PI-) cells could clearly be seen in both cell lines after AFB<sub>1</sub>-exposure (figure 5.11). The DNA intercalating PI could be seen to stain the nuclei (red), and the green annexin V-FITC staining corresponds to PS exposed on the outer cell membrane. It was seen to be unevenly distributed over the membrane in a random fashion.





**Figure 5.11:** Annexin V-FITC staining (a), PI staining (manipulated image) (b), and phase contrast image (c) of RLE cells, 2 hours after 30 minutes incubation with 5  $\mu\text{g}/\text{ml}$  AFB<sub>1</sub> in the presence of a microsomal activating system. The FITC staining is localised on the outer cell membrane and corresponds to PS exposure, while the PI is a nuclear stain. Necrotic cells are FITC<sup>+</sup>/PI<sup>+</sup>, due to loss of cell membrane integrity facilitating passage of the PI into the cell. Image (d) shows FITC and PI staining combined, on RLE cells stained on a separate occasion.



## 5.6 Discussion

### 5.6.1 Measurement of Apoptosis

Cell death can be divided into two categories; necrosis (accidental cell death) and apoptosis (programmed cell death). Necrotic cell death is a result of overwhelming cytotoxic injury due to chemical or pathological insult and as such, eliminates any affected cells. The regulation of cell death through apoptosis, however, has a critical influence on the multiple stages of hepatocarcinogenesis (Goldsworthy *et al.*, 1996). The number of DNA damaged cells may be increased by mitosis or decreased by apoptosis. An increase in apoptosis therefore may eliminate DNA-damaged cells so preventing clonal expansion of initiated cells and ultimate tumour development. A failure of apoptosis would result in inappropriate cell survival. This could therefore contribute to oncogenesis in cells possessing mutations due to adducts formed by carcinogens such as AFB<sub>1</sub>.

There is a need, therefore for the development of accurate methods for the quantitative analysis of apoptosis in conjunction with cell proliferation measurements when investigating the mechanisms of chemically induced carcinogenesis. The role of cell proliferation in the process of carcinogenesis has attracted most interest up until recent years but the area of apoptosis research is an emerging and rapidly evolving field.

Many methods have been employed for the identification of apoptosis (for a recent review see Hall, 1999); it is most easily distinguished from necrosis ultrastructurally using electron microscopy (Kerr *et al.*, 1972). However, this would prove too laborious for routine quantitative assessment, and other methods may be of use when measuring the levels of apoptosis present in a cell population or tissue sample. One such method is the presence of a DNA-ladder pattern seen on gel electrophoresis. This, in conjunction with diphenylamine, can be used to determine the percentage of DNA which has been

cleaved to oligonucleosomal fragments in a cell population (Wyllie *et al.*, 1980). Incorporation of labelled nucleotides at sites of DNA damage by *in situ* end labelling (ISEL) has been used to detect and quantify apoptotic bodies in paraffin-embedded tissue (Wijsman *et al.*, 1993). An ISEL method for the detection of apoptotic bodies in rat liver sections treated with non-genotoxic carcinogens was reported by Wheeldon *et al.*, (1995). However, morphologically evident apoptosis is not always followed by DNA fragmentation (Cohen *et al.*., 1992).

Various immunostaining techniques have been reported in the literature; rat hepatocytes undergoing cell death by apoptosis have been shown to stain positively for 'tissue' transglutaminase (tTG) (Piacentini *et al.*, 1991) and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) (Oberhammer *et al.*, 1993). Stinchcombe *et al.*, (1995) reported a novel method for the fluorescence microscopic detection of apoptotic bodies due to their high level of eosin fluorescence. It is important to note, however, that different methods may detect different stages of hepatocyte apoptosis and thus would not be expected to yield identical results.

Flow cytometry can be useful for discriminating between subpopulations of viable and nonviable cells in suspension and identifying the mode of cell death. Alterations in the forward and right angle scatter of light are seen in apoptotic cells due to cell shrinkage and chromatin condensation, and in necrotic cells following the rupturing of the cell membrane and leakage of cell constituents (Cotter and Martin 1994). Differences in staining with a DNA fluorochrome e.g. acridine orange or Hoechst dye, due to chromatin condensation, or a decrease in propidium iodide exclusion due to reduced membrane integrity (Darzynkiewicz *et al.*, 1992) are other methods for distinguishing between apoptotic and necrotic cells. However, most of the fluorescent dyes used in flow cytometry are nonvital dyes, i.e. they stain cells only after membrane damage, which presents problems when quantifying apoptotic cells. The 'Apo2.7' protein

(available from Coulter), is a new marker for direct quantitation of apoptotic cells by flow cytometry. It reacts with a 38kDa mitochondrial membrane protein exposed in cells undergoing apoptosis and its expression represents an early event of apoptosis (Zhang *et al.*, 1996a).

### 5.6.2 Development of Annexin V ELISA

Another such marker for cells undergoing apoptosis is the anticoagulant annexin V which preferentially binds to negatively charged phosphatidylserine (PS) (Andree *et al.*, 1990) exposed on the outer cell membrane during the early stages of apoptosis, once the cell is committed to dying (Fadok *et al.*, 1992). Koopman *et al.*, (1994) used FITC-labelled annexin V binding to detect apoptosis in Burkitt lymphoma cell lines by flow cytometry, reporting that DNA fragmentation and double staining with Hoechst was limited to the annexin V-positive sub-population. In addition, the percentage of annexin V-positive cells correlated well with quantification of apoptosis by Giemsa staining. No intermediate staining with annexin V was found, indicating that apoptotic cells rapidly lose their membrane asymmetry to expose PS on the cell surface in the early stages of apoptosis. Cells were double stained with annexin V-FITC and the DNA-intercalating fluorochrome ethidium bromide. This provided a method by which apoptotic and necrotic cells could be distinguished from one another, since although PS exposure was evident in both cases, only those cells with membrane damage could take up the ethidium bromide. However, it is important to take into account that cells in the later stages of apoptosis as well as necrotic cells, both of which display membrane damage, were reported to stain positive for the ethidium bromide.

Annexin V, when used in conjunction with a nonvital dye such as reported above, is useful as a marker for cells in the early stages of apoptosis. It was employed in this thesis both with, and without, propidium iodide (PI) staining. PI is a nuclear stain

closely related to ethidium bromide which cannot cross the intact plasma membrane, therefore only cells in the late stages of apoptosis and those undergoing necrosis are PI<sup>+</sup>. Flow cytometry techniques as reported by Koopman *et al.*, (1994), are only suitable for cell suspensions. The cell lines under investigation in this project; JB1 and RLE, both grow as a monolayer. It was therefore necessary to develop a technique that could utilise annexin V as a marker for apoptosis but could be employed when dealing with adherent cells. Firstly, annexin V-FITC-labelling of cells was employed as the primary antibody step of an ELISA (the PS on the cell membrane being the antigen). By using anti-fluorescein labelled with alkaline phosphatase as the secondary antibody and a phosphatase substrate, the amount of PS exposure could be evaluated colorimetrically using a spectrophotometer at a set wavelength. However, since both apoptotic and necrotic cells are detected by this method, some way of distinguishing between these two populations was required. Double staining of the cells with a nonvital dye, as described earlier, was of no use in this case since excitation of such fluorochromes is required before they emit their characteristic fluorescence for detection. This is a feature not present in the spectrophotometer, which measures the amount of light absorbed by the sample at a particular wavelength; the higher the absorbance, the greater the concentration of coloured product present. Also, if for instance, a labelled nuclear marker were used with annexin V<sup>+</sup> cells, in order for this to be detectable by ELISA, it would be necessary to use a secondary antibody that was conjugated with something other than alkaline phosphatase. Colorimetric measurements would, if this were not the case, represent the total coloured product formed by the phosphatase substrate with both secondary antibodies. However, even if the two end products to be detected were of different colours, unless they absorbed light at greatly differing wavelengths there would be interference caused by the overlap of their respective absorbance spectra. In addition, it would be undesirable to run a 'double ELISA' on one

plate due to the probability of cross reactivity between all of the antibodies used and their antigens. The possibility of running two separate ELISAs consecutively on one plate was ruled out since the BSA used in the blocking step of the first run blocks any nonspecific binding with proteins other than the one of interest, which may well include relevant proteins for the second ELISA. Therefore some other method was required to enable differentiation between apoptotic or necrotic FITC<sup>+</sup> staining cells.

Since PS exposure on the outer surface of the cell membrane is a feature of the early stages of apoptosis, such cells will therefore still be viable, and possess intact mitochondria. This meant, for this thesis, that it was possible to carry out the MTT assay on duplicate plates treated in parallel, to obtain a measure of viable cell number for each well. This allowed for the levels of annexin V staining per well (as measured by ELISA) to be expressed in relation to the number of viable cells present in the corresponding well in the duplicate plate (as measured by the MTT assay) ( $A_{405nm}/A_{495nm}$ ).

#### **5.6.2.1 Analysis of ELISA/MTT Results**

Analysis of results obtained with this technique 2 and 24 hours after treatment showed that in both cell lines, cells were exhibiting PS exposure on the outer cell membrane 2 hours after an acute dose of activated AFB<sub>1</sub>. A low level of exposure was seen in the RLE cell line with a 1% (v/v) microsome solution alone at this time point, and a higher PS index was seen overall compared with the JB1 cell line. Comparison of the MTT assay data show that cell numbers were lower by around 50% in the JB1 cell line and by around 70% in the RLE cell line in all samples including the control after 24 hours. This could be due to natural senescence of cells, but is more than likely attributable to the fact that the 2 (figure 5.4) and 24 (figure 3.12) hour procedures were carried out on separate occasions which probably led to differences in seeding out densities, length of

incubation prior to assay and other incubation conditions. For this reason, results obtained via MTT or ELISA alone would not allow for comparison between different sets of data. This was not considered to be a problem since the MTT assay results were intended to give a measure of cell number, and to allow expression of annexin V binding in relation to this, for each particular set of data. Therefore, PS indices could be directly compared between assays carried out separately on different occasions and under non-identical conditions such as previously mentioned.

An important consideration when assessing these results should be the percentage cell survival rates after 2 and 24 hours. For both cell lines 2 hours after AFB<sub>1</sub> treatment a decrease in cell number was seen in a dose responsive manner. Since this was measured via the MTT assay, we can assume that any nonviable cells have detached from the surface of the well and the readings obtained are a measure of cells that remain viable after this time lapse. The ELISA carried out on the duplicate plate is therefore measuring the amount of annexin V binding in these remaining viable cells, and therefore does not take into account any cells in the late stages of apoptosis still attached to the well, or the early or late stages of necrosis which may have become detached.

Percentage cell survival rates 24 hours after treatment did not differ significantly from those obtained after 2 hours for the JB1 cell line with microsomes alone or 1 µg/ml activated AFB<sub>1</sub>. For the higher concentrations however, a significant decrease in cell survival was seen after 24 hours compared with after 2 hours. With the RLE cell line, an initial cell loss was seen with activated AFB<sub>1</sub> which was lower than that seen for the JB1 cell line, and which remained stable after 24 hours. This indicated that cell loss due to the cytotoxic effects of AFB<sub>1</sub> was immediate and then remained stable after this time for the RLE cell line over the concentration range applied, and for the JB1 cell line for lower doses of AFB<sub>1</sub>. For the higher doses of activated AFB<sub>1</sub>, however, a decrease in cell survival was seen after 24 hours compared with after 2 hours.

However, the PS indices calculated for both cell lines show an increase after 24 hours compared with after 2 hours for both cell lines as well as an increase for each cell line in a dose responsive manner, and for the RLE cell line, in the presence of microsomes alone. This would indicate that cells are either in the early or late stages of apoptosis, or the early stages of necrosis. Those exhibiting PS exposure and possessing intact mitochondria would give a positive reading with both the MTT assay and the ELISA. Those with nonfunctional mitochondria but which are still attached would show positive with the ELISA but would not be detected by the MTT. However, the duration of visible apoptosis in hepatocytes is estimated to be only around 4 hours (Bursch *et al.*, 1990). It is likely therefore, that the increase in annexin V binding seen is due to individual cells undergoing increasing PS exposure during the course of either apoptotic or necrotic changes, rather than an increasing number of individual cells undergoing apoptosis, and the accumulation of apoptotic bodies in the absence of phagocytosis. Of note is the result obtained for the RLE cell line with unactivated AFB<sub>1</sub>. Cells treated in such a way appeared to show no effects after 2 hours. However, after 24 hours a significant decrease in cell survival rates was seen for the higher doses of unactivated AFB<sub>1</sub> used which coincided with a significant increase in PS indices in this situation. Since this result was not immediately apparent, it is possible that the RLE cell line still possesses enough P450 activity to activate the AFB<sub>1</sub> to the epoxide, albeit at a much slower rate. Koopman *et al.*, (1994) reported that there was no intermediate stage of PS exposure indicating that apoptotic cells rapidly lose their membrane asymmetry to expose PS on the cell surface. However, these results indicate that there is a gradual increase in PS exposure as the cell progresses from apoptosis into necrosis in both cell lines, although it was seen to a much greater extent in the RLE cell line.

### 5.6.3 Analysis of Flow Cytometry results

Flow cytometric analysis using annexin V-FITC and PI as markers for necrosis and apoptosis, showed that cell death seen in both the JB1 and RLE cell line two hours after an acute dose of AFB<sub>1</sub> (5µg/ml), was largely by necrosis (Figure 5.8). Although some very minor apoptotic changes were evident (around 0% to 3%), this value did not differ from the control situation. This was in accordance with the results obtained for the ELISA/MTT assay, where an initial increase in PS exposure in viable cells, along with decrease in cell number was evident after two hours (Figure 5.6). The difference in sensitivity to activated AFB<sub>1</sub> between the two cell lines, has been clearly shown by this technique; with the AFB<sub>1</sub>-sensitive RLE cell line suffering a 10% higher percentage cell loss than the more resistant JB1 cell line (Figure 5.8). In addition, the effects of an acute dose of AFB<sub>1</sub> (5µg/ml) on 'AFB<sub>1</sub>-resistant' strains of these two cell lines (method 2.3.1) was investigated. The involvement of the GST A5 subunit in protection against AFB<sub>1</sub> is illustrated by these results, since there is no apparent difference in the levels of resistance to activated AFB<sub>1</sub> between the JB1 and 'AFB<sub>1</sub>-resistant' JB1 cell line (Figure 5.8), both of which express this subunit (chapter 4). However, these results show that resistance to activated AFB<sub>1</sub> has been induced in the 'AFB<sub>1</sub>-resistant' RLE cell line, since lower levels of cell death were apparent in this cell line (26% of cell population undergoing necrosis) when compared with the RLE cell line (38% of cell population undergoing necrosis) (Figure 5.8). The absence of expression of the GST A5 subunit in the 'AFB<sub>1</sub>-sensitive' RLE cell line, and its subsequent constitutive expression in the 'AFB<sub>1</sub>-resistant' RLE cell line (chapter 4), is suggestive of this subunit's contribution towards a cells resistance to AFB<sub>1</sub>.

The results obtained when cells were incubated with unactivated AFB<sub>1</sub> were also in accordance with the results of the ELISA/MTT assay (Figure 5.6), since no change from



the control was seen after 2 hours for either cell line. In addition the 1% microsome solution was seen to have a toxic effect on the RLE cell line (as in chapter 3).

The absence of any apoptotic changes in either cell line was not in accordance with the results of the ELISA/MTT assay which indicated that the JB1 cell line appeared to possess a low level of endogenous apoptosis. This could be due to the fact that the procedure of trypsinisation necessary prior to flow cytometric analysis, led to around 10-15% of the total cell population undergoing necrosis. It is possible that any slight changes in the levels of apoptosis were masked by this, since the levels of apoptosis recorded by this procedure were comparatively low (0-3%).

#### **5.6.4 Analysis of LSC results**

Assessment of AFB<sub>1</sub>-treated JB1 and RLE cell lines using the LSC was carried out in this thesis and again, initial results obtained appear to show that the main route of cell death after 2 hours for both cell lines was by necrosis although low levels of apoptosis were apparent (Figure 5.10). Necrotic and apoptotic changes were also seen by visualisation of cells with the confocal fluorescent microscope. However, much more work is required to be carried out in this area. Optimisation of cell density is important since it is necessary to differentiate between single cells but cells must be of a sufficient density to enable statistical analysis of data to be carried out.

Of the three methods employed, the most suitable for measuring the required parameters on the cell lines under investigation was found to be the LSC. The main disadvantage was that it was not possible to carry out the procedure on 96-well plates. An alternative to growing the cells on coverslips in 6-well plates are microscope slides containing a cell culture well. There was insufficient time available in this project to enable the generation of an adequate quantity of statistical data, but this is possibly an area for further analysis.

### 5.6.5 Advantages and Disadvantages of Chosen Techniques

The main disadvantage with the ELISA/MTT assay is that it does not allow for assessment of individual cells. As mentioned earlier, although the effects of AFB<sub>1</sub> and microsomes on both JB1 and RLE cell lines were clearly demonstrated by this technique, this gave a measure of the amount of PS exposed on the outer cell membrane of cells that were still adhered to the well. It was therefore not possible to distinguish between cells in late apoptosis or early necrosis. Although the percentage cell survival rates for each set of conditions will take into account necrotic cells lost in the wash stages prior to analysis, another disadvantage is that it is only possible to compare the levels of PS exposure in each cell population and not to calculate the proportion of total cell number undergoing apoptosis or necrosis using this method.

Flow cytometry has advantages over the ELISA technique developed in this thesis as it measures a set number of cells individually and quickly. From the data generated the percentage of total cell number stained individually with one fluorochrome, or with both together could be calculated. In this way a clear distinction could be made between subpopulations of cells undergoing either necrosis or apoptosis. However, one disadvantage was that the JB1 and RLE cell lines grew as a monolayer and in order to carry out flow cytometric analysis it was necessary to obtain a suspension of these cells by way of a trypsinisation process. This is a very useful technique, as long as during the course of this, the amount of trauma the cells are subjected to (i.e concentration of, and incubation time in, trypsin/EDTA solution) is kept to a minimum for each particular cell line (method 2.7.1).

Use of the laser scanning cytometer had advantages over both of the above techniques since it enabled a set number of cells, or number of cells in a preset area, to be investigated. As with the flow cytometer, the number of cells fluorescing either green

or red, or green and red together, could be measured and expressed as a percentage of total cell number. However, this could be carried out on the cell monolayer, thus avoiding any detrimental effects that may be seen due to trypsinisation prior to flow cytometry.

### 5.6.6 Implications for Apoptosis in Carcinogenesis

All the above methods indicated that although cell death after treatment with AFB<sub>1</sub> was largely by necrosis, a low level of apoptosis was apparent in both cell lines. However, results do indicate that higher levels of apoptosis were seen in the AFB<sub>1</sub>-induced-tumour-derived JB1 cell line compared to the RLE cell line on addition of activated AFB<sub>1</sub> and would suggest that this cell line inherently displays levels of apoptosis higher than the normal background levels recorded for this set of experiments (ann V neg). Panse *et al.*, (1997), demonstrated that transformation of fibroblasts by chemical carcinogens *in vitro* leads to a phenotype characterised by its sensitivity to induction of apoptosis by neighbouring normal cells. The trigger for apoptosis is the transformed cells themselves which release the growth factor, TGF- $\beta$  (found only in hepatocytes undergoing apoptosis (Oberhammer *et al.*, 1993; Bursch *et al.*, 1993)) which in turn induces the production of an apoptosis-inducing factor, utilising a signal pathway that uses the action of reactive oxygen species (Picht *et al.*, 1995; Langer *et al.*, 1996). The apoptosis-inducing factor is then transmitted to transformed cells so causing their death. Reactive oxygen species are required in this pathway, antioxidants would therefore interfere with the elimination of transformed cells (Zeisel *et al.*, 1997) and this interference could possibly have implications in tumour progression. Whilst, however, apoptosis is induced in transformed cells by surrounding normal cells, this is not the case with the JB1 cell line since, as stated earlier, it is derived from an AFB<sub>1</sub>-induced hepatocellular carcinoma and so will contain no normal cells. However, Panse *et al.*,

(1997) also report that whereas increasing numbers of non-transformed cells leads to an increase in apoptosis due to a higher number of effector cells, an increase in numbers of transformed cells leads to sufficient concentrations of TGF- $\beta$  to trigger induction of apoptosis. This could possibly be the reason for the endogenous levels of apoptosis seen in the JB1 cell line and may represent an early anticarcinogenic control mechanism *in vivo*. Immunostaining for the presence of TGF- $\beta$  (Oberhammer *et al.*, 1993) in these cells would give an indication of whether or not this were the case. The release of TGF- $\beta$  by transformed cells seems to be of central importance for the maintenance of the transformed state (Wehrle *et al.*, 1994) but the results of Panse *et al.*, (1997) suggest that it may also represent a severe obstacle to their survival and that this sensitivity to induction of apoptosis is a general feature of transformed cell lines. The pool size of transformed cells, therefore, may be enlarged *in vivo* by substances interfering with this pathway thus protecting transformed cells, and this may be the basis for spontaneous or induced resistance later on. A tissue might also acquire or inherit mechanisms that protect its cells from induction of apoptosis which may in turn lead to the formation of tumour cells that are no longer subject to intercellular control. Cells from a wide variety of human malignancies have been shown to possess a reduced ability to undergo apoptosis (Wyllie *et al.*, 1987). The control of self-induced apoptosis by transformed cells therefore may have specific implications in the control of oncogenesis, and apoptosis may represent a mechanism for protecting the organism from cells that have acquired genetic alterations that predispose them to cell proliferation. Within hepatic preneoplastic nodules, for instance, an increase in proliferation rate is accompanied by a parallel increase in apoptosis which may account for the slow growth phenotype seen in these lesions (Schulte-Hermann *et al.*, 1995). Muskhelishvili *et al.*, (1996) reported elimination of GST-P-positive hepatocytes in rat liver preneoplastic foci due to an

increase in apoptosis induced by dietary restriction, and that GST-P-positive tumours that developed did so from apoptosis-resistant subclones of initiated hepatocytes.

Stinchcombe *et al.*, (1995) reported that the tumour promoting activity of TCDD on GST-P-positive rat liver foci, was preferentially mediated by a decrease in apoptosis in these enzyme-altered nodules. This was in accordance with observations on other tumour promoters, such as phenobarbital, in liver (Bursch *et al.*, 1984; Schulte-Hermann *et al.*, 1990). Studies along these lines may provide an insight into the regulation of and requirements for tumour progression.

Apoptosis usually inhibits carcinogenesis by eliminating initiated cells via p53-initiated mechanisms. However, in p53-defective cells, such as in HCC in humans after AFB<sub>1</sub> exposure (Ozturk *et al.*, 1991), alternative, p53-independent apoptosis pathways, such as that involving TGF- $\beta$ , may serve as a mechanism for eliminating initiated cells. This is important in the case of the JB1 cell line which may well possess mutated p53 due to formation of AFB<sub>1</sub>-DNA adducts. Immunostaining for the presence of the p53 protein in these cells would also be of interest. Acquisition by p53-deficient cells of resistance to p53-independent inducers of apoptosis such as choline deficiency (Zeisel *et al.*, 1997), TGF- $\beta$ 1 (Oberhammer *et al.*, 1993) and ROS (Picht *et al.*, 1995) may leave cells without another important apoptotic defensive barrier and may be responsible for the progression of initiated cells to carcinomas.

Errors in DNA repair that would otherwise induce apoptosis, could contribute to the high mutation rate observed in many cancers, and the inability of cells to undergo apoptosis in response to DNA damage, may underlie the enhanced resistance to chemotherapeutic agents in tumours that are deficient in p53 (Lowe *et al.*, 1994).

The finding in this thesis that the majority of cell death in JB1 and RLE cell lines is by necrosis after an acute dose of AFB<sub>1</sub> is attributable to the inhibition of RNA and protein synthesis (Clifford and Rees, 1966; McIntosh *et al.*, 1976) which represents an early

manifestation of the cytotoxic action of AFB<sub>1</sub>. This, along with AFB<sub>1</sub>-induced inhibition of glycogen accumulation (Blaude *et al.*, 1990), lipid peroxidation of membranes and generation of ROS (Shen *et al.*, 1995) leads to an overwhelming toxic injury resulting in necrosis. This will in turn lead to the elimination of any affected cells. However, induction of some apoptosis was apparent which could be due to the formation of ROS after AFB<sub>1</sub> treatment (Shen *et al.*, 1995). It is also likely that the JB1 cell line is sensitive to apoptosis triggered by the production of TGF- $\beta$  which is a feature of transformed cells and probably represents a control mechanism *in vivo* so preventing the promotion of initiated cells and ultimate tumour development. The induction of apoptosis by transformed cells may be a central regulatory step in carcinogenesis and events which lead to resistance to this, such as mutations in the p53 gene seen after AFB<sub>1</sub> treatment (Ozturk *et al.*, 1991; Mace *et al.*, 1997), overexpression of bcl-2 (Oltvai *et al.*, 1993; Jurgensmeier *et al.*, 1997) or alterations in the induction of p53-independent apoptosis, could lead to the formation of tumours from initiated cells. Certain tumour promoters may also act by suppressing the levels of apoptosis in an initiated cell or in hepatic foci, and resistance to apoptosis may be an explanation for the failure of some chemotherapeutic agents in some GST-P positive foci. Since apoptosis is tightly regulated at genetic levels, optimism exists that the process of carcinogenesis in apoptosis-resistant lesions may be controlled by inducers of apoptosis. One such inducer is perillyl alcohol (Mills *et al.*, 1995), which inhibits liver tumour growth in rats by selectively enhancing apoptosis by increasing the ability of the tumour cell to both activate and respond to TGF- $\beta$ .

### 5.6.7 Conclusion

Cell death following an acute dose of AFB<sub>1</sub> is largely by necrosis for both the AFB<sub>1</sub>-sensitive RLE cell line and the more resistant JB1 cell line. The RLE cell line however,

displayed both higher levels of necrosis and a higher PS index than the JB1 cell line both 2 hours and 24 hours after administration of the toxin. The PS index recorded by the ELISA/MTT assay after 2 hours appears to be the result of necrotic changes occurring in the cells in response to the presence of the AFB<sub>1</sub>-epoxide. Although cell death appeared to be immediate (within the first two hours) after treatment, and then tail off over the next 22 hours, the PS index increased for both cell lines, which is possibly an indication of secondary necrotic changes. A higher endogenous level of apoptosis was seen in the JB1 cell line with the ELISA/MTT assay and this may be an adaptive response of transformed cells caused by the over-expression of TGF- $\beta$  (Panse *et al.*, 1997). This possibly represents an early anticarcinogenic control mechanism *in vivo*.

A need exists for the development of more accurate and efficient methods for measuring apoptosis in cell lines and tissues. Annexin V-FITC and PI were used as markers for necrosis and apoptosis in this thesis. The ELISA /MTT assay developed in this thesis is useful in measuring the levels of PS exposure in cell populations, but does not give information on individual cells or enable fully the differentiation between necrotic and apoptotic cells. Flow cytometry requires that the cells under investigation are suspended. Providing that the amount of trauma inflicted upon them during trypsinisation is minimised, reliable and reproducible results can be obtained, although any background levels of necrosis caused by the technique may possibly mask very small levels of apoptosis. The LSC was found to be a useful method for measuring apoptosis in adherent cell lines but further work is required to be carried out to optimise this method and generate adequate statistical data.

**Chapter 6**  
**Conclusion and Further Work**



## **6 Conclusion and further Work**

### **6.1 AFB<sub>1</sub> and Carcinogenesis**

The role of AFB<sub>1</sub> in hepatocellular carcinoma is well documented, it is perceived as a serious health hazard in parts of the world where exposure to this carcinogen occurs through dietary consumption of contaminated foodstuffs, and an apparent interaction between chronic active viral hepatitis and AFB<sub>1</sub> exposure exists. The parent compound requires activation to the mutagenic 8,9-epoxide by phase I activating enzymes before it can exert its toxic effects on an organism.

Cancer is a complex, progressive biological process, which, after the administration of a carcinogen, can either evolve by itself, or can be modulated. In order to develop any real understanding of cancer, it is necessary to understand the steps through which target cells and tissues evolve during tumour development. It is evident that cancer in the rat involves a minimum of 7 or 8 steps (Farber, 1984) which involve activation of carcinogen and initiation of individual cells, cell proliferation to form resistant hepatocyte nodules (promotion), and progression of these nodules to form HCC with eventual invasion and metastasis. Chemicals act on tissues thus evoking responses by the cells or tissues, therefore the toxic manifestations of exposure to xenobiotics are the result of mutual interactions between the agent and the target tissue. It is important therefore to gain information about the physiological and biochemical nature of initiated cells as they progress through the stages of tumourigenesis.

Hepatic nodules, found in rat liver after exposure to carcinogens, including AFB<sub>1</sub>, display an acquired resistance to xenobiotics. This is due to down regulation of phase I enzymes, and over expression of phase II detoxification enzymes including the GST A5 subunit and AFAR, and such preneoplastic lesions have been proposed as end points for carcinogenicity testing. The majority of hepatic nodules regress. However, the phenotype of those remaining, has eventually become constitutive, not induced, thus

enabling such nodules to go on to form HCC, by clonally outgrowing their sensitive counterparts when treated chronically with carcinogens (Farber, 1984). This is due to their increased capacity to deal with otherwise toxic doses of xenobiotics and can therefore be considered as a physiological adaptation to survival in a hostile environment. However, in doing so, this also allows for the survival of cells possessing potentially carcinogenic genetic mutations. An increase in apoptosis has also been reported in these nodules (Schulte-Hermann *et al.*, 1995) and apoptosis may therefore represent a mechanism for protecting the organism from cells that have acquired genetic alterations that predispose them to cell proliferation.

## 6.2 Detoxification of AFB<sub>1</sub>

The GSTs are a multigene family of phase II isoenzymes that catalyse the nucleophilic attack of glutathione on electrophilic xenobiotics and toxic metabolites. The protective effect of the GST A5 subunit against AFB<sub>1</sub> toxicity has been shown in this thesis, and it can be concluded that its presence in a cell or tissue makes a major contribution to determining sensitivity to AFB<sub>1</sub>. This, along with the inducibility of the subunit in a cell line in which it is not constitutively expressed, and its expression in ethoxyquin-treated rat liver are all in agreement with the literature. In contrast to other publications, rAFAR1 was found to be expressed in cells exhibiting both the AFB<sub>1</sub>-sensitive and AFB<sub>1</sub>-resistant phenotype, although levels appeared to be lower in the AFB<sub>1</sub>-sensitive cell line than in the resistant cell line. This suggests that A-FAR does not play as vital a role as the GST A5 subunit in protection against the toxic effects of AFB<sub>1</sub>. The P-glycoprotein drug efflux pump was also found to be present in both AFB<sub>1</sub>-resistant and sensitive cell lines, although it appeared to be present at higher levels in the AFB<sub>1</sub>-resistant cell line. Results of this thesis therefore, suggest that resistance to AFB<sub>1</sub>, while achieved by a combination of different protective mechanisms is attributable largely to

the presence of the GST A5 subunit, which has a high affinity for conjugation of the *exo*-AFB<sub>1</sub>-8,9-epoxide with glutathione.

Increased detoxification is a major mechanism of chemoprotection. The induction of both the GST A5 subunit and rA-FAR1 in rat liver by chemoprotectors such as ethoxyquin, butylated hydroxyanisole and oltipraz will confer protection against hepatocarcinogenesis by effectively eliminating the harmful AFB<sub>1</sub>-epoxide from cells, thus preventing accumulation of adducts with DNA and proteins. This has implications for the human situation, and oltipraz is currently being considered for human use to protect against AFB<sub>1</sub>-induced hepatocellular carcinogenesis, although recent studies suggest that it is not as effective a chemoprotector in humans as it is in the rat. Induction of GST subunits, particularly GST-P but also members of the GST alpha family, has implications in chemotherapy and can lead to selection of hepatocytes that are resistant to chemotherapeutic agents in tumour lines (Wang and Tew, 1985).

### 6.3 Cell Death by Apoptosis or Necrosis

Results of this thesis showed that cell death following an acute dose of AFB<sub>1</sub> appeared to be largely by necrosis. However, the higher endogenous level of apoptosis seen in the AFB<sub>1</sub>-resistant JB1 cell line compared with the sensitive RLE cell line, may be an adaptive response of transformed cells caused by the overexpression of TGF- $\beta$  (Panse *et al.*, 1997) and TGF- $\beta$  receptors (Mills *et al.*, 1995), and possibly represents an early anticarcinogenic control mechanism *in vivo*. Regulation of cell death through apoptosis, has a critical influence on the multiple stages of hepatocarcinogenesis (Goldsworthy *et al.*, 1996), as an increase in apoptosis will decrease the number of DNA damaged cells preventing clonal expansion of initiated cells; liver tumour growth has been shown to be significantly inhibited by increasing the frequency of apoptosis (Mills *et al.*, 1995). Alternatively, a failure of apoptosis results in inappropriate survival of initiated cells. The promoting activity of many chemical promoters is due to their ability to inhibit

apoptosis in initiated cells (Stinchcombe *et al.*, 1995; Worner and Schrenk, 1996) and mutations in the p53 tumour suppressor gene, an initiator of apoptosis, are commonly seen in HCC after AFB<sub>1</sub>-exposure (Ozturk *et al.*, 1991; Mace *et al.*, 1997). The acquisition by a tissue, of mechanisms that protect its cells from induction of apoptosis may therefore have implications for the control of carcinogenesis at a point beyond the transformation event.

An increase in cell proliferation is apparent during the promotion stage of hepatocarcinogenesis (Hikita *et al.*, 1997), and mutations in oncogenes, such as *ras* which codes for a growth stimulatory protein, often seen after exposure to AFB<sub>1</sub> in rats (Sinha *et al.*, 1987; McMahon *et al.*, 1986) are therefore of importance in the process of carcinogenesis. Cytotoxicity of AFB<sub>1</sub> in the liver, leading to replacement division and regeneration is interrelated with genotoxicity since rapidly dividing cells are more at risk from mutations owing to limited time for DNA repair.

#### 6.4 The Human Situation

The *in vivo* contribution of protective enzymes to provide protection against xenobiotics is difficult to assess in humans, since most studies carried out in this area involve rodents. However, molecular epidemiology can be utilised to show an association between disease and the existence of genetic polymorphisms in the population, which lead to the absence of particular isoenzymes, for a limited number of GST genes. Indeed, studies in humans suggest that individuals that are homozygous nulled at the *GSTM1* locus may have an increased risk of developing cancer of the bladder, lung, colon, skin and stomach (Wiencke *et al.*, 1990).

### 6.5 Analysis of Cell Death

A need exists for the development of accurate methods for the quantitative analysis of apoptosis when investigating the mechanisms of chemically induced carcinogenesis. A novel method was devised in this thesis, the ELISA/MTT assay, which measured the levels of PS exposure on the outer membrane of adherent cell lines as an indicator of the levels of apoptosis or necrosis in response to acute exposure to AFB<sub>1</sub>. However, this failed to define adequately between the necrotic and apoptotic populations of cells, and the use of the LSC is recommended for annexin V-FITC/PI stained adherent cells.

With regard to the AFB<sub>1</sub> cytotoxicity studies, quail liver microsomes have a cytotoxic effect on the RLE cell line, possibly due to the formation of O<sub>2</sub> free radicals, and a concentration of 1% (v/v) is recommended for AFB<sub>1</sub> activation. This same cell line appears to activate the AFB<sub>1</sub> to the epoxide in the absence of an external microsomal activating system, albeit at a slower rate. Choice of AFB<sub>1</sub> diluent is of importance, and unsupplemented DMEM is recommended for the cell lines used in this thesis. The importance of optimisation of all assay conditions is stressed, particularly in the case of the MTT assay.

The ability of AFB<sub>1</sub> to produce either genotoxic or cytotoxic damage is dependent upon the intracellular levels of enzymes responsible for its activation and detoxification, the presence of efflux pumps, adequate DNA repair mechanisms and the sensitivity of the cell to apoptosis in the event of transformation, all of which have an important contribution to make towards the resistant phenotype. When evaluating the results obtained in this thesis, it must be taken into account the effects that rat strain, sex and age, and diet composition have on the levels of enzymes under investigation. Also, it must be remembered that individual homogeneous cell populations will not be subject to the same conditions as in the intact organ and that in the liver, differences in the concentrations of enzymes are seen across the acinus. In addition, although the

morphology of HCC is similar among rats and humans, major elements of etiology, molecular pathogenesis and pattern of expression differ between the two species (Almann, 1994). Therefore results of tests of chemical carcinogenicity in rodents are likely to include a significant number of false positive and false negative risks for humans (Grisham, 1996).

## 6.6 Further work

The LSC is an area for possible further research for investigation of mode of cell death, but optimisation of the technique is required in order to generate statistically valuable data.

In this study, cytotoxicity, rather than genotoxicity was employed as a biological end point, although AFB<sub>1</sub> is a proven genotoxic carcinogen, and only acute treatment with AFB<sub>1</sub> was examined. Another area for further work would be to assess the levels of DNA adducts present in cells treated both acutely and chronically with AFB<sub>1</sub>, and to investigate the levels of PS exposure on the outer membrane after chronic treatment with AFB<sub>1</sub> or over a longer time period after an acute dose.

Further work is required to be carried out to quantitate more accurately the level of expression of each gene product in both cell lines. An interesting new technique for assessing gene expression is that of microarray technology. Briefly; gene expression can be quantitatively analysed by hybridising fluor-tagged mRNA to targets on a DNA microarray (a glass slide on which has been robotically printed thousands of cDNA or oligonucleotide samples. The PCR products of two different samples of mRNA are labelled with two different fluors. These are cohybridised onto a microarray slide: the fluors are excited with lasers and fluorescence is scanned to process an image file and the differential expression of these spots is quantitated from the image file (National Human Genome Research Institute, 1996). The major advantage of this technique is that it can be used to investigate numerous genes in the same sample at once, and so in a

relatively short time, a wider range of genes can be investigated than would be possible by PCR. By comparison with control samples, expression of each gene under investigation can be analysed and quantitated. It would be interesting therefore, to obtain gene profiles for both cell lines used in this project, both before and after treatment with AFB<sub>1</sub>, and for the AFB<sub>1</sub>-resistant cell lines, with respect to the GSTs, AFARs and genes encoding for the many efflux pumps which may possibly be involved in transport of AFB<sub>1</sub>-epoxide from the cell. In addition, other genes involved in the biometabolism of AFB<sub>1</sub>, e.g epoxide hydrolase,  $\gamma$ -glutamyl transpeptidase and members of the P450 3A gene family (responsible for primary detoxification of AFB<sub>1</sub>), could be looked at, as well as DNA repair enzymes. The p53 status of the cell lines could also be investigated and compared, as could that of *bcl-2* and other proto-oncogenes. To coincide with the flow cytometry results, investigation of the expression of genes required for apoptosis, such as *bax*, *bak* and *bad*, and the various caspases, would indicate whether or not the cell lines are, in fact, inducing any apoptosis in response to AFB<sub>1</sub> exposure. This would also go towards confirming whether or not the JB1 cell line does actually possess an inherently greater capacity for induction of apoptosis than the normal RLE cell line.

In this way therefore, the regulation of these genes, before and after, exposure to AFB<sub>1</sub> could be more thoroughly and widely examined, thus enabling a more complete picture to be obtained with regard to the expression of genes involved in both cellular resistance to carcinogens, and the multistep process of carcinogenesis.

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