AN IMMUNOHISTOCHEMICAL AND MOLECULAR STUDY OF PUTATIVE PREMALIGNANT LIVER CELL POPULATIONS INDUCED BY AFLATOXIN B_1.

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

Lucinda Roberta Weir, B.Sc. (Honours) with D.I.S., (University of Ulster).

Department of Carcinogenesis

Toxicology Unit

Medical Research Council

March 1997

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

An immunohistochemistry and molecular study of putative premalignant liver cell populations induced by Aflatoxin B_1 - Lucinda Roberta Weir

Alterations in the phenotypic and genotypic expression in foci of altered hepatocytes (FAH) and tumours in livers of rats treated with aflatoxin B_1 (AFB_1) were analysed. Expression of the phase I and phase II drug metabolising enzymes, P450 2C11, glutathione S-transferase (GST) 7-7, γ-glutamyl transferase and novel aldehyde reductase, with high activity towards AFB_1-dihydrodiol, were examined. A monoclonal antibody recognising GST Yc_2, a phase II enzyme which displays high conjugating activity towards the AFB_1-epoxide, was prepared and used to determine its expression in AFB_1 treated livers. Tissues were also examined for the presence of genetic mutations in the Ki- and N-*ras* oncogenes, previously reported as occurring frequently in the livers of AFB_1-exposed rats. Codon 243 of the *p53* gene, corresponding to codon 249 of the human gene, identified as a mutation "hotspot" in aflatoxin-exposed populations, was also examined. Genetic analyses were carried out by PCR and sequencing of fixed tissue following identification of the FAH and lesions on serial sections using the phenotypic markers. Co-ordinate expression of GST 7-7 and AR was seen in single cells and FAH promptly following treatment, suggesting a similar mechanism of transcriptional control for these proteins. Subsequent induction of GST Yc_2 remained increased, despite removal of the carcinogen after twelve weeks. The final tumours were also strongly positive. A mechanism of resistance was confirmed through measurement of the level of AFB_1 adducts present in the livers of animals containing high levels of phase II drug metabolising enzymes. No mutations were detected in the *ras* or *p53* genes in the lesions at any of the stages of development examined. These mutations, therefore, cannot be essential in the process of AFB_1-induced hepatocarcinogenesis in the rat. The model used more accurately reflects the process of hepatocarcinogenesis occurring in humans following chronic exposure to low levels of AFB_1 in the diet than previously published models.

Acknowledgements

Special thanks to Dr GE Neal for being my supervisor during the period of my postgraduate studies without whose extensive knowledge, help and experience this thesis would not have been possible. Thanks also to the Medical Research Council for their generous financial support of this thesis. I would also like to give special thanks to several other people within the MRC Toxicology unit, especially Drs MM Manson and AG Smith, who provided much help and advice in their areas of expertise. In particular I would like to thank Dr MM Manson for her endless supply of support and encouragement and always finding time to talk.

Thanks to those people who proof-read various chapters of this thesis, Drs MM Manson, AG Smith, RM Lawrence and in particular Dr GE Neal for his unending supply of patience and advice and prompt return of all chapters.

Special thanks too to my family who have provided much moral support throughout the duration of my studies despite being many miles across the sea. And a very special thanks to someone who has been there from the beginning through to the end, to Ron without whose constant support, encouragement and friendship I could not have completed.

And finally I'd like to acknowledge those good friends who kept my spirits high during the writing of this thesis.

Dedication

to my husband and best friend,

Ron

"Truth is rarely pure, and never simple."

Oscar Wilde (1854-1900)

Table of Contents

Abbreviations

ABC	Avidin biotin complex
AFB_1	Aflatoxin B_1
AFL	Aflatoxicol
ASO	Allele Specific Oligonucleotide
BSA	Bovine serum albumin
CK	Cytokeratin
DAB	3'3-diaminobenzidine
DEPC	Diethylpyrocarbonate
dNTP	2'Deoxynucleoside 5'triphosphate
DTNB	5,5' -dinitrobis -(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Disodium ethylene diamine tetra acetate
ELISA	Enzyme linked immunosorbent assay
FAH	Foci of altered hepatocytes
FAPY	Formamidopyrimidine
GGT	γ glutamyltransferase
GSH	Glutathione
GST	Glutathione *S*- transferase
HA	Hypoxanthine Azoserine
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
KLH	Keyhole limpet cyanin
IMS	Industrial methylated spirits
MBS	*m*-Maleimidobenzoyl-*N*-hydroxysulphosuccinimide-3-acetate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PG	Pseudoglandular
RIA	Radioimmunoassay
RT	Room temperature
SDS	Sodium dodecyl sulphate
TEMED	NNN'N' tetramethylethylenediamine
TMB	3,3',5,5'-tetramethylbenzedine
TBS	Tris buffered solution

Chapter One

General Introduction

General Introduction

1.1 The process of carcinogenesis

1.1.1 Multistep progression

Carcinogenesis is a multistage process (Fig 1.1). Following the administration of a carcinogen there is a latency period before the appearance of tumours. During this period many steps occur. In chemical carcinogenesis mutation or loss of specific genes have been hypothesised to determine each step in this progression (see review Balmain and Brown, 1988). Initiation is the primary and essential step in the process and initiated cells can persist in the body for the life-time of the individual, remaining latent until a further stimulus forces them into the next step. Initiation occurs by the induction of alterations in a few cells some of which divide to generate small clusters of cells known as foci of altered hepatocytes. This clonal expansion is the most obvious change seen in promotion and is found consistently in liver carcinogenesis long before the appearance of hepatocellular carcinoma. Only after further mutational changes and new cell population(s) have been produced does the malignant phenotype appear. Some altered cells have the ability to remodel or redifferentiate to normal appearing cells. This is seen in chemical hepatocarcinogenesis after the removal of the carcinogen. Solt and Farber, (1976) in their resistant hepatocyte model, showed that 90% of the nodules regressed through redifferentiation by about eight weeks after initiation. The small minority of nodules that remained were the sites of further changes leading to liver cancer. Several studies have concluded that these foci represent 'initiated' hepatocytes that can be selected by secondary mutation(s) to express the malignant phenotype (Farber,1984; Bannasch and Zerban, 1992; Pitot and Sirica, 1980). Each of these successive events is likely to make the cell genetically more unstable so that the risk of subsequent changes increases.

1.1.2 Foci of altered hepatocytes (FAH)

Many of the persistent foci or nodules present after the removal of the carcinogen show remarkable similarity in cell organisation, architecture and histochemistry. They also appear phenotypically different to the normal phenotypic pattern seen in the liver during any stage in its development. This consistent pattern is seen in many different carcinogenic models (see reviews Bannasch and Zerban, 1992; Pitot *et al.,* 1982 and Farber, 1984). As a result these foci of altered hepatocytes (FAH) have become useful indicators of carcinogenic response in rat (Table 1.1).

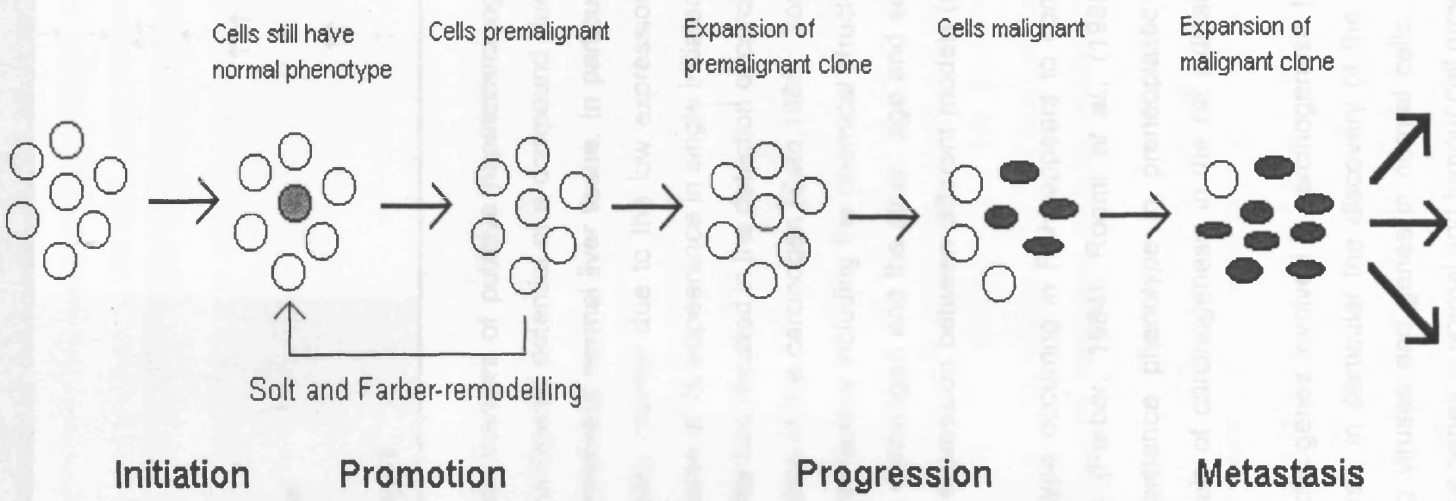


Figure 1.1 Multistage progression of cancer

(adapted from MacDonald and Ford, 1991)

Table 1.1

Common biochemical pattern of some of the phase I and phase II components seen in FAH using several different models of carcinogenesis. (Data compiled from (Roomi *et al.*, 1985; Farber, 1984)

Biochemical Phenotype	Pattern seen in FAH
Cytochrome b_5	↓
Cytochrome P450	↓
DT diaphorase	↑↑
Epoxide Hydrolase	↑
γ-glutamyl transferase	↑↑↑
Glutathione S-transferase	↑
GSH	↑↑
UDP-glucuronyltransferase I	↑

Some of these enzymatic markers of putative hepatocarcinogenesis appear to be more suitable for predicting carcinogenic potential of a compound due to their stability and low or lack of expression in surrounding normal liver tissue. In particular GST-P or 7-7 has been used extensively as an early marker due to the low expression in surrounding liver tissue in adult rats but also because of its appearance in single initiated cells long before the appearance of other markers. This has resulted in the detection of carcinogenic potential at a very early stage after administration of the carcinogen (Sato,1988). Some other markers are found to be influenced by several factors including the chemical structure of the carcinogen and duration of exposure to the carcinogen and the strain, age and sex of the animals and are therefore less consistent in expression between different models (see review Bannasch and Zerban, 1992).

The biochemical phenotype occurring in FAH appears to confer resistance to the cytotoxic effects of xenobiotics (Farber, 1984). Roomi *et al.*, (1985) found a common biochemical pattern of the resistance phenotype in preneoplastic hepatocyte nodules generated in four different models of carcinogenesis in the rat indicating this pattern as a general carcinogenic response.

The identification of specific genes involved in carcinogenesis has greatly enhanced our understanding of the process, in particular the discovery of the relationship between oncogenes in tumour producing viruses and genes in normal cells. This led to intensive research into the function of these genes in normal and tumour cell growth and development. For example, transfection of the *ras* oncogenes into cells in culture was shown to induce transformation in certain immortal cell lines, but was unable to completely transform normal, early passage cells. This suggested necessary further event(s) was required in normal

cells before they were susceptible to the effects of the *ras* oncogene. In the presence of both *myc* and *ras* oncogenes the cells were rapidly transformed (see reviews Barrett and Fletcher, 1986 and Balmain and Brown, 1988). Another group of genes, the tumour suppressor genes, have also been widely studied to determine their role in the process of carcinogenesis. Less information is available on the role of tumour suppressor genes compared with the oncogenes, primarily reflecting their more recent identification. The most widely researched tumour suppressor gene is the *p53* gene located on the short arm of chromosome 17. It is found mutated and/or deleted in a wide range and number of tumours and is therefore likely to play an important role in carcinogenesis (MacDonald and Ford, 1991).

1.2 Aflatoxins

In the 1960's a fatal disease struck turkeys in the southern and eastern parts of England (Blount, 1961). This disease was named turkey 'X' disease and reports of similar poisonings in chickens and ducklings were made soon after its discovery (Shank, 1977). All were indicated by acute hepatic necrosis, bile duct hyperplasia, weight loss and eventually death. The toxins causing these poisonings were found to be present in the groundnut meal used for feeding which was heavily contaminated by the *Aspergillus* genus of mould.

These toxins were characterised chemically and designated aflatoxins (Asao *et al.*, 1965). Aflatoxins are the secondary metabolites produced by some species of the *Aspergillus* mould group i.e. *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nominus*. They naturally contaminate foodstuffs such as corn, peanuts and cottonseed. The incidence and severity of contamination with aflatoxins, indeed any mycotoxins, depends on inoculum and environmental conditions. Although they are found world-wide, the most serious outbreaks occur in areas of high temperature and humidity and in crops damaged due to growth under stress conditions especially drought or insect damage (see review Wilson and Payne, 1994).

There are four main aflatoxins which occur in contaminated food, AFB_1, AFB_2, AFG_1 and AFG_2. *A. flavus* typically produces only B aflatoxins whereas *A. parasiticus* produces both B and G aflatoxins. AFB_1 was established as the most potent hepatotoxin followed by AFG_1, AFB_2 and AFG_2 in order of decreasing potency (Wong and Hsieh, 1976). AFB_1, as well as being the most biologically active, is also the most abundant aflatoxin found in contaminated food (see review McLean and Dutton, 1995; Wogan, 1977).

1.2.1 Physical characteristics of the aflatoxins

Aflatoxins are very stable to high temperatures, with AFB_1 having a melting point of 268-269°C and AFG_1 a melting point of 244-246°C (Asao *et al.*, 1965). They are susceptible

to degradation under alkaline conditions due to the presence of a lactone ring in the structure.

Aflatoxins are freely soluble in apolar solvents, for example, chloroform and moderately polar solvents, for example, methanol and especially dimethylsulphoxide. They can be distinguished using long-wave ultraviolet (UV) detection systems, for example, AFB_1 (λ_{max}^{ETOH} 223, 265 and 362 nm) and AFG_1 (λ_{max}^{ETOH} 243, 257, 264 and 362 nm). The B aflatoxin compounds, for example, AFB_1 fluoresce blue (λ_{max} 425 nm) and the G aflatoxin compounds, for example, AFG_1 fluoresce green (λ_{max} 450 nm). The subscripts relate to their relative chromatographic mobility (Asao *et al.,* 1965). Although UV absorption detection systems can accurately quantitate and identify the aflatoxins, other methods are available which may be suitable under certain circumstances. When antibodies to aflatoxin are available, immunochemical methods such as enzyme linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) can determine and measure aflatoxins in a short time. However the identification of the aflatoxin and the form in which it is present are dependent upon the specificity of the antibody (see reviews Truckess and Wood, 1994 and McLean and Dutton, 1995). Therefore these methods are best used in conjunction with other analytical methods.

1.3.1 Metabolism of AFB_1

A. Phase I metabolism

The biological activity of the aflatoxins depends on their ability to be metabolised. As AFB_1 is the most potent of the aflatoxins, work has concentrated on its biotransformation pathway (Fig 1.2). Almost all of its detrimental effects can be attributed to the electrophilic 8,9-epoxide species formed by the mixed function oxygenases resulting in epoxidation of the terminal furan ring double bond (Swenson *et al.,* 1974; Forrester *et al.,* 1990). Many studies have been carried out to establish the enzymes involved in the epoxidation of AFB_1 and several cytochrome P450 enzymes appear to be responsible (Forrester *et al.,* 1990; Aoyama *et al.,* 1990; Sengstad and Wurgler, 1994; Robertson *et al.,* 1983; Shimada and Nakamura, 1987). In humans the proteins expressed by two P450 gene families, the *CYP*IIIA and *CYP*IA gene families, have been postulated as important enzymes in this function. It is thought that the efficiency of their function varies depending on substrate level. Gallagher *et al.,* (1994) examined the roles of CYPIA2 and CYPIIIA4 enzymes in the bioactivation of AFB_1 and reported CYPIA2 as having a high affinity for the bioactivation of AFB_1 at low substrate concentrations and CYPIIIA4 having more activity at high substrate concentrations. In rats also several P450 forms appear to function in the bioactivation of AFB_1, in particular the CYP2C family (Shimada *et al.,* 1987). Of note is the CYP2C11 form which has a high affinity for AFB_1 and is not only rat specific but also male specific (Nelson *et al.,* 1993). The

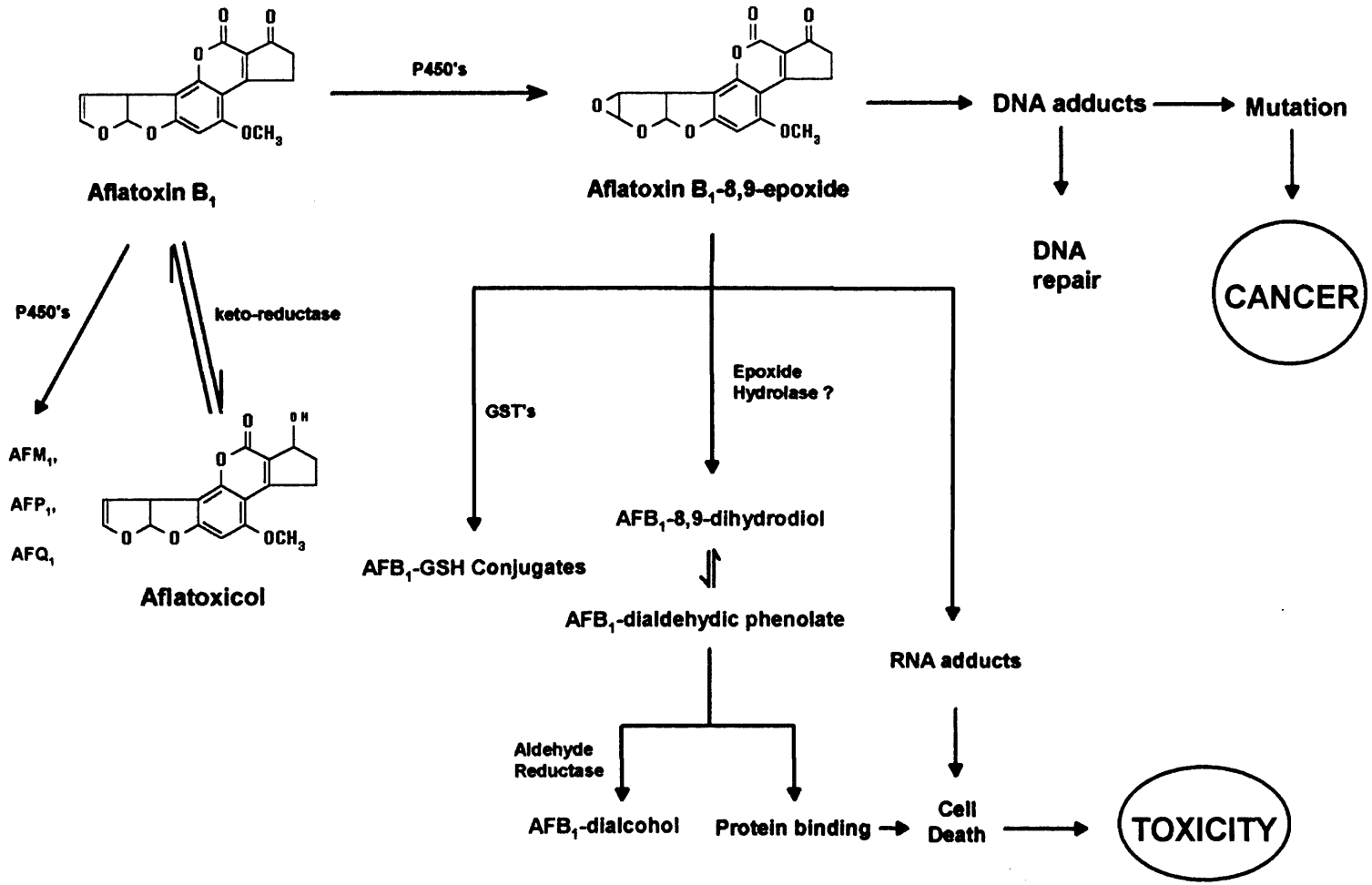


Figure 1.2 Biometabolism of Aflatoxin B_1

implications of these species differences in ability to bioactivate the aflatoxins in part explain the variation in both inter and intra-species susceptibility to the effects of AFB_1. The epoxide is capable of alkylating nucleic acids to form adducts with guanine, forming the basis of its mutagenicity and carcinogenicity. Two structural forms of the epoxide are formed by the P450 monoxygenase system *i.e.* the *exo* and *endo* isomers. Only the *exo* isomer reacts with DNA to give a *trans* adduct (Iyer *et al.,* 1994; Essigman *et al.,* 1977).

AFB_1 may also be reversibly converted by a keto-reductase in the presence of NADPH to aflatoxicol A (AFL). It is almost as potent as AFB_1 both as a carcinogen and a mutagen, as it is oxidised readily back to AFB_1. As a result it can act as a store for AFB_1 *in vivo*. AFL is found as a major metabolite in rat plasma. Other oxidations of AFB_1 by microsomal P450, form hydroxylated polar metabolites such as AFM_1, AFP_1 and AFQ_1 which are less toxic than the AFB_1-epoxide and can be, following conjugation, eliminated by hepatocytes (see review Eaton *et al.,* 1994b).

B. Phase II metabolism

As the epoxide is labile in aqueous solution it quickly hydrolyses to AFB_1-8,9-dihydrodiol which, in the phenolate resonance form, forms adducts with proteins which may cause the acute toxic effects of AFB_1 (Eaton *et al.,* 1994b). The majority of the AFB_1 adducts occurring with proteins, in both chronic or acute exposure, are formed with albumin, as shown by Wild *et al.,* (1986) in their analysis of plasma proteins using Sephadex G-200 chromatography, regardless of whether the exposure to AFB_1 was chronic or acute. In particular AFB_1 was shown to react primarily with lysine residues of albumin (Sabbioni *et al.,* 1987). A role of epoxide hydrolase in catalysing the hydrolysis of AFB_1-epoxide reaction, forming 8,9-dihydrodiol, has been suggested, but this function remains unclear. Studies carried out adding epoxide hydrolase inhibitors to microsomal incubations with AFB_1 did not result in increased dihydrodiol formation or DNA binding (see review Eaton *et al.,* 1994b). It is possible the role of this enzyme may vary from species to species helping to account for the species differences in susceptibility to aflatoxins. A recent study by McGlynn *et al.,* (1995) showed a significant association between the presence of AFB_1 adducts and the epoxide hydrolase genotype in humans. This was extended to a correlation between HCC and the epoxide hydrolase genotype and suggested the role of variation in genotype of the epoxide hydrolase gene in accounting for the different HCC incidence found in Ghana and China.

Recently it has been reported that a novel aldehyde reductase is responsible for catalysing the reduction of the AFB_1-dihydrodiol, in the dialdehyde form at physiological pH, to AFB_1 dialcohol. This enzyme is dissimilar from any previously described reductase. It is inducible by antioxidants including ethoxyquin and is present at higher levels in foci of

altered hepatocytes than in normal parenchyma. The dialcohol does not form adducts with protein (Judah *et al.,* 1993; Hayes *et al.,* 1993; McLellan *et al.,* 1994).

Detoxification involves conjugation of the epoxide mainly to glutathione through the action of glutathione *S*-transferases (GST's) (Neal *et al.,* 1987; Degen and Neumann, 1978). This conjugate is then further metabolised via the mercapturic acid pathway and excreted in the urine. One of the important enzymes in this pathway is γ-glutamyltranspeptidase (GGT). It is located on the plasma membrane of many cells including bile duct epithelium and renal epithelial cells, with the active site positioned extracellularly. Following the administration of AFB_1, GGT activity has been noted to be induced (5-10 fold over control levels) in the liver, occurring in cells not normally showing detectable levels of GGT *i.e.* foci of enzymatic altered hepatocytes (Moss *et al.,* 1984). GGT catalyses the cleavage of GSH to a γ-glutamyl moiety and cysteinylglycine which is subsequently further degraded into its two constituent amino acids. These can then be reabsorbed and subsequently recycled into GSH via the γ-glutamyl cycle (Fig 1.3). As GSH is not reabsorbed in the tripeptide state, GGT plays an important role in continuing the necessary recycling of GSH. Similarly it catalyses the cleavage of GSH conjugates to allow reabsorption and subsequent excretion of the compound via the mercapturic pathway. In the case of AFB_1, the cysteinyl moiety remains attached to the AFB_1 following cleavage by dipeptidase which is then acted upon by N-acetyl transferase to form the mercapturate (Fig 1.3) (see review Lieberman *et al.,* 1995; Neal *et al.,* 1988; Stryer,1988).

There are great differences in the substrate specificities of the glutathione *S*-transferase isomers and these differences account for some of the species variation to the toxic effects of AFB_1. For instance, adult rat liver cytosol has high activity toward the *endo* form and little detectable activity toward the *exo* form of the AFB_1-epoxide, whereas adult mouse liver cytosol almost exclusively conjugates the *exo*-epoxide. Human liver cytosols conjugate both forms of the AFB_1-epoxide, but at very low levels compared to either the mouse or rat liver cytosols (Raney *et al.,* 1992). Of particular interest are the constitutively expressed transferases in the mouse and the rat. Analysis of these enzymes in mice has revealed an alpha class glutathione *S*-transferase (GST Yc) with very high specificity towards the AFB_1-epoxide (Hayes *et al.,* 1992). No major constitutively expressed glutathione *S*-transferases in the adult rat appears to have a similar high specific activity. However Hayes *et al.* (1991) reported an alpha class transferase induced in rat showing approximately 85% homology to the constitutively expressed mouse transferase with high specific activity to the AFB_1-epoxide. This rat enzyme, GST Yc_2, was found to be inducible by ethoxyquin, an antioxidant shown to afford protection to the toxic and carcinogenic effects of AFB_1 (Cabral and Neal, 1983), and also to be present in preneoplastic lesions generated by exposure to

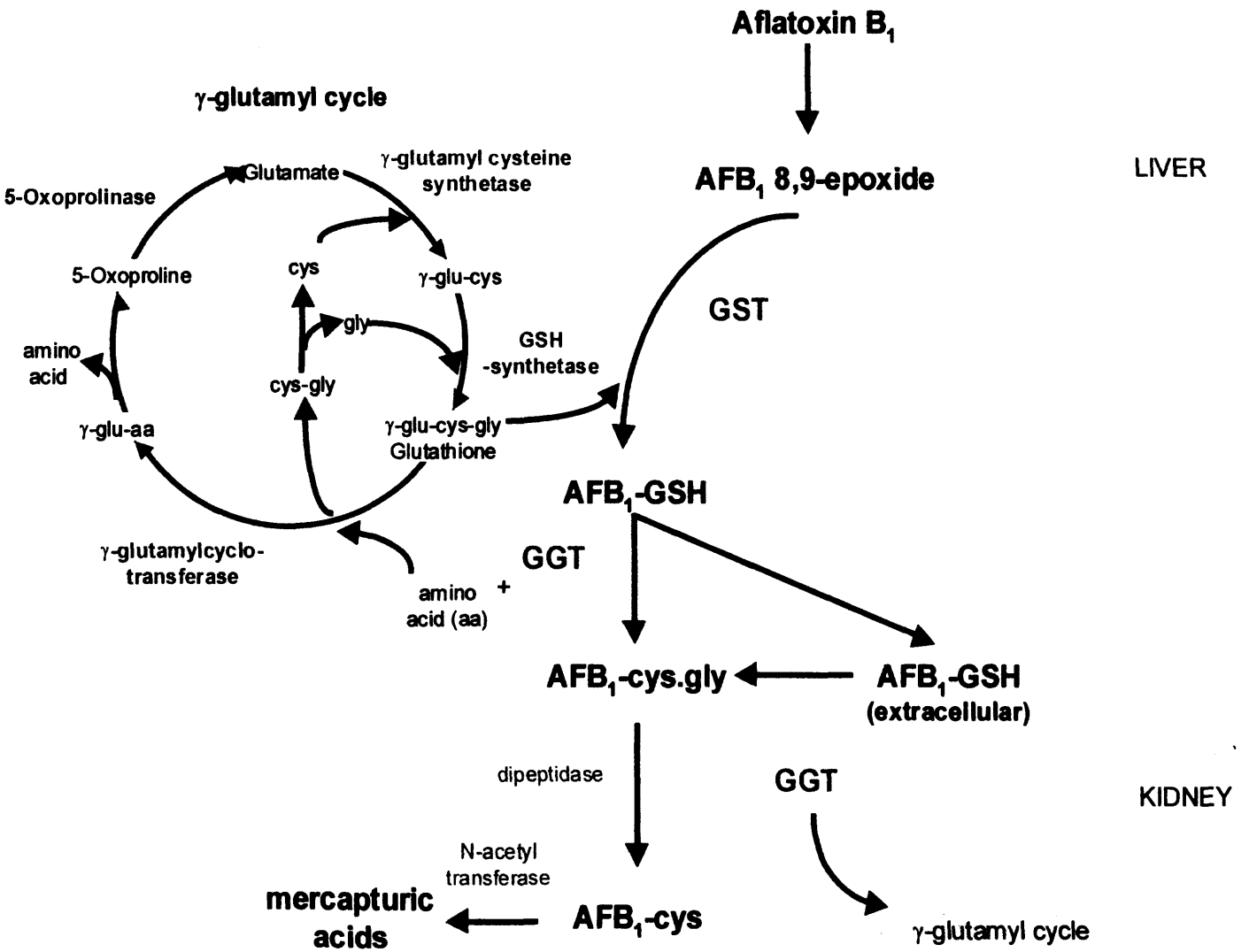


Figure 1.3 GSH conjugation and deactivation pathway

(adapted from Stryer, 1988 and Neal *et al.*, 1988)

AFB_1. In the rat one constitutively expressed alpha class transferase, Yc_1, shows very high homology (91%) to the inducible GST Yc_2 but lacks its ability to conjugate effectively the AFB_1-epoxide (Hayes *et al.,* 1994). These proteins, differing in only a few amino acids have provoked interesting studies into the mechanisms involving the specific ability of one glutathione *S*-transferase to conjugate the AFB_1-epoxide over another. The role of GST in the detoxification of AFB_1 in human liver remains to be evaluated as most of the experimental studies have concentrated on the primary P450 catalysed metabolic reactions involving AFB_1. However it would appear that GSH conjugation does not play as pivotal a role in the metabolism of AFB_1 in humans as in rodents (Kirby *et al.,* 1993; Hayes *et al.,* 1991a). Purified human μ class GST's have shown the highest conjugation activity against the *endo* AFB_1-epoxide and interestingly the μ class of GST's is polymorphically distributed in the human population with about 50% of Caucasians possessing a gene deletion for this enzyme (Eaton *et al.,* 1994a). McGlynn *et al.,* (1995) demonstrated a tentative link between the GSTM1 genotype and HCC in areas with high exposure to aflatoxins. Ghanaian men displaying the null genotype were more likely to have detectable levels of AFB_1 adducts than those men with GSTM1 present. However, the constitutive ability to conjugate the AFB_1-epoxide is low (Raney *et al.,* 1992). It is possible there may be an induced GST in humans, as in the rat, which has high specificity for AFB_1. This induced GST would presumably have high homology to the mouse or rat GST alpha class forms.

1.4.1 Toxicity, mutagenicity and carcinogenicity of aflatoxins

A. Toxicity of aflatoxins

Since the discovery of aflatoxins in their role as potent hepatotoxins in poultry and other species, many experimental studies have reproduced in laboratory and domestic animals the same hepatotoxic effects of acute structural and functional damage to the liver (see reviews Cullen and Newberne, 1994; Newberne and Butler, 1969; Barnes and Butler, 1964). These effects include necrosis in the liver and occasionally in other organs such as the heart, kidney, spleen and pancreas. The production of clotting factors, eventually resulting in hepatic failure, may be responsible for this necrosis. Liver regeneration ensues with an increase in mitotic index and DNA synthesis. Fatty infiltration of the parenchymal cells and bile duct proliferation are also common indices of acute toxicity (Dwivedi *et al.,* 1993).

Although all species examined to date have been found to be susceptible to the effects of aflatoxins, a wide variety of response exists due to the rate and ability of the species to metabolise and detoxify these toxins (see review Cullen and Newberne, 1994). Acute toxicity assessed by LD_{50} values encompasses two orders of magnitude from highly susceptible species such as ducklings or rabbits (0.34 and 0.3 LD_{50} mg/Kg respectively) to

very resistant species such as hamster or chicken (10-12 and 15-18 LD_{50} mg/Kg respectively). Intraspecies variation also occurs, rats are more sensitive to the effects of aflatoxins than mice but within the rat species the Fischer 344 strain is more sensitive to acute toxicity than the Sprague-Dawley strain (Wogan, 1977). Gender also influences sensitivity to aflatoxin toxicity. There is a greater sensitivity of males compared with females, implying hormonal influences. Several studies have been carried out to investigate such effects (see reviews McLean and Dutton, 1995 and Cullen and Newberne, 1994; Neal and Judah, 1978). Righter *et al.,* 1972 showed complete protection from aflatoxins in the diet in castrated four week old rats, an effect which disappeared with testosterone treatment prior to and during exposure. The age of the animal also determines its sensitivity, although mice are generally more resistant than rats to the acute toxic effects of AFB_1, the neonatal mouse has a very low LD_{50} value (2.0 mg/kg)(Vesselinovitch *et al.,* 1972).

In humans the first case of death proven to be due to aflatoxin exposure was reported in 1970 by Serck-Hanssen in Uganda. A 15 year old African boy died after ingesting cassava heavily contaminated with aflatoxin. As awareness of the problem grew further reports suspected aflatoxin as the cause of death in many cases where symptoms of Reye's syndrome occurred. Reye's syndrome symptoms in humans are very similar to those seen with acute aflatoxicosis in monkeys (Cullen and Newberne, 1994). Shank *et al.,* (1971) reported aflatoxins to be present in specimens from children in Thailand who died of an acute disease of unknown aetiology, showing encephalopathy and fatty degeneration of the viscera, symptoms similar to that seen in Reye's syndrome, manifested by vomiting, convulsions and coma. The most conclusive data for aflatoxicosis in humans however was reported in 1975 by Krishnamachari *et al.* who reported an outbreak of hepatitis in Western India apparently as a result of aflatoxicosis through the ingestion of contaminated maize. Almost 200 widely scattered villages were involved, in which the males were affected twice as commonly as females.

Many factors affect aflatoxin toxicity (see review Cullen and Newberne, 1994), gender and age have already been mentioned, another is diet. Many studies have indicated protein content as an important factor in determining the sensitivity to AFB_1 exposure. A low protein diet results in increased number of toxic liver lesions and vice-versa (Mandel *et al.,* 1992; Wogan, 1977 and Nigam and Ghosh, 1994). But a low level of choline or methionine protects against AFB_1 toxicity (Salmon and Newberne, 1962). Vitamin A deficiency has also been shown to increase the susceptibility of AFB_1 but only in male rats and this can be overcome by supplementation of vitamin A. The addition of certain compounds which affect glutathione levels also alter sensitivity, for example, Oltipraz which elevates GSH levels, induces detoxification of AFB_1 and hence protects against AFB_1 toxicity (Maxuitenko *et al.,* 1993).

Similarly diethylmaleate or buthionine sulphoxamine which decrease GSH levels cause an increase in mortality rates in animals after exposure to AFB_1 (Gopalan-Kriczky *et al.*, 1994).

B. Mutagenicity

In vitro studies have proved the genotoxic capabilities of the aflatoxins in particular AFB_1 (IARC, 1987). Although *in vitro* systems are very limited since the hepatic enzyme systems required to metabolise aflatoxins are difficult to maintain in culture, they have been of use in identifying the cytological effects of aflatoxins. AFB_1 produces unscheduled DNA synthesis, chromosomal aberrations and adduct formation in both rodent and human cell lines.

The principle covalent adduct formed with DNA is the 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-aflatoxin B_1 (N^7-guanyl) adduct which constitutes 80-90% of the total AFB_1 adducts formed with DNA (Essigmann *et al.*, 1977; D'andrea and Haseltine, 1978). Maximum levels of modification occur two hours after exposure to AFB_1 corresponding to 1% of the total AFB_1 exposure. A dose dependent relationship exists between the level of its occurrence in liver DNA and AFB_1 doses over the range 0.125-1.0mg/kg (Croy *et al.*, 1978; Croy and Wogan, 1981). It has a short life of 7.5 hours and 70% of this adduct is removed from DNA during the first twenty-four hours after exposure to AFB_1. Spontaneous depurination at adducted guanine residues, as shown *in vitro,* has been proposed as one method by which the high incidence of G:C → A:T transitions induced by AFB_1 can be accounted (Bailey, 1994). The presence of the apurinic sites at the guanine residues can result the insertion of adenine into these sites which become fixed during replication and result in this specific base substitution. Formation of the second major AFB_1 adduct, 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydro-pyrimid-5-yl formamido) 9-hydroxy AFB_1 (FAPY) adduct, has been reported, formed by alkaline hydrolysis, of 20% of the remaining 30% of the N^7-guanyl adduct, resulting in imidazole-ring opening (Essigmann *et al.*, 1982; Hertzog *et al.*, 1982). This adduct has a much longer half-life as the bond linking it to deoxyribose of the DNA is chemically more stable. Due to the nature of the bulky adduct positioned at a guanine, mispairing can occur during replication resulting in an adenine being inserted opposite these sites giving a high incidence of G:C → T:A transversions.

Primary cell cultures also demonstrate AFB_1-induced cytotoxicity with 50% of human embryo liver cells showing cytotoxic effects after exposure to AFB_1 at 1μg/ml for 24 hours (Zuckerman *et al.*, 1966). In the Ames test AFB_1 was found to be mutagenic using several strains of *Salmonella typhimurium* following activation of the AFB_1 by rat or human microsomal preparations (Wong and Hsieh, 1976; Halvoroson *et al.*, 1988). Due to the mutagenic effects of AFB_1, attempts have been made to identify the genes which may play

an important role in the carcinogenic properties of AFB_1. In particular, genes involved in general cell cycle control or signalling pathways have been examined.

C. Carcinogenicity

Shortly after the discovery of aflatoxins through the hepatotoxicity in turkeys an incidence of hepatocellular carcinomas (HCC) in rainbow trout occurred which was found to be caused by exposure to aflatoxin contaminated diet (Halver, 1969). Following this, many studies were carried out on laboratory animals, particularly rats, demonstrating the potent carcinogenic effects of aflatoxins. Again species variation occurs in the sensitivity to the carcinogenic effects as with the acutely toxic effects of AFB_1 (Newberne and Wogan, 1968). The most sensitive species is the rainbow trout and the most resistant is chickens. Therefore it would appear that the toxicity of AFB_1 is important in its carcinogenic potential. Looking at the hepatotoxic effects of AFB_1 one sees cell death resulting in compensatory cell replication. This cell replication could in itself be the contributing factor linking toxicity and carcinogenesis. Cell replication is occurring in carcinogen-altered hepatocytes which have the ability to survive and replicate in the presence of a general hepatotoxin. In the mouse, typically resistant to the carcinogenic effects of AFB_1, hepatocarcinogenesis is induced by exposure to AFB_1 in the neonatal mouse which has a high rate of cell replication and also after partial hepatectomy where cell replication is induced (Vesselinovitch *et al.,* 1972; Dix, 1984).

Typically, chronic low doses of AFB_1 are most efficient in producing hepatocellular carcinoma (HCC) in rats and this correlates well with the realistic exposure of humans to AFB_1 (Wogan, 1976). Godoy *et al.* (1976) carried out a timecourse study examining the effects of prolonged feeding (6 weeks) of AFB_1 (4 ppm) on adult rat liver in which they examined the histology, zonal centrifugation profile of nuclei and the levels of DNA and RNA synthesis. This feeding regime resulted in 100% incidence of HCC forty weeks later. During the first three weeks of the feeding study, histological observations indicated acute toxicity with inhibition of RNA and DNA synthesis and cell necrosis affecting predominantly the tetraploid hepatocytes situated in the periportal areas. The second three weeks of the study indicated cell proliferation and mitogenesis especially of a high ploidy population of parenchymal cells and oval biliary cells. The third three weeks of the study indicated an increase in this regeneration and re-establishment of the cells damaged by the initial toxic dose. In parallel with this study Neal *et al.,* (1981) reported lower covalent binding of AFB_1 to DNA in animals given a prolonged carcinogenic feeding regime. Inhibition of DNA and RNA synthesis reported as a toxic effect of AFB_1 exposure has been related to the level of covalent binding of AFB_1 to DNA. Therefore the ability to replicate and regenerate in the presence of the toxin implies a mechanism of resistance in these replicating cells. Neal *et al.,*

(1981) suggests three possible mechanisms as to how this process could be occurring: a) reduced metabolism or activation of AFB_1, b) increased rate of repair and/or c) increased rate of detoxification of the AFB_1-epoxide. This and other studies have shown no decrease in the metabolism of AFB_1 to the AFB_1-epoxide. Evidence exists for the increased rate of repair in other carcinogenic systems (Montesano *et al.,* 1980), but Neal *et al.* in this study reported their data as being inconsistent with this theory. However other studies have shown up to 40% repair of AFB_1 adducts after six hours indicating that significant repair does occur. In addition interspecies variation has been reported in the repair capacity of AFB_1 adducts which may contribute to the differences in species susceptibility (see review McLean and Dutton, 1995). Despite this it would appear that this resistance mechanism is largely due to the increase in detoxification ability toward AFB_1. Histochemical analysis indicated increasing numbers of foci of altered hepatocytes containing elevated levels of GGT and possessing high levels of GSH.

Ethoxyquin (EQ), an antioxidant widely used in animal food preservation, has a protective effect toward AFB_1 carcinogenesis. This protective effect afforded by EQ has been reported to be due to changes in the ability to metabolise and detoxify AFB_1 (Kensler *et al.,* 1986). Mandel *et al.,* (1987) described increases in both cytochrome P450 and GST activity in animals treated with 0.5% EQ in the diet. If these animals were also exposed to AFB_1 reduced DNA-adduct formation occurred. This effect appeared to be dose related and present only while EQ was being administered. In the absence of ethoxyquin the effect quickly 'wore off' (Cabral and Neal, 1983). Tsuda *et al.,* (1988) reported levels of P450's, GST's, GGT and epoxide hydrolase, induced in the periportal region by EQ and another antioxidant, butylated hydroxyanisole (BHA), which resulted in inhibition of hepatocarcinogenesis after treatment with *N*-ethyl-*N*-hydroxyethylnitrosamine, indicating this response is a general one. Dietary components have been shown to also induce the detoxification enzymes and hence protect against the carcinogenic effects of AFB_1, in particular the cruciferous vegetables such as broccoli, cabbage and brussel sprouts. The components inducing this effect remain largely unknown but one component, isothiocyanate sulphorane, has been postulated due to its known ability to induce GST's in rodent tissues (see review Kensler *et al.,* 1994). Indole-3-carbinol, another natural constituent of cruciferous vegetables, is also known to be a component causing this protection as it is an inhibitor of carcinogenesis in animal models when given either before or during carcinogen exposure (see review Nigam and Ghosh, 1994).

1.5.1 Social Implications of Aflatoxins

A. Geographical distribution of human liver cancer and aflatoxin

If one looked at the world as a whole the incidence of liver cancer would be seen to be a relatively rare condition with a rate as low as two cases per 100,000 per year for the general population (Shank, 1977). However among certain populations in Africa, Southern India, Japan, South America and Southeast Asia the rate of liver cancer is unusually high. This brings liver cancer into the category of one of the three most common types of cancer resulting in at least 250,000 to 1,000,000 deaths per year (see review Buetow, 1992). The type of cancer noted to be primarily increased is that of hepatocellular carcinoma although cholangiocarcinomas and mixed-cell tumours are also seen. In general the sex ratio for primary liver cancer is between 1.0 and 1.5 to 1, with males having the higher incidence but in these areas of high risk for liver cancer this ratio is much increased from between 3 and 6 to 1, depending on the area of discussion, indicating a sex ratio bias. In addition the age at which the liver cancer occurs is much lower in these high risk areas peaking at ages as low as 35 years. In the low risk areas liver cancer is extremely rare before the age of 45 years and the rate increases with age rather than peaking (Shank, 1977).

In an effort to reconcile these differences in the incidence of liver cancer several factors were examined. If one compares a world map indicating the areas where the rate of human liver cancer is unusually high and plots the areas where high levels of aflatoxin contamination in food and exposure have been noted, the two factors correlate very well indeed (see reviews Shank, 1977; Busby and Wogan, 1984)(Fig 1.4). However, it is unlikely that exposure to aflatoxin alone is sufficient to account for the large disparity in incidences of liver cancer and other risk factors have to be considered, for example, that of the role of cirrhosis. In areas having a low incidence of liver cancer, cirrhosis leading to hepatocarcinoma is relatively rare, however in areas with high levels of hepatocarcinoma, cirrhosis appears to play a larger role, usually appearing concomitantly with the liver cancer. Of particular interest in this link between cirrhosis and liver cancer is viral hepatitis, which often leads to cirrhosis and is common in most of the areas where high incidences of hepatocarcinoma occurs. Many studies have indicated the presence of hepatitis-associated antigens in the samples obtained from patients with hepatocarcinoma (Wild *et al.,* 1992; Hatch *et al.,* 1993). In an attempt to calculate the relative risk factors for exposure to the two major risk factors for liver cancer - hepatitis B and AFB_1, Ross *et al.,* (1992) analysed data from urine samples collected from over 18,000 healthy males between the ages of forty-five and sixty-five years from Shanghai. Of these individuals twenty-two developed liver cancer and their samples were analysed for AFB_1 biomarkers and hepatitis B virus surface antigen (HBVsAg) status. The relative risk was found to be approximately 5 in those patients

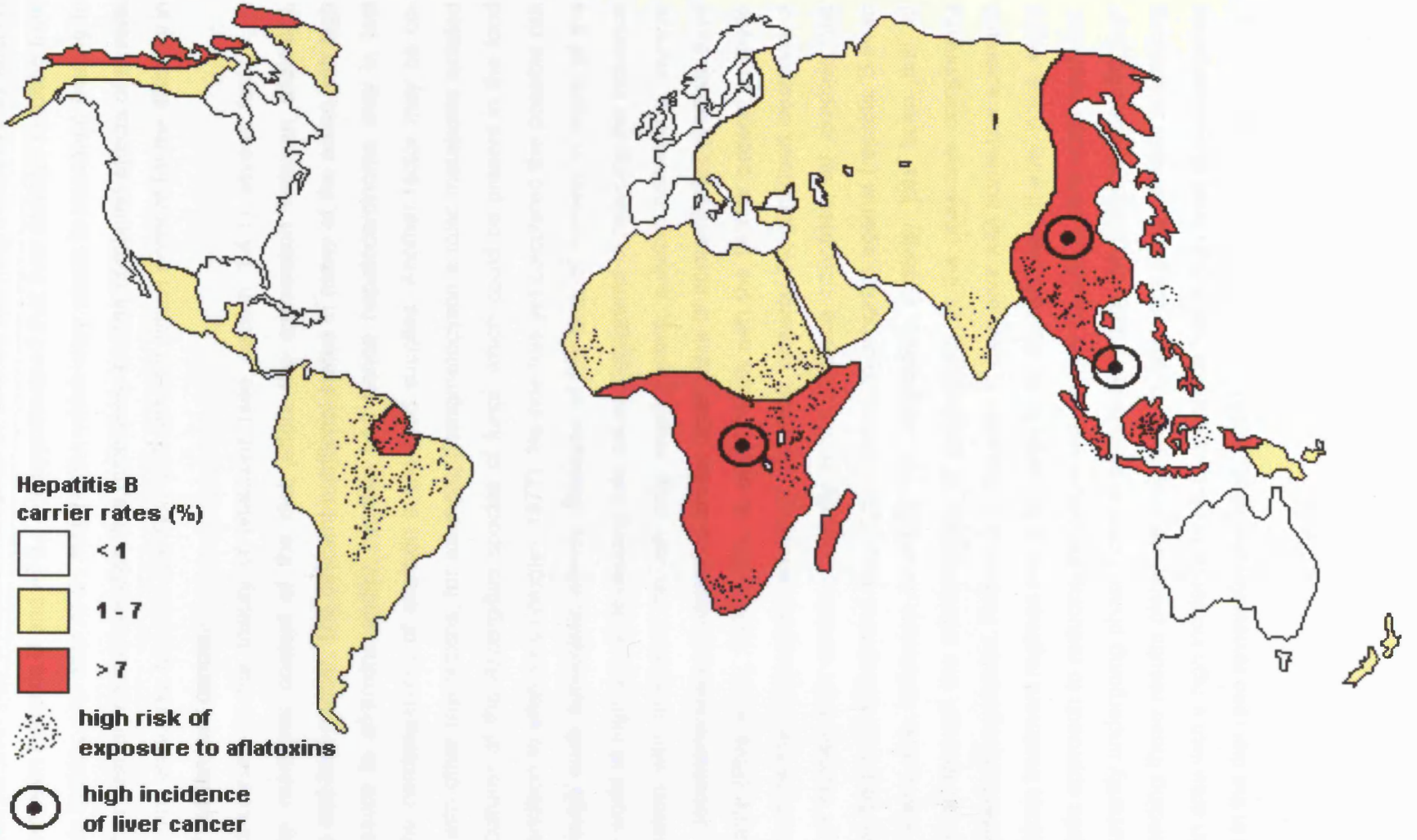


Figure 1.4 World incidence of hepatitis B, aflatoxins and human liver cancer

displaying either AFB_1-N^7 guanine adducts or who tested positive for HBVsAg. But those who tested positive for both had a relative risk factor of over 60 showing conclusive synergistic interaction between hepatitis B and AFB_1. It is hypothesised that liver damage resulting from HBV or other factors, can alter AFB_1 metabolism which may result in increased binding to DNA and other cellular macromolecules. It is most likely it is the combined effects of several factors, including aflatoxins, hepatitis infection and cirrhosis, which account for the disparity in incidence of human liver cancer.

As discussed in the toxicity of aflatoxins (see section 1.4.1) several factors, in particular the nutritional content of the diet, affect the association between aflatoxins, aflatoxicosis and liver cancer. The deficient nutritional status in many of the areas with high risk of exposure to aflatoxins and/or hepatitis and hence hepatocarcinoma may in fact potentiate the carcinogenicity of aflatoxin in the food supplies. Another factor may be co-intoxication with other mycotoxins, for example, Sterigmatocystin a toxic metabolite isolated from other cultures of the *Aspergillus* species of fungi, which could be present in the food supplies in addition to aflatoxins (Wogan, 1977). No-one has yet calculated the possible risk involved through such synergistic effects. Because of the lack of funding in many of the areas of the world at high risk of developing liver cancer, programs of reducing the incidence of contamination with aflatoxins through crop management, suitable harvesting, storage procedures, decontamination of aflatoxins and/or destruction of contaminated produce have not been viable (Piva *et al.,* 1995). This, in combination with the factors present in these areas to potentiate the carcinogenic effects of aflatoxins, ensures the continued existence of high levels of human liver cancer. Recently some of these countries, for example, the Gambia have begun a widespread long term vaccination program against hepatitis B in an attempt to lower the incidence of HCC. An alternative strategy has been that of chemoprotection through the administration of antioxidants or the protective components found in cruciferous vegetables. Research in this area is extensive with numerous scientists exploring various treatment regimes and is too large to be covered here. It may, however, be a more realistic approach to reducing the risk of human liver cancer in third world countries. Oltipraz is currently undergoing phase I clinical trials to determine the potential side effects or toxicity's. Pending these results plans are underway to carry out phase II trials in Quidong city, China an area with a high incidence of HCC and which has a high level of contamination of Aflatoxins in the diet (see review Kensler *et al.,* 1994)

1.3 Early Markers, Oncogenes and Tumour Suppressor genes

1.3.1 RAS genes

The *ras* gene family is a group of cellular oncogenes consisting of three members; Ha-*ras* (the oncogene of Harvey murine sarcoma virus), Ki-*ras* (the oncogene of Kirsten murine sarcoma virus) and N-*ras* (detected in tumours but not in retroviruses). All three mammalian forms of *ras* are localised on the inner surface of the plasma membrane and are ubiquitously found. Although the spliced junctions of all three *ras* genes are identical, the intron regions vary greatly giving rise to vast size differences, for example, Ha-*ras* is 4.5 kbp whereas Ki-*ras* is 50 kbp in length. All three genes have four encoding exons (Ki-*ras* possesses two alternative fourth coding regions) and express a 21 kDa protein (see reviews Hesketh, 1994; MacDonald and Ford, 1991 and Barbacid, 1987). As is seen in the case of AFB_1 exposure in rodents a single point mutation in any of the *ras* genes is sufficient to confer transforming-inducing properties. Many point mutations have been found in *ras* genes, both occurring naturally or induced in *in vitro* mutagenesis studies, localised in many codons, for example, codons 12, 13, 61, 63, 116 and 119 all of which confer transforming properties to *ras* genes. In particular the substitution of glycine at codon 12 by any other amino acid (except proline) or its deletion results in its oncogenic activation (see review Barbacid, 1987). The normal products of *ras* genes are known to bind guanine nucleotides. It functions like a binary switch, in its OFF position it binds the nucleoside diphosphate GDP. In this state it sits on the plasma membrane until it receives a signal which activates it into shedding GDP and binding the nucleoside triphosphate GTP where it then exists in the ON state. Normally the ON state is short-lived as the bound GTP rapidly converts to GDP by the intrinsic GTPase activity possessed by Ras, returning to the OFF state. This process is enhanced by interaction with a cytoplasmic protein, GTPase activating protein, which can stimulate the intrinsic GTPase activity and speed up the transition from Ras-GTP to Ras-GDP. Ras functions as part of a cellular signalling cascade and its position in this cascade has only recently been elucidated (Fig 1.5) (Egan and Weinberg, 1993, Satoh *et al.,* 1992). It changes intracellular metabolism by releasing growth stimulating signals, in the ON state, into the cell in response to signals from the cell surface receptors. Upstream of Ras, in response to a signal, for example polypeptide growth factor binding to its tyrosine kinase receptor, many different effector proteins may be activated. One example is Grb2 which then transduces the signals to Ras. This may be done through the regulation of location or activation of several proteins, for example Sos. This protein probably has many functions, one of which is to activate Ras. Activated Ras in turn activates several effector systems including Raf. Downstream Raf, once activated, phosphorylates a second kinase, MAP kinase kinase or MEK, which controls a third kinase the mitogen-activated kinase MAP

kinase (MAPK). This kinase and one further down the cascade send the signals into the cells by phosphorylating transcription factors which then activate the appropriate gene(s). The signals received by Ras to pass down the signal cascade are varied, of most importance appear to be the growth and differentiation signals, for example, epidermal growth factor, which when disfunctioning could easily induce transformation in a cell.

It must also be remembered that the Ras pathway is only one of many complex cellular signalling cascades which have the ability to cross-talk and components of these pathways such as Ras donate signals to many effectors or targets. Therefore changes in Ras may effect many other signalling pathways. The oncogenic forms of Ras proteins have a greatly reduced GTPase activity and therefore do not turn off. Instead they remain on for long periods of time flooding the cell with growth stimulatory signals (Egan and Weinberg, 1993; Marshall, 1993; Satoh *et al.,* 1992; McCormick, 1993).

A. Activation of ras by AFB_1

The ability of AFB_1 to activate the *ras* oncogene in F344 rats was initially demonstrated by McMahon *et al.,* in 1986 using transfection studies, where Ki-*ras* mutations were found in 20% of the original tumours produced and increased levels of mutant p21 protein expression were also seen. Subsequent studies indicated the preference for these mutations to occur in codon 12. All three *ras* genes have been found to be mutated after exposure to AFB_1 but in particular Ki-*ras* appears to be most prevalent (McMahon *et al.,* 1990; Sinha *et al.,* 1988). Recent studies using more sensitive techniques have found an increase in the percentage of rat tumours, induced by AFB_1, containing ras mutations. Soman and Wogan, (1993) illustrated Ki-*ras* mutations at codon 12 in all of the aflatoxin induced hepatocellular tumours in the F344 rats used in their study. A variety of mutations were noted but all were in the same codon. The predominant mutation observed was a G:C $\rightarrow$ A:T transition in the second base of the codon. At a lower frequency a G:C $\rightarrow$ T:A transversion was seen in the first base and finally a G:C $\rightarrow$ T:A was also seen, but in the second base of the codon. As already indicated, this type of mutation correlates well with the N^7-guanyl adducts formed by AFB_1 with DNA. Mutations in other codons, however, have also been noted, for example, in the c-Ha-*ras* oncogene codon 61 mutations have been found in liver tumours induced by AFB_1 in neonatal mice (Bauer-Hoffman *et al.,* 1990). An increase in c-Ha-*ras* transcription has also been shown in hepatomas induced in male Fischer rats by treatment with AFB_1. In the same study Tashiro *et al.,* 1986 examined the transcription levels of other oncogenes including c-N-*ras*, c-*fos* and c-*myc*, but of these only c-*myc* showed an elevation in expression. Of note is that none of these studies have found an increase in copy number of the gene indicating the gene was not amplified in these tumours (Tashiro *et al.,* 1986; McMahon *et al.,* 1986).

In humans, although *ras* mutations are frequently found in a significant number of a variety of tumour types, little evidence exists as to their importance in hepatocarcinogenesis. As reported in animal studies, mutations in codons 12 and 61 are sufficient for transformation (see reviews Bos, 1988; Barbacid, 1987 and Teich, 1993). Challen *et al.*, 1992 reported infrequent point mutations in codon 61 of c-Ki-*ras* (5.3%) and c-N-*ras* (15.8%) in hepatocellular carcinomas from Britain and Tada *et al.*, 1990 reported no mutations at all in HCC samples from Japan. Nikolaidou *et al.*, 1993 reported infrequent point mutations in codon 12 of Ki-*ras* (12.2%) from Greece. These three studies from diverse geographical locations with dissimilar diet and exposure to environmental factors may indicate that point mutations of the *ras* genes do not play a significant role in the development of human HCC. And more interestingly Leon and Kew, (1995) report no mutations in codons 12,13, and 61 of the three c-*ras* oncogenes in South African Blacks despite the fact that dietary exposure to AFB_1 is a high risk factor in this population.

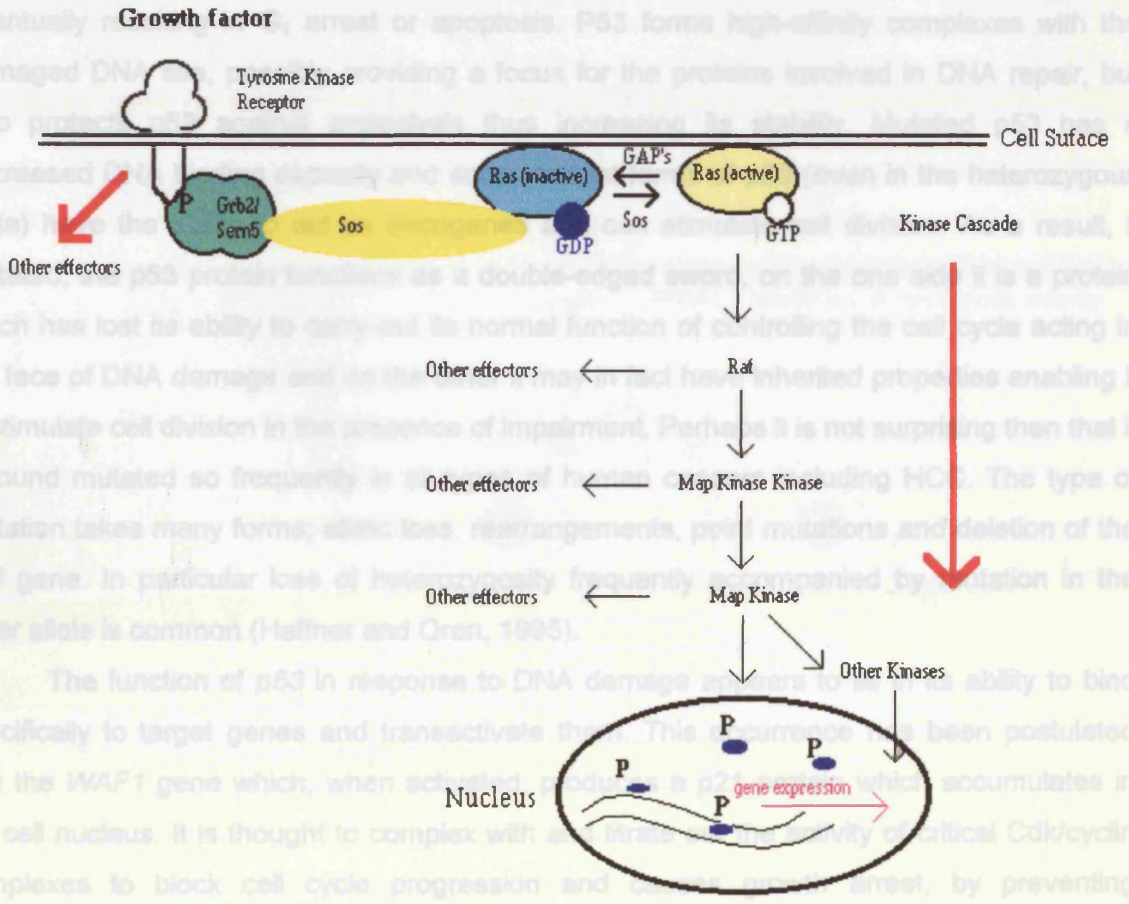


Figure 1.5 Ras Pathway (simplified)

(adapted from Egan and Weinberg, 1993)

1.3.2 P53 *gene*

A. Function(s) of p53

Another gene, this time a tumour suppressor gene, which has received much attention with regard to its role in the progression of human HCC, is the *p53* gene. The *p53* tumour suppressor gene is located on chromosome 17 and is becoming known as the most frequently mutated gene in human cancers. The normal allele of this gene encodes a 53 kDa nuclear phosphoprotein which has its functional role in the control of cell cycle proliferation (see reviews Levine *et al.*, 1991 and Donehower and Bradley, 1993). As a sequence specific transcription factor it activates genes possessing a *p53* consensus site and over-expression of the wild-type can lead to repression of many genes probably through interaction with the basal components of transcription. *P53* is thought to be an important response to DNA damage, which causes accumulation and an increase in the stability of wild-type p53 eventually resulting in G_1 arrest or apoptosis. P53 forms high-affinity complexes with the damaged DNA site, possibly providing a focus for the proteins involved in DNA repair, but also protects p53 against proteolysis thus increasing its stability. Mutated p53 has a decreased DNA binding capacity and some mutant forms of p53 (even in the heterozygous state) have the ability to act as oncogenes and can stimulate cell division. As a result, if mutated, the p53 protein functions as a double-edged sword, on the one side it is a protein which has lost its ability to carry out its normal function of controlling the cell cycle acting in the face of DNA damage and on the other it may in fact have inherited properties enabling it to stimulate cell division in the presence of impairment. Perhaps it is not surprising then that it is found mutated so frequently in all types of human cancers including HCC. The type of mutation takes many forms; allelic loss, rearrangements, point mutations and deletion of the *p53* gene. In particular loss of heterozygosity frequently accompanied by mutation in the other allele is common (Haffner and Oren, 1995).

The function of *p53* in response to DNA damage appears to lie in its ability to bind specifically to target genes and transactivate them. This occurrence has been postulated with the *WAF1* gene which, when activated, produces a p21 protein which accumulates in the cell nucleus. It is thought to complex with and titrate out the activity of critical Cdk/cyclin complexes to block cell cycle progression and causes growth arrest, by preventing phosphorylation of cyclin dependent kinase substrates through inhibition of cyclin dependent kinase activity. In addition the p21 protein is thought to inhibit directly DNA synthesis by binding to proliferating cell nuclear antigen and blocking its interaction with polymerase δ (see reviews Selivanova and Wiman, 1995; Haffner and Oren, 1995) (Fig 1.6). In addition to enhancing transcription from the *WAF1* gene, wild-type *p53* is thought to enhance

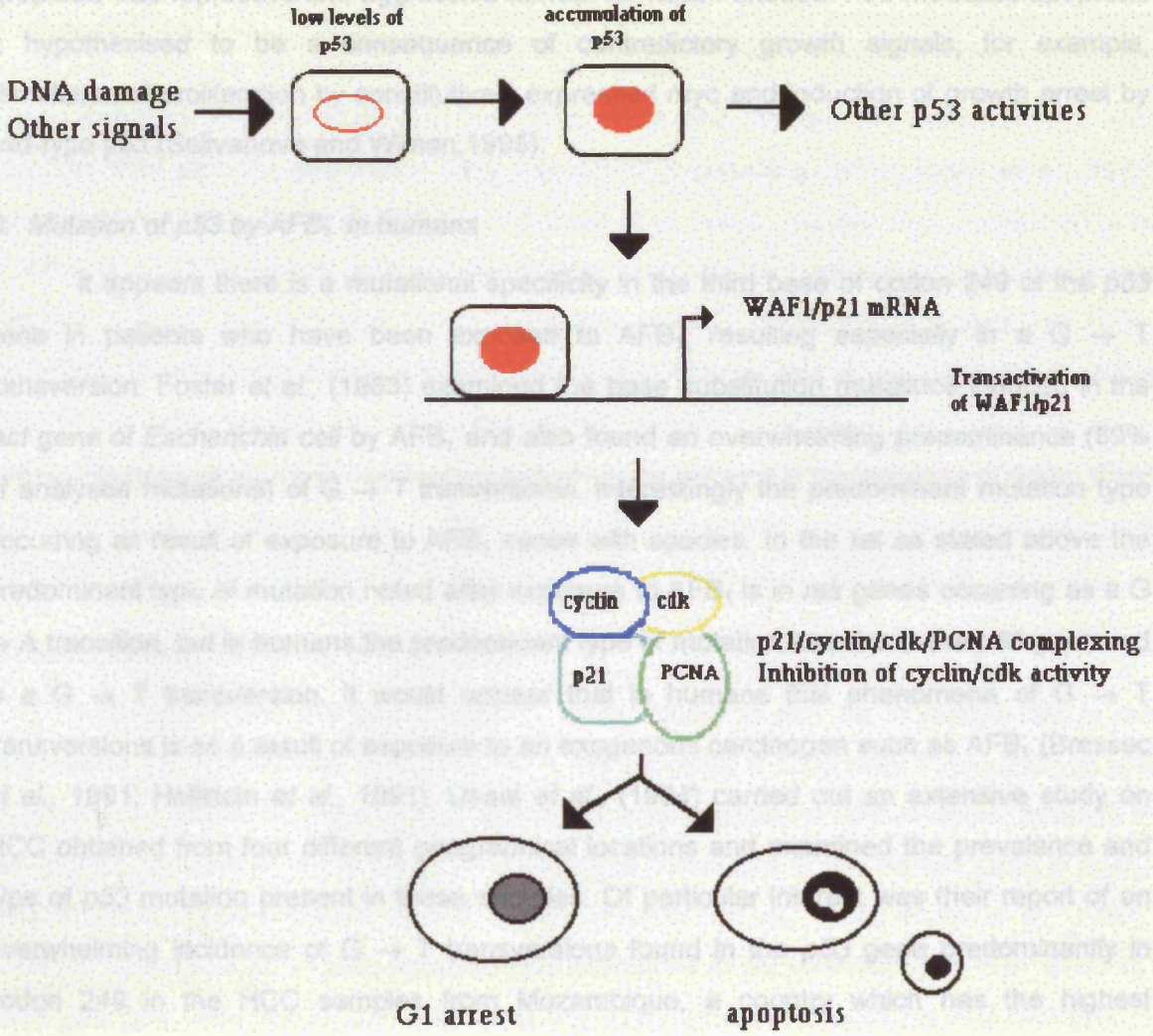


Figure 1.6 Possible processes involving the induction of G_1 arrest and apoptosis by *p53*.

(adapted from Selivanova and Wiman, 1995)

transcription from the *Rb* gene and activate the *bax* gene as well as down-regulate *bcl-2* expression, the combined effects of which result in the promotion of *p53*-mediated apoptosis. Symonds *et al.,* (1994) in his transgenic mouse model indicated a central role for loss of p53-dependent apoptosis in tumorigenesis. In this model inactivation of the pRB family but not p53 resulted in the development of slow growing tumours. If p53 was also knocked out apoptosis was repressed and aggressive tumour formation ensued. P53-mediated apoptosis is hypothesised to be a consequence of contradictory growth signals, for example, stimulation of proliferation by constitutively expressed myc and induction of growth arrest by wild-type p53 (Selivanova and Wiman,1995).

B. Mutation of p53 by AFB_1 in humans

It appears there is a mutational specificity in the third base of codon 249 of the *p53* gene in patients who have been exposed to AFB_1, resulting especially in a G → T transversion. Foster *et al.,* (1983) examined the base substitution mutations induced in the *lacI* gene of *Escherichia coli* by AFB_1 and also found an overwhelming predominance (89% of analysed mutations) of G → T tranversions. Interestingly the predominant mutation type occurring as result of exposure to AFB_1 varies with species. In the rat as stated above the predominant type of mutation noted after exposure to AFB_1 is in *ras* genes occurring as a G → A transition, but in humans the predominant type of mutation found is in the *p53* gene and is a G → T transversion. It would appear that in humans this phenomena of G → T transversions is as a result of exposure to an exogenous carcinogen such as AFB_1 (Bressac *et al.,* 1991; Hollstein *et al.,* 1991). Unsal *et al.,* (1994) carried out an extensive study on HCC obtained from four different geographical locations and examined the prevalence and type of *p53* mutation present in these samples. Of particular interest was their report of an overwhelming incidence of G → T transversions found in the *p53* gene predominantly in codon 249 in the HCC samples from Mozambique, a country which has the highest incidence of HCC in the world and the presence of only G → A transitions in variable codons in the HCC samples from Germany, a country which has low incidence of HCC. Mozambique is known to have a high incidence of AFB_1 exposure and it is thought this is accounts for the differences in results between these countries. Buetow (1992) suggests that an average *p53* mutation rate in HCC of 25% is assumed for areas with high AFB_1 exposure which is double the rate seen in other areas. Cerutti *et al.,* (1994) studied the mutability of codons 247-250 of the *p53* gene in human hepatocytes following exposure to AFB_1. They found a preferential induction of a transversion of guanine to thymidine in the third base of codon 249 supporting the *in vivo* findings in humans with high risk of exposure to AFB_1.

Numerous studies have been carried out examining the presence of *p53* mutations in HCC taken from humans with and without the risk of having been exposed to AFB_1. Analysis

of these studies would appear to support the evidence for mutational specificity in codon 249 (Hsu *et al.,* 1991; Unsal *et al.,* 1994; Hollstein *et al.,* 1993; Fujimoto *et al.,* 1994; Coursaget *et al.,* 1993; Aguilar *et al.,* 1994). Ozturk *et al.,* (1991) carried out a study examining the incidence of *p53* mutations in human HCC's obtained from fourteen different countries and found the presence of codon 249 mutation correlated well with high risk of exposure to aflatoxins. Coursaget *et al.,* (1993) has reported the highest mutation frequency in the *p53* gene at codon 249 (67%) in Senegal where the incidence of HCC is one of the highest in the world and exposure to AFB_1 is also high. Conversely studies carried out in Japan, Hong Kong and Europe failed to record this incidence of *p53* mutation in codon 249 in HCC samples from patients who had a very low risk of exposure to AFB_1 (Nose *et al.,* 1993; Hayashi *et al.,* 1993; Ng *et al.,* 1994a; Volkmann *et al.,* 1994). Aguilar *et al.,* (1994) showed that this codon 249 mutation can also be present in apparently normal liver and he suggests that this mutation may precede the development of HCC in individuals exposed to AFB_1, indicating an early role in the progression of hepatocarcinogenesis. Point mutations of *p53* at sites other than codon 249 have been documented in HCC (Volkmann *et al.,* 1994; Ng *et al.,* 1994b; Fujimoto *et al.,* 1994) and would appear to be random in the absence of AFB_1. Even in the presence of hepatitis B virus (HBV) there does not appear to be a relationship between HBV and specific *p53* mutations (Hayashi *et al.,* 1993; Nose *et al.,* 1993; Ng *et al.,* 1994a).

C. Mutation of p53 by AFB_1 in nonhumans

Although codon 249 *p53* mutations would appear to play an important role in the progression of AFB_1-induced HCC in humans, the converse is apparently true in rodents and non-human primates. No mutations at codon 249 were detected in AFB_1-induced tumours in non-human primates or in the site corresponding to codon 249 of the human gene in AFB_1-induced hepatic preneoplastic lesions in rats (Fujimoto *et al.,* 1992; Hulla *et al.,* 1993). However Lilliberg *et al.,* (1992) reported *p53* alterations in rat AFB_1-induced liver tumours, these changes occurred in one allele only and both structural alterations and changes in expression were noted.

In an attempt to characterise the phenotype of the *p53* gene containing a G $\rightarrow$ T transversion at codon 249, Dumenco *et al.,* (1995) reported the properties of this gene in an hepatocyte-derived cell line. A mutant murine *p53* gene p53Ser246 corresponding to the human mutant p53Ser249 was introduced into a nondifferentiated nontransformed murine hepatocyte cell line. Expression of this gene appeared to confer a growth advantage when compared to the control vector or another mutant murine p53 or when expressed in a nonhepatocyte line. The cells also exhibited a more heterogeneous appearance, but did not appear fully transformed as shown by the lack of focus transformation, tumour formation in nude mice or anchorage-independent growth. Therefore although this mutation conferred a

nude mice or anchorage-independent growth. Therefore although this mutation conferred a growth advantage it was not sufficient to transform hepatocytes completely, at least in the mouse.

D. Hepatitis B virus and p53

Many studies have begun to examine the synergistic effect between AFB_1 and hepatitis B virus to try and determine its function, in particular the possible relationship between hepatitis B virus and the p53 protein. Slagle *et al.,* (1991) reported integration of the hepatitis B virus into host DNA causing gross chromosomal alterations which may alter the expression of adjacent cellular genes. One such location for this integration was in human chromosome 17p near the *p53* gene where the deletion of at least one allele of the *p53* gene was found to be common (53% LOH). Additional allele deletions were noted in other parts of the genome, for example, in chromosome 8q where the *myc* gene is located. Furthermore the hepatitis B virus X protein, a small protein of 154 amino acids required for viral infection, has been found to complex with wild type p53 protein causing inhibition of its sequence-specific DNA binding and transcriptional activation properties (Feitelson *et al.,* 1993; Wang *et al.,* 1994). This ability may therefore influence a wide range of p53 functions and contribute to the process of hepatocarcinogenesis.

Of note have been the recent reports of the prevalence of hepatitis C virus (HCV) infection in those areas of the world where hepatitis B virus infection is found. Although little work has been done yet to determine the role of this virus in hepatocarcinogenesis Teramoto *et al.,* in 1994 examined the role of infection with hepatitis C virus in conjunction with the presence of *p53* mutations and found, in the presence of HCV infection, frequent induction of p53 mutations. The type of mutation most commonly seen was a transitional change of nucleotide, especially at the CpG site. In general this type of mutation is not normally seen in the liver.

1.3.3 Cytokeratins

A. Cytokeratin expression in human and rat epithelia and tumours

The cytokeratins are a family of proteins ranging in size from 40-68 kDa which form intermediate filaments (with diameters 7-11 nm) of the intracellular cytoskeleton in almost all epithelial cell types. They are a complex family of many different polypeptides (of which 19 have been identified to date) related to epidermal α keratin's. They are expressed in different epithelia in various combinations of polypeptides depending on cell type, origin of epithelium and state of histological differentiation. A given epithelium or epithelial cell can therefore be

Table 1.2

Distribution of Cytokeratin 8, 18 and 19 polypeptides in human and rat liver epithelia and carcinomas (data from Green *et al.*, 1990; Carthew *et al.*, 1989; Moll *et al.*, 1982)

Cytokeratin type molecular weight (x 10^{-3}) rat (human)	8 55 (52)	18 47-49 (45)	19 39-40 (40)
bile duct epithelia	+	+	+
oval cells	+	+	+
hepatocytes	+ (+)	+(+)	-(-)
cholangiocarcinoma	+	+	+
pseudoglandular	+	+	+
hepatocellular carcinoma	+(+)	+(+)	-(-)

characterised by the specific pattern of its cytokeratin components, for example, human cytokeratin 8 and 18 are markers of simple epithelial cells such as hepatocytes (Table 1.2). Similarly, most tumours can be keratin typed relying on the generalisation that tumour cells continue to express the cytokeratin polypeptide of the cell type from which they originated. However, some tumours will express only a subset of keratin's present in the cell of origin or an additional one not found in normal tissue. This may indicate the origin of the tumour from a precursor in the cell-lineage forming a minor population or even absent in the adult tissue. In addition tumours appearing morphologically homogeneous may in fact be subdivided using keratin typing (Osborn and Weber, 1986).

Green *et al.*, (1990) examined the expression of cytokeratins 8, 18 and 19 during AFB_1-induced carcinogenesis in rat liver and found that not only did hepatocellular carcinomas begin to express cytokeratin 19, but that the carcinomas were also heterogeneous in their expression. Franke *et al.*, (1981) found in culture that changes in expression of cytoskeletal proteins occurred even in cloned cell populations resulting in a heterogeneous cell population. They reported these changes to occur in a given cell population, without obvious effects on cell growth rate or cell morphology and suggested these changes to simply represent small alterations in differentiation not necessary for cell growth. Green *et al.*, (1990) suggested the possibility of pseudoglandular tumours acquiring their cytokeratin phenotype throughout hepatocarcinogenesis. However, Carthew *et al.*, (1989) demonstrated continuity of phenotypic expression in cells recognised as bile duct in origin through normal development in the rat and in AFB_1-induced tumour development. Green *et al.*, (1990) also suggested that in view of the fewer number and later appearance of cytokeratin 19 positive foci obtained using their AFB_1 feeding regime, analyses of the

expression of cytokeratin 19 may be a more useful marker for transformation as these foci may not be remodelled after withdrawal of the carcinogen.

1.4 Aims

The purpose of this thesis was to examine the processes occurring during hepatocarcinogenesis induced by the potent hepatocarcinogen AFB_1, in male Fischer 344 rats. Several aspects of cellular and genetic function were examined through the analysis of protein and RNA expression and gene mutation analysis. Male Fischer 344 rats were used as they have been reported to be more sensitive to the effects of AFB_1 than mice or other rat strains. Male rodents are more sensitive than females to the effects of AFB_1. A stop-protocol was employed to enable examination of the liver after withdrawal of the carcinogen. Frequent time-points during the study were examined. Hepatocellular carcinomas from a study carried out previously in this laboratory were also included for analyses in some parts of the project. Sections of the carcinomas were available which had been prepared from nude mice which had been injected subcutaneously with a cell line which had been derived from liver tumours induced in male Fischer 344 rats treated with AFB_1.

Early focal lesions were identified and characterised and these changes were correlated with the subsequent development of hepatocellular carcinoma. Biochemical changes occurring in the foci of altered hepatocytes (FAH) were examined using antibodies to proteins established as early key markers of hepatocarcinogenesis for example, glutathione *S*-transferase 7-7, γ-glutamyl transferase and cytokeratins 18 and 19. The enzymic activity of γ-glutamyl transferase was determined at each of the time-points during the AFB_1 study. Proteins having an important role in the metabolism of AFB_1 were also examined for example, glutathione *S*-transferase Yc_2, P450 2C11 and aldehyde reductase. The role of the alpha class GST Yc_2, induced in rat, during hepatocarcinogenesis in the detoxification of AFB_1 has been well established. In view of the probable importance of this enzyme in the carcinogenic process and the lack of a specific antibody to detect this protein, it was decided to produce a specific monoclonal antibody which could be used to examine the changes in glutathione *S*-transferase Yc_2, occurring in the FAH through to hepatocellular carcinoma. A synthetic peptide for use as an antigen to produce this antibody, was designed carefully in an attempt to prevent crossreactivity with the constitutively expressed glutathione *S*-transferase Yc_1, which has 90% homology with the Yc_2 protein.

The total level of AFB_1 adducts present in nucleic acids was determined for each of the time-points on the study during feeding of AFB_1 and compared to the level seen in control animals fed an AFB_1-free diet. These results were compared to the level of detoxifying enzymes at each time-point to determine the presence of a mechanism of resistance induced by chronic feeding of the AFB_1 diet. During the AFB_1 feeding period one

test and one control animal at each time-point was injected with [^{3}H]AFB_1 two hours before culling. Genomic DNA was extracted, hydrolysed and the hydrolysates were fractionated using analytical HPLC and the level of radioactivity determined in each sample. Assuming the development of a mechanism of resistance, the level of [^{3}H]AFB_1 adducts present in the animals fed the AFB_1 diet would, with increasing time, be expected to show a decreased level of adducts compared to the control animals. The level of [^{3}H]AFB_1 adducts present on genomic DNA was compared to the level seen on total nucleic acids to determine the specificity of the AFB_1-epoxide for DNA compared to RNA. The location of these adducts in the liver was to be determined using autoradiography on the acetone fixed liver sections. The location of the radioactivity was expected to be excluded from the FAH which displayed the phenotypic pattern induced in the mechanism of resistance.

Specific point mutations in exons of the *ras* oncogene occur frequently in AFB_1 induced tumours in the rat, however, their place in the multistep progression of hepatocellular cancer is unknown. The presence or absence of mutations, located in exons one and two of *N-ras* and *Ki-ras* genes, was determined, using PCR and sequencing, at various time-points in this model and related to the genotypic pattern of the hepatocellular carcinomas produced.

Point mutations in many exons of the p53 tumour suppressor gene occur frequently in a large number of human tumours including hepatocellular carcinomas. Reports suggest a specific codon 249 mutation (a G → T transversion) as a late event occurring in human hepatocellular carcinoma as a result of exposure to AFB_1. The final hepatocellular carcinomas produced in this study and a previous study were examined for the presence of a G → T transversion in the corresponding codon of the p53 gene in rat.

Human liver biopsy samples from the Gambia, an area of high aflatoxin incidence, were obtained and also examined for the presence of a G → T transversion in codon 249 of the p53 gene, using immunohistochemistry. It had been intended to carry out more work on the human samples for example, examining the presence or absence of hepatitis B virus and correlating these results to the mutations at codon 249 of the *P53* gene. However, only a few human samples were obtained and the necessary controls were not available, despite repeated attempts to acquire them, therefore, only a limited amount of work was possible using these samples. Some immunohistochemistry work was carried out on the human samples using the anti-rat antibodies to determine possible crossreactivity for a use in correlating the phenotypic pattern of the rat and human hepatocellular carcinomas. Western blotting analysis was used to confirm the specificity of such crossreactivity.

It was envisaged the results of this thesis would suggest a possible sequence of events, encompassing biochemical and molecular changes, occurring in the development of hepatocellular carcinomas in the rat induced by exposure to AFB_1. And some correlation could be made between the rat and human models of carcinogenesis.

Chapter Two

Materials and Methods

2.1 Materials

Antibodies

Anti-Aldehyde reductase was obtained from Dr JD Hayes, BRC, University of Dundee

Anti-Cytochrome P450 2C-11 kindly donated by Dr CR Wolf, ICRF, University of Edinburgh

Anti-Cytokeratin 19 (anti-rat)- Amersham International plc, Bucks

(anti-human)- Dako Ltd., High Wycombe, Bucks

Anti-Cytokeratin 18 was kindly donated by Dr MM Manson, MRC, Univ. of Leicester

Anti-Glutathione-S-transferases

7.7 was kindly donated by Dr MM Manson, MRC, University of Leicester

Yc_1/Yc_2 was kindly donated by Dr JD Hayes, BRC, University of Dundee

Anti-P53 ab-6- Oncogene Science, Cambridge Bioscience, Cambridge

Anti-Mouse IgG peroxidase conjugated - Dako Ltd., High Wycombe, Bucks

Rabbit AFB_1 antiserum prepared in this laboratory previously using AFB_1-adducted BSA.

[^{3}H] AFB_1 - Moravek Biochemicals Inc., Brea, California

DAB substrate peroxidase kit- Vector Laboratories, Peterborough, Cambs

dATP ^{35}S - Amersham International plc, Bucks

DNA ladder - Gibco BRL, Paisley

dNTP's - Pharmacia Biotechnology Ltd., St.Albans, Herts

Dynabeads® Oligo dT_{25} - Dynal (UK) Ltd., New Ferry, Wirral

ECL™ detection kit - Amersham International plc., Bucks

Genescreen plus® nylon membrane - Amersham International plc., Bucks

HaeIII and HinfI - New England Biolabs, CP Laboratories, Herts

Keyhole Limpet Haemocyanin (KLH) - Pierce & Warriner (UK) Ltd., Chester

Kwiksep™ Dextran plastic desalting columns - Pierce & Warriner (UK) Ltd., Chester

Magnetic particle concentrator MPC-E - Dynal (UK) Ltd., New Ferry, Wirral

Microsep-30 - Flowgen Instruments Ltd., Sittingbourne, Kent

Multiprime labelling kit - Amersham International plc., Bucks

N-(L-γ-glutamyl)-4-methoxy-2-naphthylamide- Novabiochem, Nottingham

Nap™ 5 column - Pharmacia Biotechnology Ltd., St. Albans, Herts

Nunc 96 well immunoplates - Life Technologies Ltd. Gibco BRL, Paisley

Photographic developer Phenisol™ - Ilford Ltd., Mobberley, Cheshire

Photographic Emulsion K5™ - Ilford Ltd., Mobberley, Cheshire

Precipitate-II cartridge filters - Applied Biosystems, Birchwood Sci. Nth.,Warrington

Protein assay kit - Bio-rad Laboratories Ltd., Bio-rad House, Hemel Hempstead, Herts

Proteinase K - Applied Biosystems, Birchwood Scientific North, Warrington

Qiaquick-spin PCR purification kit - Qiagen Ltd., Dorking, Surrey

RNase-it - Stratagene Ltd., Milton Road, Cambridge

RNasin® ribonuclease inhibitor - Stratagene Ltd., Milton Road, Cambridge

RNazol™ B - Biogene Ltd., Bolnhurst, Bedfordshire

Sequenase version 2.0 DNA sequencing kit - Cambridge Bioscience, Cambridge

Sulpho-MBS (*m*-Maleimidobenzoyl-*N*-hydroxysulphosuccinimide-3-acetate) - Pierce & Warriner (UK) Ltd., Chester

Superscript™ II RNase H- reverse transcriptase - Gibco BRL, Paisley

Taq DNA polymerase -Promega Corporation, Southampton

Ultima Gold - Canberra Packard, Pangbourne, Berks

Vectastain elite ABC kit- Vector Laboratories, Peterborough, Cambs

Visigel - Stratagene Ltd., Cambridge

All other reagents were of best quality available from either Merck-BDH, Lutterworth, Leics, Sigma Chemical Company, Poole, Dorset or Fisons Scientific Equipment, Loughborough, Leics.

Methods

2.2 Safety Precautions

When handling aflatoxin and radioactive material a double layer of gloves, safety spectacles and the correct laboratory coat were worn. Aflatoxin was weighed and dispensed in a fume-hood and whenever possible in the biohazard laboratory. All work areas were swabbed with 0.5% sodium hypochlorite after use. Radioactivity was dispensed and administered in the appropriate radioactivity laboratories. Aflatoxin solutions and contaminated glassware and plasticware were decontaminated by soaking in 0.5% sodium hypochlorite, radioactive glassware was decontaminated in a solution of Decon 90 detergent. After decontamination all plastics were incinerated.

2.3 Animals

The aflatoxin feeding experiments used males of the inbred Fischer rat strain F344. Fischer rats, especially males, are particularly sensitive to the biological and biochemical effects of aflatoxin B_1 and therefore provide a suitable model for use in a study investigating the effects of AFB_1. Using an inbred strain of rat ensures genetic similarity. The induction of certain hepatic enzymes (for example, aryl hydrocarbon hydroxylase) are under the control of a single Mendelian gene giving reasonable reproducibility of enzyme levels present after induction. Genetic isogenicity also allows for a smaller sampling group of animals (Festing, 1975).The overall number of animals used in the study was restricted by the availability of animal house facilities.

2.3.1 Preparation of AFB_1 diet

Reagents

Aflatoxin B_1 (Sigma)
Amber storage bottles 100 ml volume
Dimethyl sulphoxide
Peanut oil
Sodium hypochlorite 0.5%

Method

NOTE: This method was carried out with great care and attention to the safety procedures outlined in section 2.2.

The tin containing AFB_1 (50 mg) was opened and the bottle seal removed taking great care to avoid creating particle dispersion due to static. The AFB_1 was dissolved in the

original bottle using 5 ml aliquots of DMSO. This solution was transferred to a universal and DMSO added to give a concentration of 2 mg/ml AFB_1 in DMSO. The AFB_1/DMSO solution (8 ml) was then carefully pipetted into labelled amber bottles containing 80 ml of peanut oil. This was then mixed with 4 kg of RPM1 diet for at least 20 min using a foodmixer until homogeneous.

2.3.2. Animal procedure

The animals were housed two or three animals per cage, in an environmentally controlled room at 20°C ± 2°C with a humidity level of 50% ± 5%, and a lighting cycle of twelve hours (0700-1900). Identification of each animal was by electronic tagging. Animals were allowed food (RM1 complete dry diet alone as the normal diet and containing AFB_1 4 p.p.m. as the test diet) and water *ad libitum*. Certain parameters were measured routinely; the animals were weighed twice weekly and the weight of diet received and consumed per cage was recorded three times per week. Weekly health inspections of the animals were carried out to ensure well being of the animals.

2.4 Tissues

Some sections from a previous study were donated by Dr GE Neal for the purpose of a pilot study. These were liver tumour sections obtained from nude mice which had been injected subcutaneously with a cell line either JB1 or BL10 which had been derived from liver tumours induced in male Fischer 344 rats treated with the carcinogenic compound AFB_1. The different cell lines represented different tumour types which were induced by AFB_1 (Neal *et al.,* 1983). JB1 represented a pseudoglandular type liver tumour and BL10 represented a poorly differentiated type of hepatocellular carcinoma after classification according to standard histologic criteria (Cotran *et al.,* 1989). The tumours had been fixed in cold acetone and paraffin embedded.

The majority of samples used in this study, however, were obtained from an AFB_1 feeding study (Fig 2.1). This was carried out using 120 male Fischer 344 rats, following a stop protocol. AFB_1 was administered in the diet, after two weeks acclimatisation, to the test animals at 4 p.p.m., for a limited period (twelve weeks) after which the test animals were returned to the normal diet. The control animals were maintained on the normal diet throughout the study. This protocol type is of most use for investigating the important aspects of the sequential mechanisms of hepatocarcinogenesis (Bannasch *et al.*, 1986). Animals were culled at frequent timepoints providing samples for analysis of the progression of the study from early foci through to HCC (Table 2.1).

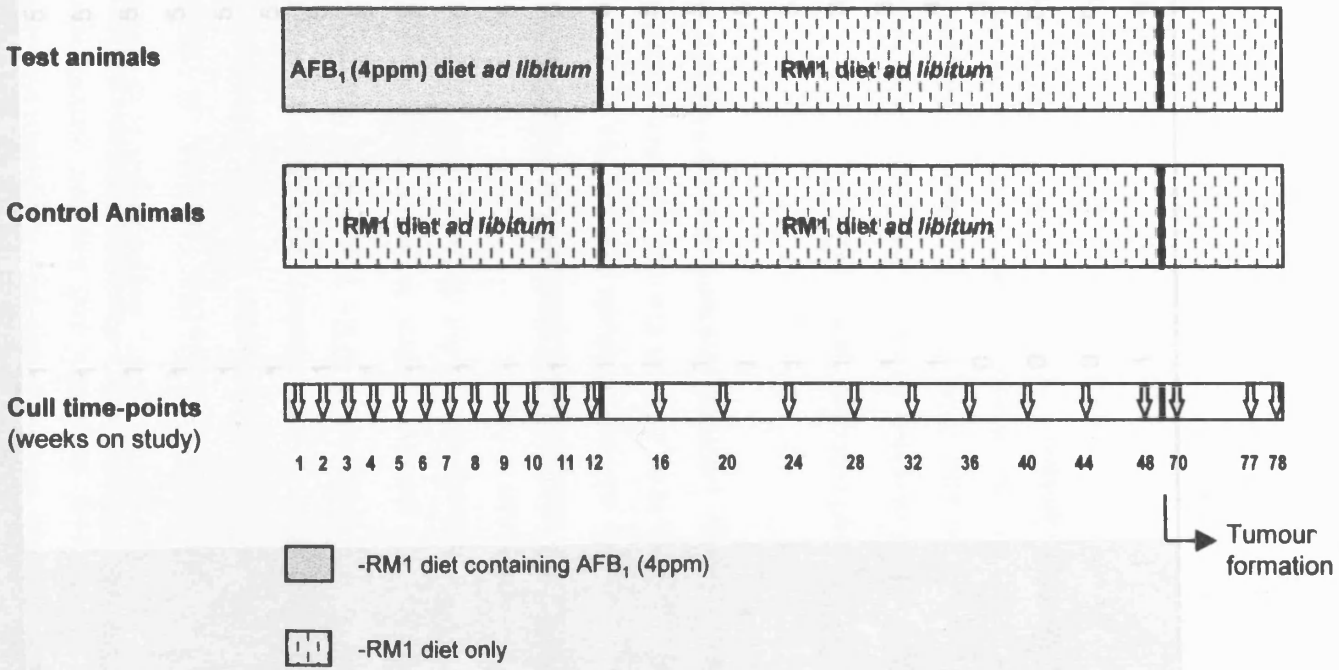


Figure 2.1 Aflatoxin B_1 feeding study

Table 2.1 Culling procedure of animals on AFB_1 feeding study

Week no. of study	Number of animals culled	
	Control animals	Test animals
1	1	5
2	1	5
3	1	5
4	1	5
5	1	5
6	1	5
7	1	5
8	1	5
9	1	5
10	1	5
11	1	5
12	1	5
16	1	5
20	1	5
24	1	5
28	1	5
32	1	5
36	1	3
40	1	3
44	1	4
[a]48	0	3
70	0	[b]1
77	0	[c]1
78	1	4

[a] In view of the lack of the anticipated incidence of tumour development it was decided to keep the remaining animals on the study until tumours developed.

[b] This animal was culled because of loss of weight and apathy.The liver of this animal contained two gross necrotic lesions on two separate lobes which were each a third of the volume of the lobe.

[c] This animal died unexpectedly. A postmortem revealed up to six small tumours within the liver parenchyma. In view of this it was decided to cull the remaining animals on the study.

In all cases the liver was taken and when the presence of tumours was observed these were dissected away from the surrounding tissue and treated as separate tissue. Slices were embedded in paraffin wax, following fixation in buffered formalin for conventional histology or cold acetone for immunohistochemical analysis. The remaining liver/tumour was snap-frozen in liquid nitrogen and stored at -80°C. In animals which showed obvious liver tumours the lung, spleen, kidneys and pancreas were also taken and treated as the liver.

One test animal from each time-point and selected controls were injected i.p. two hours before cull with 100-465 mCi/100 g body weight [^{3}H]AFB_1 in saline (500 µl), to assay changes in the level of formation of AFB_1-DNA adducts, at different stages in the carcinogenic process, using HPLC and scintillation counting. This would provide a measure of the development of resistance to AFB_1 toxicity. One control fed weanling animal was injected i.p. two hours before culling with AFB_1 in saline (1 mg/kg) to provide an AFB_1 adducted nucleic acids standard (positive control) for use in the ELISA experiments.

The experimental procedures carried out on the samples provided by this study are summarised in the scheme outlined in Fig 2.2.

The human liver biopsy samples were kindly donated by Dr A Price, Northwick Park Hospital. These samples were all hepatocellular carcinomas from patients living in the Gambia, an area of high exposure to AFB_1 in the diet. Pathology reports were prepared by Dr. K West of the Royal Infirmary, Leicester, following the haematoxylin and eosin staining of these sections.

2.5 Enzyme linked immunoabsorbent assay (ELISA)

ELISA is a specific and sensitive assay for the detection of compounds or adducts formed between the compound and DNA, RNA or protein, if antibodies which detect the compound are available. It can also be used to identify and quantify an antibody in sera of animals exposed to a particular antigen.

The direct ELISA system is based on a sandwich technique, in which a primary antibody binds to an antigen immobilised on a multiwell plate. Following binding of antigen to the plate any unbound antigen is washed away and the plate blocked with an excess of an irrelevant protein to prevent nonspecific binding of proteins before the primary or test antibody is bound to the antigen. A secondary antibody with an enzyme covalently-linked (or ligand) is bound to the primary antibody. This second antibody is raised against the species in which the primary antibody was formed and addition of the appropriate substrate after washing away free ligand results in the formation of a coloured product which can be measured using a spectrophotometer. Colour formation is proportional to the amount of test antibody bound to the antigen absorbed onto the plate (Fig 2.3). Conversely this assay can

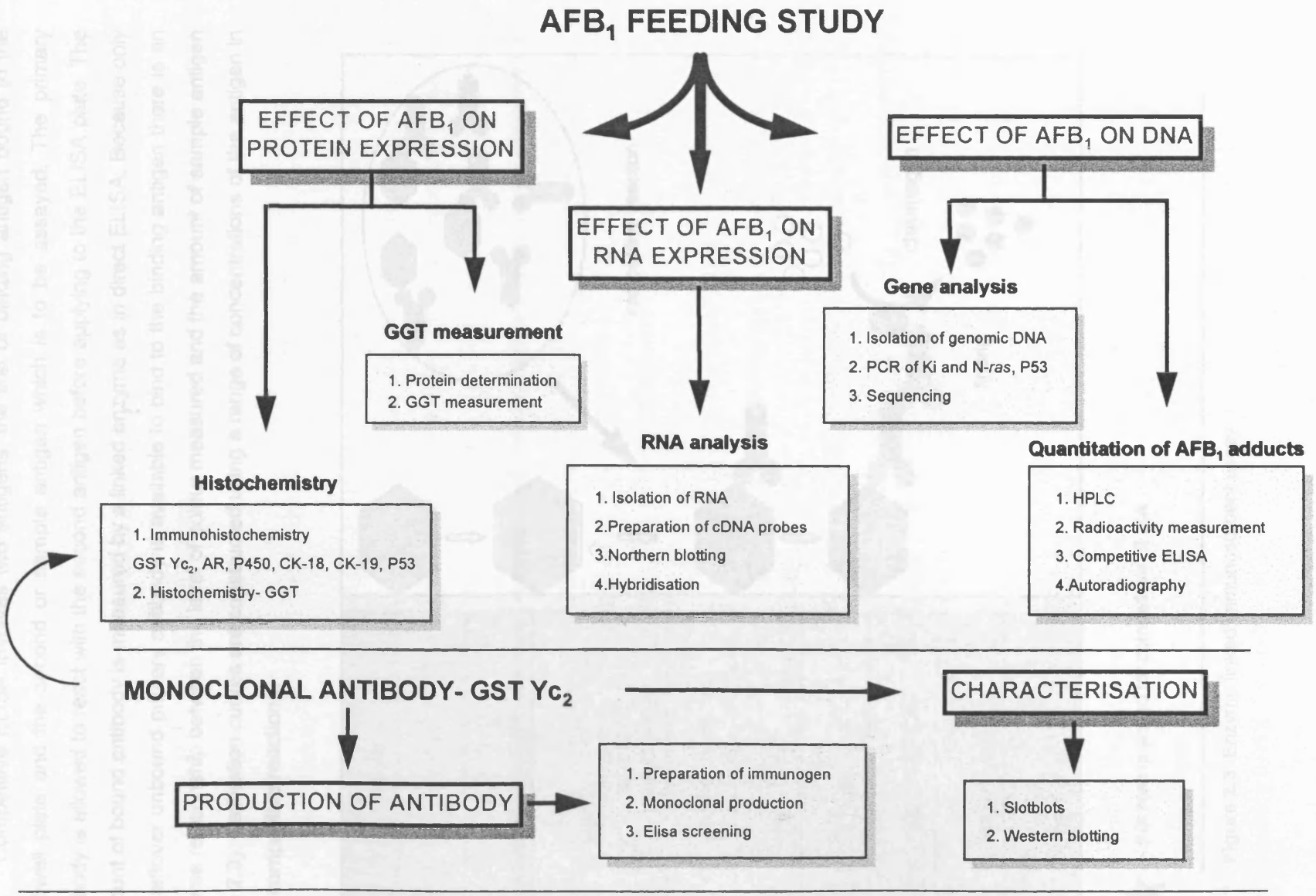
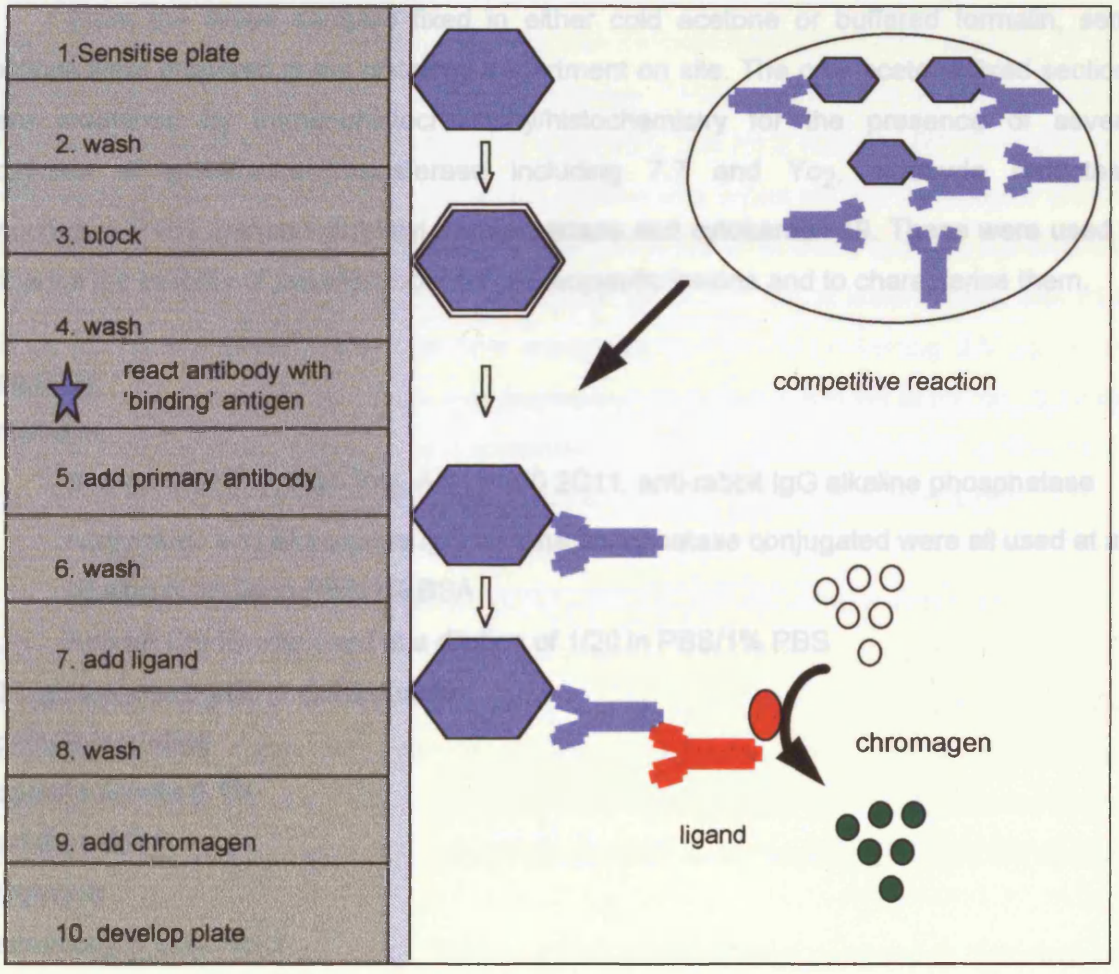


Figure 2.2 Overall scheme of AFB_1 feeding study

also be used to identify and quantify an antibody to a specific antigen as stated above by binding the antibody to the multiwell plate.

Competitive ELISA involves two antigens, the first or binding antigen bound to the multiwell plate and the second or sample antigen which is to be assayed. The primary antibody is allowed to react with the second antigen before applying to the ELISA plate. The amount of bound antibody is measured by a linked enzyme as in direct ELISA. Because only the leftover unbound primary antibody is available to bind to the binding antigen there is an inverse relationship between the level of colour measured and the amount of sample antigen (Fig 2.3). Calibration curves are constructed using a range of concentrations of the antigen in the competitive reactions.



- this step is added for competitive ELISA

Figure 2.3 Enzyme linked immunosorbent assay

To establish the most suitable dilution of antigen to use for coating the ELISA plates and the most suitable dilution of antisera containing the primary antibody a chequerboard experiment was routinely carried out. In this, a chequerboard layout of serial dilutions of the antigen and the antibody were tested in duplicate and compared to results obtained using BSA or other irrelevant protein as the binding antigen. Where there was strongest colour development and greatest diversity from BSA, this indicated the most suitable dilutions to use.

2.6 Immunochemical Protocols

2.6.1 Histochemistry

From the tissue samples fixed in either cold acetone or buffered formalin, serial sections were prepared in the histology department on site. The cold acetone fixed sections were examined by immunohistochemistry/histochemistry for the presence of several isozymes of glutathione-*S*-transferase including 7.7 and Yc_2, aldehyde reductase, cytochrome P450, gamma-glutamyl transpeptidase and cytokeratin 19. These were used to enhance the visibility of possible foci and preneoplastic lesions and to characterise them.

Reagents

Antibodies

Anti-rat GST 7.7, GST Yc_2, AR , P450 2C11, anti-rabbit IgG alkaline phosphatase conjugated and anti-mouse IgG alkaline phosphatase conjugated were all used at a dilution of 1/100 in PBS/1% BSA

Anti-rat CK-19 was used at a dilution of 1/20 in PBS/1% PBS

20% glacial acetic acid in distilled water

Apathy's mountant

Copper sulphate 0.1M

Fast Blue BBN

Glyglycine

Haematoxylin Gill's No. 2

HCl 0.1 M

Histoclear

IMS 100% and 70%

L-gamma glutamyl GMN A

PBS containing 0.05% Tween-80

PBS containing 1% BSA

Veronal Acetate buffer pH 9.2 (0.1 M Na acetate/ 0.1 M barbitone Na, mix 1:1 and adjust pH to 9.2 with HCl) containing 0.5 µg/ml of fast red TR and 0.5 µg/ml Sodium Napthol AS-BI

Method

For the visualisation of glutathione-*S*-transferase, cytochrome P450 2C11, cytokeratin 19 and aldehyde reductase, an indirect method was used where an unconjugated antibody was bound to the antigen in the specimen to which, in turn, was bound with an alkaline-phosphatase conjugated antibody. A suitable substrate was then used to visualise the reaction (Ormerod and Imrie, 1992). The cold acetone fixed, paraffin embedded sections were dewaxed in histoclear and rehydrated through graded alcohols to water. The endogenous phosphatase activity was blocked with 20% acetic acid for 15 min. After washing in PBS-Tween 80 the primary antibody ([d]100µl, diluted 1 in 50-100 in PBS/1% BSA) was applied to the slide and placed in a humidity box for 2 hours at RT. The slide was gently washed 3-4 times with PBS-Tween 80, excess was wiped off and antispecies alkaline phosphatase conjugate (100 µl, diluted 1 in 50-100 in PBS/1% BSA) applied, the slide was then replaced in the humidity box for 1 hour at RT. After washing at least 4 times with PBS-Tween 80 the substrate solution (veronal acetate buffer pH 9.2 containing 0.5 µg/ml Fast Red TR and sodium naphthol AS-BI) was filtered on to the slides and left at RT for 10-30 min to give a coloured end-product. As the substrate is light sensitive it was made up just before use. Sections were counterstained with Gills No. 2 Haematoxylin-blue and mounted using an Apathy's aqueous mountant (Ormerod and Imrie, 1992; Bourne, 1993).

Gamma glutamyl transpeptidase (GGT) was measured using a direct histochemical method of Rutenburg *et al.,* 1969. The substrate was applied to the section and GGT cleaved the γ-glutamyl group from the substrate N-(γ-L-glutamyl)-4-methoxy-2-naphthylamide which subsequently binds to the acceptor molecule glyglycine. 4-Methoxy-3-naphthylamine, liberated enzymatically, coupled with the diazonium salt (fast blue BBN) to form a red-orange dye which when chelated with copper, yielded an even more insoluble, intense red dye (Fig 2.4).

The cold acetone fixed, paraffin embedded sections were dewaxed in histoclear and rehydrated through graded alcohols to water. A solution of the substrate N-(γ-L-glutamyl)-4-

[d] The optimal working dilution of the antibody is determined by its titre and purity and the incubation time to be used. This will give the most intense specific staining and least amount of background interference (Bourne, 1993; Ormerod and Imrie, 1992).

GGT

N-(γ-L-glutamyl)-4-methoxy-2-naphthylamide + glyglycine → 4-methoxy-2-naphthylamine + Glutamyl-Glycylglycine

copper salt

4-methoxy-2-naphthylamine + fast blue bb salt → azo dye

Fig 2.4 Chemical reactions involved in histochemical measurement of GGT

methoxy-2-naphthylamide dissolved in 0.1 M NaOH (6 ml) containing 1.8% NaCl/ 0.1 M sodium phosphate buffer at pH 6.7 (8 ml) (NaH_2PO_4 and 0.1 M Na_2HPO_4) and 0.1 M HCl (6 ml) containing glyglycine (58.8 mg) and fast blue (Sigma) (10 mg) was filtered onto the slides and incubated for 20 min at RT. The chemical reaction which occurred under these conditions resulted in an orange colour at the site of enzyme activity. After washing twice with saline, 0.1M copper sulphate solution was applied for 5 min at RT. This not only stabilised and fixed the colour by preventing leaching into the surrounding tissue but also intensified the colour to a more easily visible brick-red colour. After removing the copper sulphate by washing twice with saline the tissue was counterstained with Gills No. 2 Haematoxylin for 30 sec. The slide was then washed four times with tap water and mounted with Apathy's or Gurr's aqueous mountant. Positive control was a rat kidney tissue section which constituitively expresses GGT in the cortex or a liver tissue section containing preneoplastic tissue which had previously been demonstrated to stain positively for GGT.

The numbers and areas of positive/negative foci more than 10 cells in cross-section were examined using a Zeiss light microscope. The foci were expressed as numbers of foci/mm^2. Areas were calculated by superimposing the sections on graph paper and counting the number of mm^2. Photographs were taken using a Zeiss camera with Kodak Ectakrome 64 colour slide film.

2.6.2 Immunohistochemistry using Vector ABC kit (Vector Laboratories)

The Vectastain ABC system is a sensitive and reliable immunoperoxidase system. The technique uses unlabelled primary antibody followed by biotinylated secondary antibody and then a preformed avidin biotinylated horseradish peroxidase macromolecular complex which is visualised using the DAB substrate system. This system was found to be most useful when using the human sections, as the proteins being examined were present in very small quantities. The Vectastain and DAB substrate working dilutions were prepared according to the manufacturer's recommendations.

Reagents

Antibodies

Anti-human P53 ab-6 used at 1/25 dilution

Anti-human CK-19 used at 1/50 dilution

0.3% H_2O_2 in methanol

Apathy's mountant

DAB substrate kit for peroxidase containing:

buffer stock solution

3'3 -diaminobenzidine stock solution

hydrogen peroxide solution

Haematoxylin Gill's No. 2

PBS

Vectastain elite ABC kit containing:

horse serum

biotinylated anti-mouse IgG

ABC reagent

All solutions were prepared according to manufacturer's instructions.

Method

Routinely, formalin fixed human liver biopsy samples were deparaffinised and hydrated through histoclear and graded alcohol series as above. The sections were rinsed for 5 mins in distilled water. Distilled water was boiled in the microwave in a covered container on high setting for 10 mins. The sections were added to the superheated solution and left in the solution until cool, approximately 15 min. This step was used as an antigen retrieval system which had been demonstrated to expose epitopes in fixed materials thus facilitating access by the antibody. Endogenous peroxidase activity was quenched by incubating the sections for 30 mins in H_2O_2 (0.3% in methanol). After washing in PBS for 20 mins, the sections were incubated for 20 mins with dilute horse serum. Excess serum was blotted from sections and the sections incubated for 30 mins with primary antibody (100 µl diluted 1:25-50 depending on the antibody in PBS-1% BSA). After washing for 10 mins in PBS the slides were incubated for 30 mins with diluted biotinylated anti-mouse or anti-rabbit antibody (100 µl) and then washed for a further 10 mins in PBS. The sections were incubated for 30 mins with Vectastain ABC reagent before a further 10 min wash in PBS. The substrate was prepared using DAB substrate kit for peroxidase and the sections incubated at RT until a suitable intensity of stain developed, usually 5-10 mins. After washing

the sections for 5 mins in water the sections were counterstained using haematoxylin and mounted using Apathy's mountant.

2.7 Protein determination and gamma-glutamyltranspeptidase measurement

Protein determination was carried out by the Bradford method (Bradford, 1976) using a kit available from Bio-rad. The Bio-rad protein assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Measurement of GGT was carried out using a colorimetric method for determining GGT activity (Naftalin *et al.*, 1969) available in a kit form from Sigma (Fig 2.5). The assay is based on the transfer of the glutamyl group from L-glutamyl-p-nitroanilide to glyglycine catalyzed by GGT. The p-nitroanaline liberated is diazotised using the Bratton-Marshall reaction. The absorbance of the pink azo-dye measured at 530-550 nm is proportional to GGT activity.

$$\text{L-Glutamyl-p-Nitroanilide + Glyglycine} \xrightarrow{\text{GGT}} \text{p-Nitroaniline + Glutamyl-Glycylglycine}$$

$$\text{p-Nitroaniline + } NaNO_2 \xrightarrow{\text{Acid}} \text{Diazo compound + N-(1-Naphthyl)ethylenediamine} \rightarrow \text{pink azo dye}$$

Figure 2.5 Colorimetric assay for determining GGT activity

2.7.1 Protein determination

Reagents

BSA (5,10,15,20 μg/ml concentrations)

Protein assay kit

Method

Experimental procedure was carried out as described by Bio-rad.

Protein calibration

Several dilutions of protein standard BSA containing 5-20 μg/ml were prepared.

0.8 ml of standards and 0.8 ml of sample buffer (distilled water) as reagent blank in triplicate were placed in 2 ml cuvettes. Dye concentrate reagent (0.2 ml) was added, the samples were vortexed and the absorbance read (approximately 20 min later) at 595 nm vs reagent blank.

To determine the protein concentration of each of the samples a 1:1000 dilution was prepared in distilled water, 0.8 ml was added to triplicate cuvettes and Dye concentrate reagent (0.2 ml) added. The mixture was vortexed and the absorbance read at 595 nm approximately 20 min later. The average absorbances were read off the calibration curve using the Microsoft excel package to determine the protein concentration.

2.7.2 GGT measurement

Reagents

Acetic acid solution 10%

GGT kit (Sigma diagnostics) :

- GGT substrate solution
- Trizma buffer
- Sodium nitrite tablets
- Ammonium sulphamate solution
- N-(1-Naphthyl)ethylenediamine DiHCL
- GGT calibration solution

All reagents were prepared according to manufacturer's instructions. GGT substrate solution was prepared and stored at -20^{o}C in 0.5 μl aliquots.

PBS/ 0.1% Triton X-100

Method

Experimental procedure was carried out as described by Sigma.

GGT Calibration

Into five 15 ml tubes labelled 1-5, volumes of solutions were pipetted as detailed in table 2.2. To each tube acetic acid solution (2 ml) was added and mixed by gently swirling. In timed sequence to each tube sodium nitrite solution (1 ml) was added, mixed quickly and left at room temperature for 3 min. In the same timed sequence ammonium sulfamate solution (1 ml) was added to each tube, mixed quickly and left at room temperature for 3 min. N-(1-Naphthyl)ethylenediamine solution (1 ml) was added and shaken vigorously to encourage the bubbles to rise to the top. The solution was aliquoted in triplicate 1 ml volumes into cuvettes and the absorbance read at 540 nm. The calibration curve was plotted as average absorbance vs corresponding GGT activity using Microsoft excel software.

Table 2.2

Tube no.	GGT calibration solution	Distilled water	GGT activity (units/ml)
1	0.0 ml	0.5 ml	0
2	0.1 ml	0.4 ml	20
3	0.3 ml	0.2 ml	60
4	0.4 ml	0.1 ml	80
5	0.5 ml	0.0 ml	100

Sample assay

Note: A blank is required for each sample.

Frozen liver samples (one liver for each control and a pool of three livers for each test time-point) were homogenised in a solution of PBS/ 0.1% Triton X-100 at a concentration of 50 mg/ml. The appropriate number of tubes containing GGT substrate were thawed at 37°C to equilibrate, liver homogenate (100 µl) was added and mixed gently by swirling. The tubes were incubated at 37°C for exactly 20 min. After incubation acetic acid solution (2 ml) was added to each tube and mixed by gently swirling. To blank tubes corresponding liver homogenate (100 µl) was added. In timed sequence to all tubes sodium nitrite solution (1 ml) was added, the solution was mixed quickly and allowed to remain at room temperature for 3 min. In the same timed sequence ammonium sulphamate solution (1 ml) was added, mixed quickly and allowed to remain at room temperature for 3 min. To each tube N-(1-naphthyl)ethylenediamine solution (1 ml) was added and shaken vigorously so that the bubbles rose to the top. One ml was transferred in triplicate to disposable cuvettes and the absorbance of test vs blank read at 540 nm. Activity was determined from the calibration curve using Microsoft excel package. The expression of GGT activity was expressed as units/mg protein.

2.8 Isolation of RNA

RNA analysis was carried out in order to examine the level of expression of ras genes in tumour samples and untreated liver controls. cDNA probes were prepared using RT-PCR and used to probe both total and messenger RNA. mRNA was prepared using Dynabeads either directly from the tissue or from isolated total RNA. Figure 2.6 illustrates the manipulations performed.

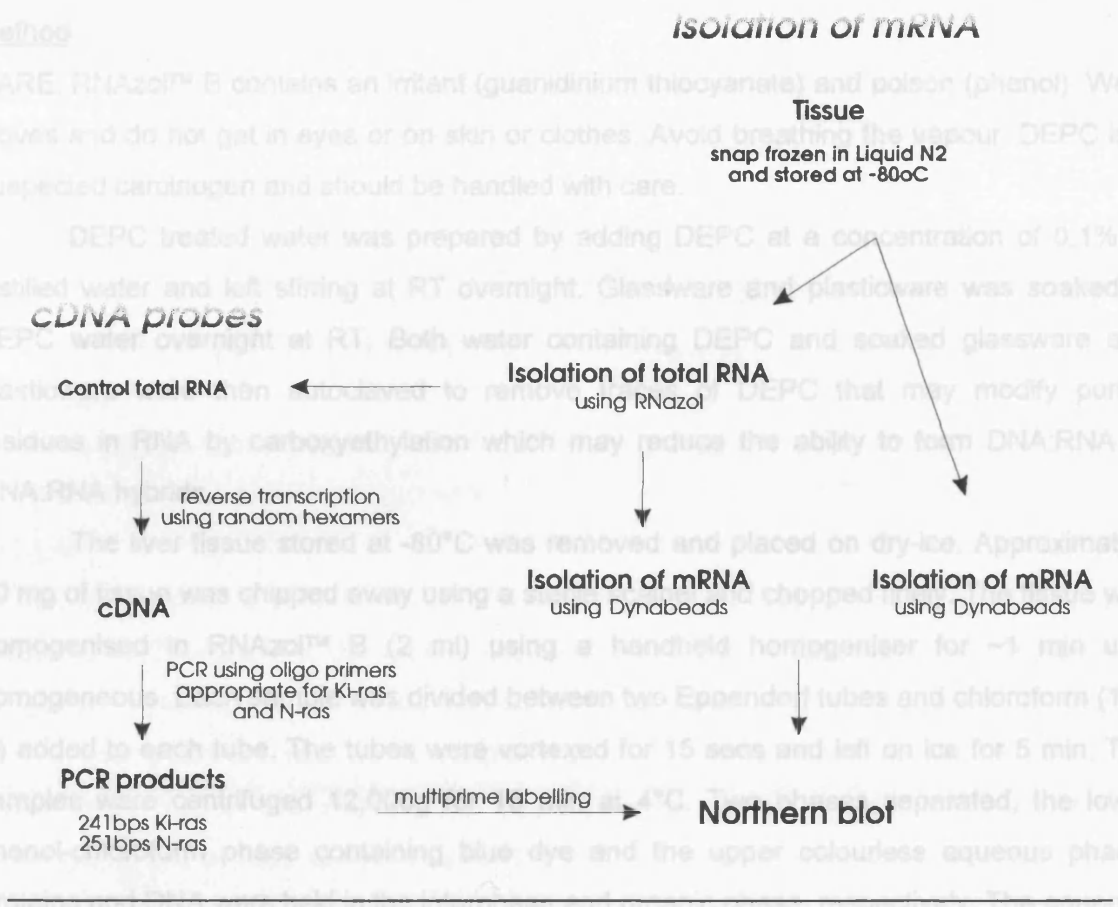


Figure 2.6 Manipulation of RNA : Isolation of RNA and preparation of cDNA probes

NOTE: Prevention of RNase contamination is necessary for any work where RNA is required intact. Hands and dust may be major sources of RNase contamination. Use gloves, keep tubes closed and use sterile, disposable polypropylene tubes. Diethylpyrocarbonate (DEPC) treated RNase free solutions and treated tubes and glassware should be used for all RNA procedures. DEPC is a strong but not complete inhibitor of RNases.

2.8.1 Isolation of total RNA

Reagents

Chloroform

DEPC 0.1%

EDTA 1 mM pH 7.0

Ethanol 75%

Isopropanol

RNAzol™ B

Method

CARE: RNAzol™ B contains an irritant (guanidinium thiocyanate) and poison (phenol). Wear gloves and do not get in eyes or on skin or clothes. Avoid breathing the vapour. DEPC is a suspected carcinogen and should be handled with care.

DEPC treated water was prepared by adding DEPC at a concentration of 0.1% to distilled water and left stirring at RT overnight. Glassware and plasticware was soaked in DEPC water overnight at RT. Both water containing DEPC and soaked glassware and plasticware were then autoclaved to remove traces of DEPC that may modify purine residues in RNA by carboxyethylation which may reduce the ability to form DNA:RNA or RNA:RNA hybrids.

The liver tissue stored at -80°C was removed and placed on dry-ice. Approximately 50 mg of tissue was chipped away using a sterile scalpel and chopped finely. The tissue was homogenised in RNAzol™ B (2 ml) using a handheld homogeniser for ~1 min until homogeneous. Each sample was divided between two Eppendorf tubes and chloroform (100 µl) added to each tube. The tubes were vortexed for 15 secs and left on ice for 5 min. The samples were centrifuged 12,000g for 15 min at 4°C. Two phases separated, the lower phenol-chloroform phase containing blue dye and the upper colourless aqueous phase. Proteins and DNA were held in the interphase and organic phase, respectively. The aqueous phase was transferred to a fresh tube for precipitation of the RNA with an equal volume of isopropanol. The samples were stored at 4°C for 15 min. After centrifugation at 12,000g for 15 min at 4°C a white-yellow pellet of RNA became visible. The RNA was washed with 75% ethanol (0.8 ml) after removing the supernatant, was vortexed and subsequently centrifuged for 8 min at 7,500g at 4°C. The pellet was dried ~10 min under vacuum and dissolved in 1 mM EDTA pH 7.0 (50µl). If the RNA pellet became too dry, the solubility was greatly decreased, so to aid dissolution, the RNA was vortexed and/or incubated for 5 min at 60°C.

2.8.2 Isolation of mRNA

Dynabeads® Oligo $(dT)_{25}$ provide an easy and rapid purification of polyadenylated mRNA (poly A^+ mRNA). They are uniform, superparamagnetic, polystyrene beads, 2.8 µm in diameter that contain 25 nucleotide long chains of deoxythymidylate covalently attached to the bead surface via a 5' linker group. Base pairing occurs between the poly $(A)^+$ residues at the 3' end of mRNA and the oligo (dT) residues attached to the beads. Other RNA species e.g. rRNA and tRNA do not have a poly $(A)^+$ tail and therefore are washed away (Jakobsen *et al.*, 1990; Hornes and Korsnes, 1990).

A. Purification of mRNA from total RNA

Reagents

Binding buffer 2x concentration (20 mM Tris HCl pH 7.5, 1 M LiCl, 2 mM EDTA)

Dynabeads® Oligo $(dT)_{25}$

Elution buffer 2 mM EDTA pH 7.5

Washing buffer (10 mM Tris HCl pH 7.5, 0.15 M LiCl, 1 mM EDTA)

Apparatus

Magnetic particle concentrator

Method

The volume of total RNA (75 μg) was adjusted to 100 μl with elution buffer and heated to 65°C for 2 min to disrupt the secondary structures. Meanwhile, Dynabeads® Oligo $(dT)_{25}$ were concentrated in the magnetic concentrator for 30 secs, the supernatant removed and the beads washed once with binding buffer (2x concentration 100 μl). Binding buffer (2x concentration 100 μl) was added to the beads and the total RNA added. The solution was mixed gently and allowed to hybridise for 3-5 min. The beads/RNA mixture was then placed in the magnetic concentrator, the supernatant removed and the beads washed twice with washing buffer (100 μl). Care was taken to remove all the supernatant after the final washing step. The desired amount of elution buffer (usually 5 μl) was added to the beads and then heated to 65°C for 2 min. The tubes were placed immediately in the magnetic concentrator and eluted mRNA transferred to a clean RNase-free tube. The eluted mRNA was then stored at -80°C until required (Hornes and Korsnes, 1990).

B. Isolation of mRNA directly from solid tissue

Reagents

Homogeniser

Pestle and Mortar DEPC treated and autoclaved

Dynabeads® Oligo $(dT)_{25}$

Washing buffer (10 mM Tris HCl pH 7.5, 0.15 M LiCl, 1 mM EDTA, 0.1% SDS)

Elution buffer 2 mM EDTA pH 7.5

Lysis/binding buffer (100 mM Tris HCl pH 8.0, 500 mM LiCl, 10 mM EDTA pH 8.0, 1% SDS, 5 mM DTT)

Method

Approximately 100 mg of tissue taken from liquid nitrogen was ground up in a pestle and mortar stored on dry-ice. The frozen powder was then transferred to a RNase free universal containing lysis/binding buffer (1 ml) and homogenised using a hand-held homogeniser. The lysate was spun for 30 secs in an Eppendorf centrifuge, the supernatant transferred to an Eppendorf tube containing Dynabeads® Oligo $(dT)_{25}$ (1.5 mg) suspended in lysis/binding buffer (100 µl) and annealing was allowed to occur for 3-5 min at RT or on ice. The tubes were then placed in the magnetic concentrator for at least 30 secs and the supernatant removed. The beads were washed three times with washing buffer each time concentrating the beads in the magnetic concentrator and removing the supernatant. Elution was carried out at 65°C for 2 min with elution buffer (10 µl). The tube was then placed in the magnetic concentrator, the eluted mRNA removed and stored at -80°C in an RNase-free tube (Jakobsen *et al.*, 1990).

C. Spectrophometric determination of RNA

The concentration of the RNA was determined by measuring the A_{260} of the final preparation. A_{260} = 1 corresponds to approximately 40 µg/ml of RNA. The purity of RNA was estimated by determining the ratio between the readings at 260 nm and 280 nm. An A_{260}/A_{280} absorbance ratio in the range 1.8 to 2.0 indicated a pure preparation of RNA. To check that the RNA was intact and not degraded the total RNA/mRNA was run on a 1% denaturing agarose gel at 22 volts overnight and visualised using ethidium bromide 0.5 µg/ml. mRNA appeared as a smear from approximately 10 Kb down with no evidence of ribosomal RNA. Electrophoresis of total RNA revealed two major rRNA bands at 18S and 28S with no evidence of smearing below the 18S band (Sambrook *et al.*,1989).

D. Regeneration of Dynabeads® Oligo $(dT)_{25}$ for reuse

As the oligo $(dT)_{25}$ residues are covalently attached to the bead surface it was possible to regenerate the beads for reuse. However due to reduction in binding capacity it was not recommended to do this more than five times, in practise this was no more than twice.

Reagents

Binding buffer 2x (20 mM Tris HCl pH 7.5, 1 M LiCl, 2 mM EDTA)

Dynabeads® Oligo $(dT)_{25}$ to be regenerated

NaOH 0.1 M

Water bath 65°C

Method

The used beads were resuspended in O.1M NaOH solution (200 µl) in the same Eppendorf tube. The suspension was then transferred to a new RNase-free tube and heated to 65°C for 2 min. The beads were concentrated in a magnetic concentrator for at least 30 secs and the supernatant removed. Two more rinses with NaOH were performed and the beads were resuspended in binding buffer (2x concentration). Two more washes with binding buffer (2x concentration) were carried out until the pH was less than 8.0 (approximately three times). The Dynabeads® Oligo $(dT)_{25}$ were then resuspended in a volume of binding buffer (2x concentration) to give 0.2 mg/ml concentration and stored at 4°C.

2.8.3. Preparation of Ki-ras and N-ras probes for Northern blotting

A. Preparation of cDNA

Reagents

Agarose 1%

DNase I

dNTP's 10 mM each

DTT 0.1 M

Ethidium bromide 0.5 µg/ml

First Strand buffer 5x (250 mM Tris HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$)

H_2O DEPC treated

Random hexamer primers 100 ρmol/µl

RNasin® ribonuclease inhibitor

Superscript™ II RNase H^- Reverse Transcriptase

Method

To total RNA (5 µg in 10 µl volume), DNase I (0.5 µl) and RNasin® (0.5 µl) was added to remove contaminating DNA ensuring the integrity of the RNA and to destroy RNases which would digest the RNA. DNase I is an endonuclease that degrades double-stranded or single-stranded DNA. RNasin® ribonuclease inhibitor acts by binding noncovalently to RNases in a 1:1 ratio. The following reagents were then added sequentially: H_2O (3 µl), buffer (5x concentration 4 µl), DTT (2 µl), random hexamer primers (1 µl), dNTP's (1 µl) to a total volume of 20 µl. The mixture was gently vortexed. A negative control was included which did not contain any RNA. The reaction was carried out on a BioMetra thermocycler under the following cycling conditions: 65°C 5 min, 37°C 10 min (1 µl of reverse

transcriptase was added after this step), 37°C 50 min and 94°C 5 min. The cDNA was then either stored at -20°C until required or used immediately for PCR.

B. Amplification of cDNA

The cDNA was then amplified using primers designed to give products of suitable length for use in northern blotting to examine the expression of Ki-*ras* and N-*ras* in rat liver after exposure to AFB_1. The PCR primers chosen spanned exon 1 and exon 2 of either rat Ki-ras or N-ras genes and gave products of 241bps and 251bps respectively.

Reagents

dNTP's 10 mM each

Ethidium Bromide 0.5 μg/ml

H_2O deionised

PCR buffer 10x (Promega)

Primer 1 forward 50 ng/μl

Primer 2 reverse 50 ng/μl

Taq polymerase (Promega)

Visigel

Method

To cDNA (1 μl) prepared as above the following reagents were added sequentially: H_2O (41.2 μl), PCR buffer (10x concentration 5 μl), dNTP's (0.5 μl), primer 1 (1.0 μl), primer 2 (1.0 μl) and Taq polymerase (0.2 μl) to a final volume of 50 μl. Controls included one reaction containing a known or commercial cDNA sample as a positive control and one reaction containing the 'no RNA' control from the preparation of the cDNA as a negative control to check for contamination. The reaction was carried out on a Perkin Elmer thermocyler under the following cycling conditions: 94°C 2 min, 94°C 1 min/ 50°C 1 min/ 72°C 1.5 min 40 cycles and soak file 4°C. The integrity of the PCR product was assessed by running of the PCR product (9 μl) with loading buffer (1 μl) on a Visigel with 2 μg of a 100bp DNA ladder to check the purity and size of the product. As the primer sets spanned two exons, contaminating genomic DNA containing introns could be detected.

2.8.4 Northern Blotting

Reagents

Agarose

Alkaline solution (NaOH 50 mM, NaCl 10 mM)

Ethidium bromide 0.5 μg/ml

Formaldehyde 40% (v/v) analytical grade

Formamide deionised

GeneScreen® *Plus* nylon membrane

H_2O_2 3%

Mops buffer 10x concentration (0.2 M mops, 50 mM NaOAc, 0.01 M EDTA, pH 7.0 with acetic acid)

Neutralising solution (Tris HCl 0.1 M pH 7.4)

RNA loading buffer (50% glycerol, 1 mM EDTA, 0.01% bromophenol blue)

RNA size marker 0.24-9.5 Kb

SSC (150 mM sodium chloride/ 15 mM trisodium citrate/ 0.1 mM EDTA)

Transfer solution SSC 20x concentration

Vacugene XL blotting system

Water (Milli-Q) DEPC treated and autoclaved

Method

Electrophoresis tanks used for electrophoresis of RNA were soaked in a solution of 3% H_2O_2 for 10 min at RT. The tank was then thoroughly rinsed with DEPC treated water.

The 1% agarose gel was prepared by dissolving agarose (1 g) in 10 ml Mops 10x concentration and DEPC treated water (76 ml) in a microwave. Once the agarose had cooled to ~50°C, formaldehyde (17 ml) was added. Formaldehyde was used to ensure the RNA remained single-stranded as it reacts with free amines. The resulting Schiff base is not capable of hydrogen bonding between complementary bases which reduces the likelihood of two strands of RNA reannealing or forming secondary structures. It is less toxic and more stable than other denaturants such as glyoxal and methylmercuric hydroxide. However since formaldehyde is toxic the gels were poured in the fumehood.

The RNA samples were prepared by adding to either 30 μg of total RNA or all of the prepared mRNA in 12 μl volume the following: deionised formamide (25 μl), Mops (10x concentration 5 μl), formaldehyde (8 μl) and ethidium bromide (1 μl). The samples were vortexed briefly before incubating at 65°C for 5 min. After chilling on ice, RNA loading buffer (5 μl) was added and mixed gently. Approximately 5 μg of RNA ladder was prepared in exactly the same way as the RNA samples. The RNA samples were loaded into the appropriate wells and the marker loaded in the end lane. Electrophoresis was carried out overnight at ~22 volts with the buffer being circulated using a peristaltic pump. After electrophoresis the gel was removed and rinsed five times in five volumes of DEPC treated water to reverse the formaldehyde reaction (Mason *et al.*, 1993; Williams *et al.*, 1991).

GeneScreen® *Plus* hybridisation membrane was used for the northern transfer of RNA. It is a supported, positively charged nylon membrane which is resistant to cracking or tearing. Its charged surface gives it a higher nucleic acid binding capacity than uncharged membranes. The membrane was carefully cut wearing gloves to the exact size of the gel and soaked in DEPC treated water for a few seconds until fully hydrated. The membrane was then soaked in 10x concentration SSC for 15 min. A mask was chosen which was approximately 5 mm larger than the membrane and was placed over the membrane on the porous screen. The frame was positioned and secured and the gel was carefully placed on the membrane avoiding trapped air bubbles ensuring an overlap on the mask of at least 2 mm. Small cracks in the gel were filled with low melting point agarose to ensure a good seal, the pump was switched on and stabilised between 50-55 mbar. Immediately, enough water to cover the gel was applied and left for 5 min. The gel must always be covered with solution when the pump is on. The unit was tilted and the liquid removed using a pipette. Any residual liquid was removed by gently wiping the surface of the gel with a gloved finger. To improve transfer the RNA was partially hydrolysed by alkaline hydrolysis. Approximately 30 ml of alkaline solution was poured onto the gel and left for 5 min. The excess liquid was removed as described above. To neutralise the reaction approximately 30 ml of neutralising solution was poured unto the gel and left for 5 min, removal of excess liquid occurred as above. Transfer was allowed to occur for 4 hours with the gel covered to twice its depth in transfer solution. After transfer the liquid was removed, the pump switched off, the gel removed and the outline of the gel marked with a pencil on the membrane. The membrane was removed and rinsed briefly in 2x concentration SSC to remove residual agarose.

The RNA was fixed to the membrane using UV light 254 nm (UV Stratalinker™ 24000, Stratagene) 0.12J/cm^2 on 'autocrosslink' which formed crosslinks between a small fraction of the bases in the RNA and the positively charged amine groups on the surface of the membrane. Care was taken not to over-irradiate the membrane as this results in covalent attachment of a higher proportion of thymines which reduces the hybridisation signal. Baking the membrane at 80°C for 1-2 hours to reverse the formaldehyde reaction was recommended but this was found to reduce the hybridisation signal. The membrane was then stored at -80°C wrapped in SaranWrap™[f] until required.

2.8.5 Labelling of cDNA probes

Multiprime labelling was used to label the cDNA probes for use in hybridisation to northern blots. This method developed by Feinberg and Vogelstein in 1983 provides a reliable way to radiolabel small quantities of DNA to very high specific activity. The method

[f] SaranWrap™ is a registered trademark of the Dow Chemical Co.

uses random sequence hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length. It uses the 'klenow' fragment of DNA polymerase I which, since it lacks the 5'→3' exonuclease activity, would result in the labelled nucleotides incorporated by the polymerase being subsequently removed as monophosphates.

The multiprime kit from Amersham provides a method of preparing high specific activity probes (~2 x 10^9 dpm/μg) which give excellent sensitivities and low backgrounds in hybridisation, in thirty minutes.

Reagents

Multiprime DNA labelling systems kit containing:

Enzyme solution (1 unit/μl DNA polymerase I 'Klenow' fragment in potassium phosphate 50 mM, pH 6.5, 2-mercaptoethanol 10 mM and glycerol 50%)

Primer solution (random hexanucleotides in an aqueous solution)

Reaction buffer (dATP, dGTP and dTTP in Tris HCl pH 7.8, $MgCl_2$, 2-mercaptoethanol)

cDNA probe

[α-^{32}P] dCTP (~3000 Ci/mmol)

Nap™-5 column

TE buffer (Tris HCl 10 mM pH 8.0, EDTA 1 mM)

Method

The cDNA to be labelled was dissolved to a concentration of 25 μg/ml in TE buffer and 1 μl of DNA denatured by heating to 95-100°C in a boiling water bath for 2 min and then chilled on ice. The following reagents were then added to the denatured DNA on ice sequentially: buffer solution (10 μl), primer solution (5 μl), water (27 μl), [α-^{32}P] dCTP (5 μl) and enzyme solution (2 μl). The components were mixed by gently pipetting up and down and spun briefly in a microfuge. The reaction was allowed to proceed at 37°C for 30 min. To remove unincorporated nucleic acids the sample was run through a Nap™-5 column. These are prepacked disposable columns containing Sephadex® G-25 medium.The storage buffer was poured from the column and the column equilibrated with TE buffer (2 ml). The sample volume was increased to 200 μl with TE buffer and applied to the column, the probe eluted with 200 μl volumes of TE buffer and approximately ten fractions collected. The fraction containing the labelled probe was identified using a bench top scintillation counter.

2.8.6 Hybridisation of RNA

Reagents

Deionised Formamide

Denhardt's solution 100x concentration (polyvinylpyrrolidone MW 40,000 (1 g), BSA (1 g), Ficol 400 (1 g), add H_2O to 50 ml, filter sterilise and store 4°C.)

Hybridisation solution (identical to prehybridisation solution except nonhomologous DNA is omitted)

Prehybridisation solution (SSC 5x, deionised formamide 50% w/v. Denhardt's solution 5x, SDS 1%, Dextran Sulphate, Na salt MW 500,000 10%, salmon sperm denatured 100 μg/ml)

Salmon sperm DNA

SDS 10%

SSC 20x (NaCl 3 M, sodium citrate dihydrate 0.3 M)

Method

The membrane was soaked in 2x SSC prior to prehybridisation. The membrane was then prehybridised in 50 μl/cm^2 of prehybridisation buffer at 42°C for at least one hour in a Techne Hybridiser HB-1D hybridisation oven. Sheared salmon sperm DNA was added as denatured nonhomologous DNA to act as a blocking agent to reduce background. It was denatured by heating to 95-100°C in a boiling water bath for 5 min and chilled on ice. The prehybridising solution was poured off and fresh hybridisation solution preheated to 42°C added to the membrane (approximately 50 μl/cm^2). Approximately 5x10^5 dpm purified probe per ml hybridisation buffer was denatured by boiling for 5 min at 95-100°C and then chilled on ice. This was added to the membrane and hybridisation was allowed to occur overnight at 42°C.

The hybridisation buffer was poured off and the membrane washed with SSC (2x 200 ml) at RT for 15 min. This washing procedure was repeated until non-specifically bound probe was removed by monitoring the blot using a Gieger counter. The membrane was washed twice in SSC 2x, SDS 2% (400 ml) at 65°C for 45 min. The final two washes were in SSC 0.1x (200 ml) at RT for 15 min. Excess moisture was blotted from the membrane, it was then covered in SaranWrap™ and autoradiographed overnight at -80°C using intensifying screens. Note: The membrane was not allowed to dry out as drying caused the probe to become irreversibly bound to the membrane.

A. Stripping DNA probes

As the target RNA was covalently bound to GeneScreen® *Plus* nylon membrane and was not allowed to dry out it was possible to strip the membrane for reprobing. However some loss of target RNA did occur.

Reagents

Sodium chloride 0.015 M

Sodium citrate 0.0015 M (0.1x SSC)

SDS 1%

Method

The solution comprised of the reagents above was boiled in a covered container in a microwave oven on high setting for 10 min. The membrane was added to the superheated solution and left in the solution until cool, approximately 10 min. The membrane was then wrapped in SaranWrap™ and stored at -20°C until required for further probing.

2.9 Quantitation and determination of AFB_1 adducts

2.9.1 Isolation of Genomic DNA

Reagents

Chloroform/isoamylalcohol 24:1

Ethidium bromide

IMS 100%, ice cold

IMS 70%, ice cold

Phenol/Chloroform/isoamylalcohol

Proteinase K

SDS 10%

SSC (150 mM sodium chloride/ 15 mM trisodium citrate/ 0.1 mM EDTA)

TE (Tris HCl pH 8.0 10 mM/ EDTA 1 mM)

TNE (Tris HCl pH 7.5 10 mM/ NaCl 150 mM/ EDTA 2 mM)

Method

Genomic DNA was isolated from normal rat liver tissues by the SDS/proteinase K digestion method (Davis, 1986). A small piece of tissue (1-2 mm^2) was chipped off the frozen liver samples. This was chopped finely with a sterile scalpel and digested at 37°C overnight in 0.5 ml Tris HCl pH 7.5 10 mM/ NaCl 150 mM/ EDTA 2 mM (TNE), 60 µl 10% SDS and 60

μg proteinase K. A Phenol/chloroform extraction was carried out. If DNA only was required the sample was RNased, RNase A (10 μg) was added and incubated at 37°C for 2hrs. A Phenol/chloroform extraction was repeated and the DNA was ethanol precipitated and resuspended in Tris EDTA (100 μl). An aliquot was run on a 2% agarose gel, stained with ethidium bromide (0.5 μg/ml) to confirm that a satisfactory sample of DNA had been obtained (i.e. that it was of high molecular weight and had not been sheared or degraded through the manipulations and there was no RNA present). This high molecular weight DNA, because of its size does not move easily through an agarose gel but remains close to the negative terminal. DNA prepared in this way from untreated animals fed the control diet provided the wild type control for each of the experiments.

Nucleic acids extraction from control and test liver for use with the ELISA technique was carried out using the Applied Biosystems 341 Nucleic Acid purification system, Genepure. The frozen liver tissue (200-300 mg) was chopped up finely using sterile scalpel blades and homogenised in 2 ml of SSC buffer (150 mM sodium chloride, 15 mM trisodium citrate, 0.1 mM EDTA) on ice using a hand held homogeniser (Omni international 1000). The homogenate was then added to the extractor using a fine plastic pasteur pipette. After lysis using lysis buffer and proteinase K (Applied Biosystems), three extractions were performed, two using equal volumes of a 70% phenol/water/chloroform mixture and the third using just chloroform to remove residual phenol. Precipitation was performed using sodium acetate buffer pH 4.5 and isopropanol. DNA/RNA was then eluted onto 25 mm precipitette-II cartridge filters (Applied Biosystems) and solubilised in SSC buffer (1 ml) by vortexing.

A. Spectrophotometric determination of DNA

The concentration of the DNA only samples was determined by measuring the A_{260} of the final preparation. A_{260} = 1 corresponds to approximately 50 μg of double-stranded DNA per ml. The purity of DNA was estimated by determining the ratio between the readings at 260 nm and 280 nm. An A_{260}/A_{280} absorbance ratio in the range 1.8 to 2.0 indicated a pure preparation of DNA (Sambrook *et al.*, 1989).

2.9.2 Methods of hydrolysis

Nucleic acids prepared using the SDS/proteinase K digestion method were hydrolysed either using hydrochloric acid or potassium acetate.

A) Hydrochloric acid method: HCl (1 M) was added to the sample at a final concentration of 0.1 M and the sample depurinated optimally by heating to 75°C for 15 min followed by placing immediately on ice. The sample was then spun briefly to remove residual nucleic acids (Croy and Wogan, 1981). This method was used to prepare the samples used in the HPLC experiments.

B) Potassium acetate method: KOAc pH 5.2 was added to a final concentration of 0.05 M and $ZnCl_2$ to a final concentration of 0.1 mM. The sample was depurinated/denatured by heating to 95°C for 15 min, placed on ice and then spun briefly (Croy *et al.*, 1978). This method of hydrolysis was used in the ELISA experiments to quantify AFB_1 adducts as it was found to release more purines as measured using radioactivity (see section 2.9.4).

2.9.3 High Pressure Liquid Chromatography (HPLC) separation

The fractionation of DNA hydrolysates was achieved on a chromatograph comprising two LDC ConstaMetric III pumps, LDC FluorMonitor III and either a SpectroMonitor III UV detector or an ABI 1000s diode array detector set at 365 nm for aflatoxin detection. The cartridge column used was a chromopack C18 CP-Sphere (μ particle size) 100mm by 3 mm. Gradient elution was used to separate the hydrolysis products using reverse phase chromatography. Solvent A: 15% methanol/ 8% acetonitrile/ 0.02% H_3P0_4 and solvent B: 40% methanol/ 8% acetonitrile/ 0.02% H_3P0_4. Gradient development was 0 to 100% B in 7 min, hold for 2 min and return to initial conditions over 10 min at 1 ml/min with a 12.5 min cycle between assays. In general 5 μl of standards and 100 μl of DNA hydrolysates were injected via a Rheodyne 7125 injection port. Analytical HPLC was carried out in collaboration with Mr D Judah at the Medical Research Council Toxicology Unit, Leicester.

2.9.4 Radioactivity Measurement

Tritium measurements of hydrolysates obtained using preparative HPLC were obtained in a Canberra Packard 1500 Tri Carb liquid scintillation analyzer. Ultima Gold scintillant (10 ml) was added to samples in minivials. Counts were an average of two readings of five minutes each. Counts were converted from cpm to dpm using a quenching factor of 61% as determined by running a tritium standard <0.2 mCi (supplied with the scintillation analyzer).

2.9.5 Competitive Enzyme Linked Immunosorbent Assay (ELISA)

Competitive ELISA was used to quantify AFB_1 adducts present on the total nucleic acids extracted from livers of test and control animals.

Reagents

AFB_1 in PBS 0.001-100 ng/ml

AFB_1-BSA conjugate

ELISA microtitre plates

H_2SO_4 10 M

HCl 0.1 M

KOAc pH 5.2 0.05 M

PBS

PBS containing 0.05% Tween 20

PBS containing 0.25% gelatin

Sodium hypochlorite 0.5%

3,3',5,5'-tetramethylbenzedine (TMB) liquid substrate

$ZnCl_2$ 0.1 mM

Method

AFB_1-BSA conjugate was prepared in the laboratory previously using the method of Sizaret *et al.,* (1982) at a molar ratio of AFB_1:BSA in the order of 7:1. The conjugate was diluted to 10 ng/ml in PBS and 50 µl used to coat ELISA microtitre plates (F96 polysorp Nunc-immunoplate) by adding to each well and allowing to dry at 37°C overnight. The ELISA plates were then wrapped in foil and stored at -20°C until required. The plates were washed four times with PBS-Tween 20 (0.05%) using an immersion technique. The ELISA plates were completely submerged in the PBS-Tween 20 in a plastic box and the surface of the plate gently rubbed to avoid air locks in the wells. Two minutes soaking time was allowed for the first wash. The liquid was shaken out and the plates dried by vigorously banging the inverted plate on several layers of tissue. The nonspecific sites were blocked by incubating each well with of PBS-gelatin (0.25% gelatin) (100 µl) for 60 min at room temperature. A concentration range of AFB_1 standards (0.001-100 ng/ml) were prepared and 150 µl pipetted into sterile microtubes, two tubes of PBS were also prepared as an uninhibited standard. The unknown samples were diluted appropriately and hydrolysed using potassium acetate (KOAc).

Rabbit anti-AFB_1 serum prepared in the MRC Toxicology Unit previously was diluted 1:10,000 having already determined in previous experiments that this dilution was most suitable using direct ELISA. The diluted antiserum was added to the AFB_1 concentration standards and unknown samples using a 1:1 ratio of antibody to sample. The plate was sealed and then incubated at 37°C with continuous shaking for 60 min. After incubation to block nonspecific sites on the plate the solution was discarded and the plate washed twice with PBS-tween 20, then dried as before. Of the antibody-sample/AFB_1 concentration standard mixture, 50 µl was added to the plate in replicates of four and sealed. It was then incubated with continuous shaking for 90 min at RT. The second antibody, rabbit anti-IGg-peroxidase conjugate diluted 1:5,000 in PBS-gelatin, was prepared and stored on ice about 5 min before use. The plate was washed five times with PBS-Tween 20 and dried as before.

The diluted anti-rabbit-IGg-peroxidase conjugate was added into each well in 50 μl volume, the plate sealed and incubated at RT for a further 90 min with continuous shaking. The plate was washed five times with PBS-Tween and once with distilled water before adding 50 μl of 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system (Sigma) to each well. After incubation at RT for 30 min the reaction was stopped by adding 50 μl of 10 M sulphuric acid which changed the blue colour to yellow. The absorbance was then measured at 450 nm using a microtitre plate reader (Labsystems Multiskan plus).

The percentage inhibition was calculated using the mean of the 'PBS only' wells as the uninhibited control and the mean of the four replicates for each sample. Once the competition curve had been plotted the sample concentration could be calculated. Only readings obtained in the proportionate part of the curve were used (usually 0.01-1 ng/ml) each sample being diluted accordingly to bring it within this range. For each plate the results were normalised using a positive control sample for which the concentration of AFB_1 was known.

2.9.5 Autoradiography of Fixed Tissue

Liver sections from animals injected with [^{3}H] AFB_1 100-465 μCi/100g body weight, two hours before cull, were examined to determine the sites of radioactive incorporation within the fixed tissue section. This experiment was designed to examine the theory of the mechanism of resistance of enzymatically altered foci to further insult by AFB_1. The induction of the enzymes involved in detoxification by antioxidants shows striking parallels with altered foci present after AFB_1 exposure, in their enzymatic pattern of expression. By injecting [^{3}H] AFB_1 and determining the sites of radioactivity in the liver one could establish whether the resistance mechanism present in the areas of altered enzyme expression, was reflected in a reduced formation of AFB_1 adducts in those areas.

Reagents

Acetic acid 1%

Coplin jar

Coverslips

Fixed tissue sections

Forceps

Glycerol 2% aqueous solution

Haematoxylin

Lightproof boxes

Mounting media

Photographic developer 10% phenisol solution

Photographic emmulsion K5
Photographic fixer 20% sodium thiosulphate solution
Safelight (Kodak GBX-2 red light or equivalent)
Silica gel dessicant
Water bath 42°C

Method

NOTE: Carried out in dark room with safety light.

The slides were dewaxed in histoclear and rehydrated through graded alcohols to water. Photographic emulsion was prepared by placing emulsion granules in a coplin jar and standing it in a water bath (42°C) until molten. The emulsion was diluted 1:1 with 2% aqueous glycerol and stirred carefully avoiding bubbles. The slides were dipped individually into the molten emulsion/glycerol solution, removed carefully allowing excess to drain and the back of the slide wiped with tissue. The slides were laid out on the bench until the emulsion gelled, after which they were placed in a slide rack and the racks stored in a light proof box with dessicant at 4°C, not less than 1 week, before development.

After exposure, the slides were removed from the lightproof box and placed in developer at RT for 5 min, rinsed in distilled water at RT and placed in 1% acetic acid for 1 min. The slides were fixed for ~4 min at RT, the time required for fixing was twice the time it took for the opaque emulsion to become clear. The slides were washed in running tap water for 3 min and rinsed in distilled water, then counterstained in haemotoxylin briefly for 30 secs and rinsed four times in tap water. The slides were mounted and examined microscopically and assessed using a Zeiss light microscope (Ashby *et al.*, 1985).

2.10 PCR amplification

Reagents

2-mercaptoethanol 6.7 mM
Ammonium sulphate 11 mM
BSA 113 μg/ml
dNTP's (dATP, dGTP, dCTP, dTTP) 1 mM of each
EDTA pH 8 4.4 mM
Molecular weight marker (100bp DNA ladder)
Magnesium chloride 4.5 mM
Mineral oil
Oligonucleotide primers (Table 2.3a-b)
Taq DNA polymerase

Tris HCl pH 8 45 mM

Qiaquick-spin PCR purification kit

PB binding buffer

PE wash buffer

PCR columns

collection microtubes 2 ml

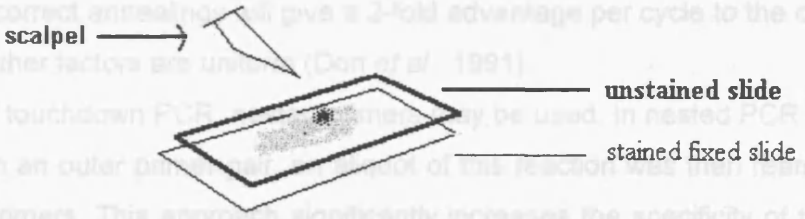
Method

After staining the slides, the areas of interest (positive or negatively stained for histochemical and immunohistochemical markers see section 2.6) were marked with a marker pen and orientated under an unstained formalin fixed serial section. A small scraping of this area was taken using a sterile scalpel blade (No. 15) and added to the PCR mixture (20 µl) containing 45 mM Tris HCl pH 8, 11 mM ammonium sulphate, 4.5 mM $MgCl_2$, 6.7 mM 2-mercaptoethanol, 4.4 µM EDTA pH 8, 1 mM each of dNTPs (dATP, dGTP, dTTP, dCTP), 113 µg/ml BSA, 50 ng PCR primers and 2 units Taq DNA polymerase enzyme and overlayed with 40 µl filtered mineral oil (Lord *et al.*, 1992; Whetsell *et al.*, 1992). For each of the *ras* genes, segments spanning the areas of interest were amplified via two rounds of PCR using the primers listed in Table 2.3a in a Perkin-Elmer/Cetus DNA thermal cycler.

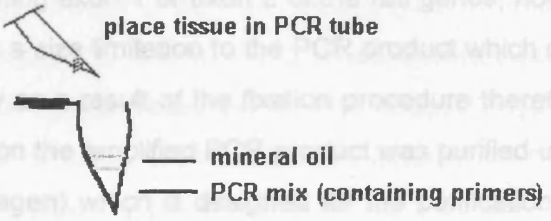
Table 2.3a

Position	Ki-ras	N-ras
Exon 1	$^{5'}$gagtataaacttgtggtag$^{3'}$ catattcatccacaaagtg	$^{5'}$cctacagatttttgcaggtg$^{3'}$ ctatggtgggatcatattc
Exon 2	$^{5'}$caagtagtaattgatggaga$^{3'}$ cacaaagaaagccctcccca	$^{5'}$agtggtgattgacggtgagacc$^{3'}$ atggcaaacacacagaggaacc

For the second round PCR, 2 µl of the first round PCR were transferred to a tube containing the PCR mixture (20 µl). Both first and second round PCRs were put through a program comprising of 10min at 94°C then 20 cycles of 1 min at 94°C, 1.0min at X°C (where X represents a value from 70 to 52 with every second cycle reducing the annealing temperature by 2°C), 1.5 min at 72°C then 20 cycles of 1 min at 94°C, 1.0 min at 50°C and 1.5 min at 72°C. There was one final incubation at 72°C for 10 min which allows for the completion of PCR products. This format of PCR amplification is known as 'touchdown' PCR (Fig 2.7) and was used to prevent spurious priming of non-specific sequences. This method uses the exponential factor in PCR reactions. It begins above the expected annealing temperature which is then decreased every second cycle by 2°C to a 'touchdown' of 50°C, at



scrape section



two rounds

touchdown cycle

denaturing step	94°C	4 mins	1 cycle
annealing step	94°C	1 min	
	X°C	1min	
	72°C	1.5 mins	20 cycles
	94°C	1 min	
	50°C	1 min	
	72°C	1.5 mins	20 cycles
primer extension step	72°C	10 mins	1 cycle

(X°C = 70°C to 52°C, decreasing 2°C every second cycle)

Qiaquick-spin PCR purification kit (Qiagen)

Electrophoresis on 2% agarose gel

Figure 2.7 'Touchdown' PCR (Don *et al.*, 1991)

which 20 cycles were carried out. Any difference in the melting temperature between the correct and incorrect annealings will give a 2-fold advantage per cycle to the correct product, assuming all other factors are uniform (Don *et al.*, 1991).

Instead of touchdown PCR, nested primers may be used. In nested PCR a reaction was performed with an outer primer pair, an aliquot of this reaction was then reamplified with an inner pair of primers. This approach significantly increases the specificity of the PCR, since two pairs of primers were required to amplify the target sequence for a final product to be generated. This method was attempted using an outer primer pair spanning exons 1 and 2 and inner primer pairs spanning exon 1 or exon 2 of the *ras* genes, however as Whetsell *et al.*, 1992 also found, there is a size limitation to the PCR product which can be obtained from formalin fixed slides possibly as a result of the fixation procedure therefore this method was abandoned. After amplification the amplified PCR product was purified using a QIAquick-spin PCR purification kit 250 (Qiagen) which is designed for the purification of single or double-stranded PCR products away from primers, nucleotides and polymerases. A proportion of the purified products eluted in 50μl TE buffer was then run on a visigel, stained using ethidium bromide and visualised using ultraviolet light. A 100bp DNA ladder was run alongside the samples to establish the product was of the correct size.

Controls Negative or "No DNA" control PCRs were included to check for contamination where sterile distilled water was used in place of the DNA sample. As a positive PCR control, genomic DNA isolated from normal rat liver (200ng) was amplified as for the fixed tissue. This also provides 'normal DNA' as a control for the sequencing experiments.

2.10.1 Assay for P53 mutations

Rat tissue was scraped from unstained tumour sections and amplified by touchdown PCR (as described above, section 2.10) using primers covering exon 6 containing the region corresponding to codon 249 of the human p53 gene (Table 2.3b) (Hulla *et al.*, 1993a). The 274bp product was purified using Qiaquick-spin PCR purification kit and sequenced.

Table 2.3b

Position	p53 rat
exon 6	$^{5'}$gcctctgacttattcttgctcttag$^{3'}$ cccaacctggcacacagcttcctac

2.10.2 Sequencing PCR fragments

Sequence determination of amplified DNA fragments was performed by the chain-termination DNA sequencing method as described by Sanger *et al.*, 1977 using T7 DNA polymerase, the primers used for amplification were also used for sequencing. The Sequenase version 2.0 DNA sequencing kit (USB) was used with Mn buffer to facilitate reading of sequences close to the primer region.

The principle of chain-termination sequencing is based on the synthesis of a DNA strand by a DNA polymerase using a single-stranded DNA template initiated at a single site where an olionucleotide primer anneals to the template. The reaction is terminated by the incorporation of a chain-terminating nucleotide *i.e.* one of the 2',3'-dideoxynucleoside 5'- triphosphates (ddNTP's) which lack the 3'-OH group necessary for DNA chain elongation. For each of four separate reactions a mixture of deoxynucleoside triphosphates (dNTP's), one of the four ddNTP's and a radioactively labelled nucleotide is included. This results in the formation of different lengths of radioactively labelled DNA strands terminating where the ddNTP has encorporated. By running the results of each of the four reactions, after separation using high-resolution electrophoresis and visualising using autoradiography, the complete sequence can be obtained (Sanger *et al.*, 1977).

Version 2.0 DNA sequencing kit uses Sequenase version 2.0 to synthesis the DNA strand.This enzyme has high processivity and no 3' to 5' exonuclease activity producing bands of more uniform intensity and less background radioactivity. The DNA synthesis is performed in two steps, the first is the labeling step where the primer is extended using limiting concentrations of dGTP, dTTP, dCTP and radioactively labelled dATP. This allows complete incorporation of the radioactively labelled dATP into DNA chains varying in length from several nucleotides to hundreds of nucleotides. The second step involves increasing the concentration of all the dNTP's, adding a ddNTP and allowing DNA synthesis to continue until all the DNA strands have been terminated by a ddNTP. The addition of EDTA and formamide terminates the reaction and the samples are denatured by heating before running on an acrylamide gel.

Reagents

Alpha ^{35}S dATP

Fixing buffer: acetic acid 10%/methanol 10%

Polyacrylamide gel 6% prepared using acrylamide/bisacrylamide stock solution 25:1 w/v/ 7 M

urea/ 1xTBE using sequencing concentrate diluent

Sequenase version 2.0 DNA sequencing kit:

dGTP labelling mix (5x concentration diluted 1 in 15 in distilled water)

dNTP's dATP, dGTP, dTTP, dCTP

DTT 0.1 M

Mn buffer sodium isocitrate 0.15 M/ $MnCl_2$ 0.1 M

sequenase diluted 1 in 7 in enzyme dilution buffer (Tris HCl pH 7.5/ DTT 5 M /BSA 0.5 mg/ml)

Stop mix formamide 95%/ EDTA 20 mM/ bromophenol blue 0.05%/ xylene cyanol FF 0.05%

T7 DNA polymerase

Method

The ANNEALING REACTION consisting of DNA (0.5 pmol), primer (10 pmol), Mn buffer (2 µl) (0.15 M sodium isocitrate/0.1 M $MnCl_2$) made up to 10 µl using H_2O was boiled for 5 min then rapidly placed in a dry ice/methanol bath for 45 sec and spun for 15 sec. LABELLING REACTION 5.5 µl of the labelling mix [2 µl of 1 in 15 dilution of dGTP labelling mix 5x concentration] (7.5 mM dGTP/ 7.5 mM dCTP/ 7.5 mM dTTP), 1 µl 0.1m DTT, 0.5 ml alpha ^{35}S dATP, 2.0 µl of 1 in 7 dilution of sequenase in enzyme dilution buffer (10 mM Tris HCl pH 7.5/ 5 mM DTT/ 0.5 mg/ml BSA)] was added to the annealing reaction and incubated at room temperature for 45 sec. TERMINATION REACTION To 2.5 µl of prewarmed individual dNTPs, 3.5 µl of the reaction mix was added and incubated at 37°C for 5 min. The reaction was terminated by the addition of 4 µl of stop mix (95% formamide/20 mM EDTA/0.05% Bromophenol Blue/0.05% Xylene Cyanol FF). The samples were heated to 80°C for 5 min and placed on ice before loading 2.5 µl of each of the 4 reactions on a 6% PAGE denaturing gel and running at 60 W. After electrophoresis, the gel was fixed for 15 min in 10% acetic acid/10% methanol. This treatment also removes urea. If not removed, urea prevents the gel from drying out completely and contributes to a higher background and loss of resolution. Drying the gel is essential because ^{35}S is a weak beta emitting isotope which is easily quenched by the wet gel. The gel was then dried onto 3 MM Whatman using a vacuum gel drier and exposed to X-ray film (Fuji NIF new RX) using intensifying screens and then developed.

2.11 Production of Yc_2 monoclonal antibody

2.11.1 Preparation of an immunogen for use in monoclonal antibody production

In the initial stages of the study, the anti-GST Yc_2 antibody available had high crossreactivity against rat GST Yc_1. It was therefore decided, because of the importance of GST Yc_2 in the study, to develop a more specific anti-GST Yc_2 antibody. To provide an antigen for such a study two possibilities existed either to use the already available recombinant Yc_2 protein or to identify and make a suitable polypeptide exhibiting maximum possible disparity to the Yc_1 sequence. Both approaches were attempted.

As short oligonucleotides are poor immunogens it is necessary to conjugate them covalently to immunogenic carrier proteins. Keyhole Limpet Hemocyanin (KLH) is a suitable carrier which provides major histocompatiblility (MHC) class II/ T-cell receptor epitopes while the peptides serve as B-cell determinants. Two methods are most commonly used to couple the peptide to the carrier. Glutaraldehyde conjugation which links amino groups on both carrier and peptide (Avarameas, 1969) or *m*-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) coupling which crosslinks the thiol group of cysteine on the peptide to an amino group on the carrier. The number of lysine molecules (available NH_2 groups) in KLH is very high, 300-600 are usually available for binding. The latter method generates a better defined conjugate. The stable thioether linkage between, the maleimide group and the reacted sulphydryl (cysteine) that is formed, cannot be cleaved under physiological conditions (Fig 2.8).

$$\text{MBS} + \text{KLH-NH}_2 \rightarrow \text{KLH-NH-MBS}$$

$$\text{KLH-NH-MBS} + \text{peptide-SH} \rightarrow \text{KLH-NH-MBS-S-peptide}$$

Figure 2.8 MBS coupling reaction

Reagents

5,5' -dinitrobis -(2-nitrobenzoic acid) DTNB pH 7.0 4 mg/ml

Conjugation buffer (0.083 M sodium phosphate, 0.9 M NaCl, .01 M EDTA, pH 7.2)

DTT 0.025 mM

KLH reconstituted 10 mg/ml in distilled water

KwikSep™ Dextran plastic desalting columns

MBS dissolved 2 mg/ml in conjugation buffer

Microsep-30

Sephadex G-10

Synthetic peptide (PFDDEKCVEDAKK) commercially synthesised at Dept. of Biochemistry, Queens Medical Center, Nottingham, UK.
Tris HCl pH 8.0 100 mM

Method

KLH 10 mg/ml (200 μl) was reacted with MBS 2 mg/ml (100 μl) at RT for 1 hour. A desalting column with an exclusion limit of 5000 mW was prepared by pouring off storage buffer and equilibrating with conjugation buffer (15 ml). The reaction volume 300 μl was applied to the column and eluted with 0.5 ml aliquots of conjugation buffer. Fractions of 0.5ml were collected. Protein elution was monitored by reading the absorbance at 280 nm. The maleimide-activated KLH eluted in fractions 3-5 (cloudy greyish colour) were pooled.

DTT was used to reduce all SH groups in the peptide and remove residual thiols present from the cleavage/deprotection reaction in the peptide synthesis. DTT (1.96 mg) was added to the peptide (2.5 mg) dissolved in 500 μl conjugation buffer and reacted for 1- 1.5 hrs at RT.

The peptide (500 μl) was applied to the column and eluted with 0.5 ml aliquots of 1.5 hours at RT. To remove residual DTT which would interfer with the SH conjugation step a 5 ml G-10 sephadex column was prepared and equilibrated in 15 ml conjugation buffer. conjugation buffer. 0.5 ml fractions were collected and peptide elution monitored by reading the absorbance at 216 nm. As the conjugation buffer also absorbs at 216 nm it is necessary to blank against the first eluted sample. The peptide eluted in fraction 5. Free SH was determined using the 5,5'-dinitrobis-(2-nitrobenzoic acid) (DTNB) assay in the eluate containing the peptide. DTNB or Ellman's reagent reacted with the cysteine thiol side chain at pH 8.0 to give a thionitrobenzoic acid product that had a strong absorbance at 412 nm (Ellman, 1959). Tris HCl pH 8.0 100 mM (925 μl) was mixed with DTNB 4 mg/ml pH 7.0 (50 μl) and the absorbance read at 412 nm. The eluate containing peptide (50 μl) was then added and the final absorbance at 412 nm taken (Fig 2.9).

$$[SH]\ \text{mM in fraction is:} \quad \frac{(\text{final } A_{412} - \text{initial } A_{412}) \times 20\ (\text{dilution})}{13.6\ (\text{extinction coefficient})}$$

Peptide 5 mg/ml ~ 2.5 mM SH

(Ellman, 1959)

Figure 2.9 Determination of [SH] using DTNB assay

Since reoxidation of the peptide SH would occur, the peptide was mixed with activated KLH immediately and incubated for 2hrs at RT stirring continuously. The efficiency of conjugation was determined using DTNB assay as described above to assess removal of free SH groups (Fig 2.9). Conjugated peptide concentration was determined by reading the absorbance at 280 nm (Fig 2.10).

Abs_{280} 1.33 = 1 mg/ml protein

(Sambrook *et al.*,1989)

Figure 2.10 Spectrophotometric conversion of protein

As EDTA was included in the conjugation buffer to prevent reoxidation of the disulphides, the coupled peptide was finally spun through a Microsep-30 size exclusion column to remove the EDTA before injection into mice.

2.11.2 Monoclonal antibody production

Injection of an antigen into an animal usually results in the production of antibodies in the blood. However, a complex mixture of antibodies occurs in the antiserum due to the number of antigenic sites available on a single protein. Monoclonal antibody technology (Kohler and Milstein, 1975) has provided a method of producing antibodies with defined specificity and in unlimited quantities (Fig 2.11). Most monoclonal antibodies are produced from mouse cells as the appropriate myeloma cell lines are freely available for this species.

The animal husbandry and cell culture used in this experiment to produce a specific monoclonal antibody to GST Yc_2 was carried out at the The Leicester Royal Infirmary, Department of Surgery where there were established facilities for carrying out such work.

Four animals were exposed intraperitoneally to 10-25 μg of antigen (two were given the recombinant Yc_2 protein and two the KLH-coupled peptide) in the presence of an adjuvant (Titremax Research Adjuvant, Vaxcei) in 100 μl volume. After three weeks a single boost of 10 μg of the antigen in 250 μl PBS was given intraperitoneally. Tail bleeds were taken after seven days and GST Yc_2 antibody production was assessed, in the animals, using direct ELISA. The serum was tested for production of antibodies to GST Yc_1, Yc_2 and BSA in both the preimmunized and in the immunized serum. One animal was chosen as showing the highest specific production of Yc_2 antibody.

The spleen of this animal was then resected and disaggregated into multiple cell suspensions. Within this population of lymphoid cells there were antibody producing plasma or B cells. The spleen cells were fused with a mouse myeloma cell line, NSO, which conveys immortality to the cells. Fusion occurred with the addition of polyethylene glycol (PEG) which

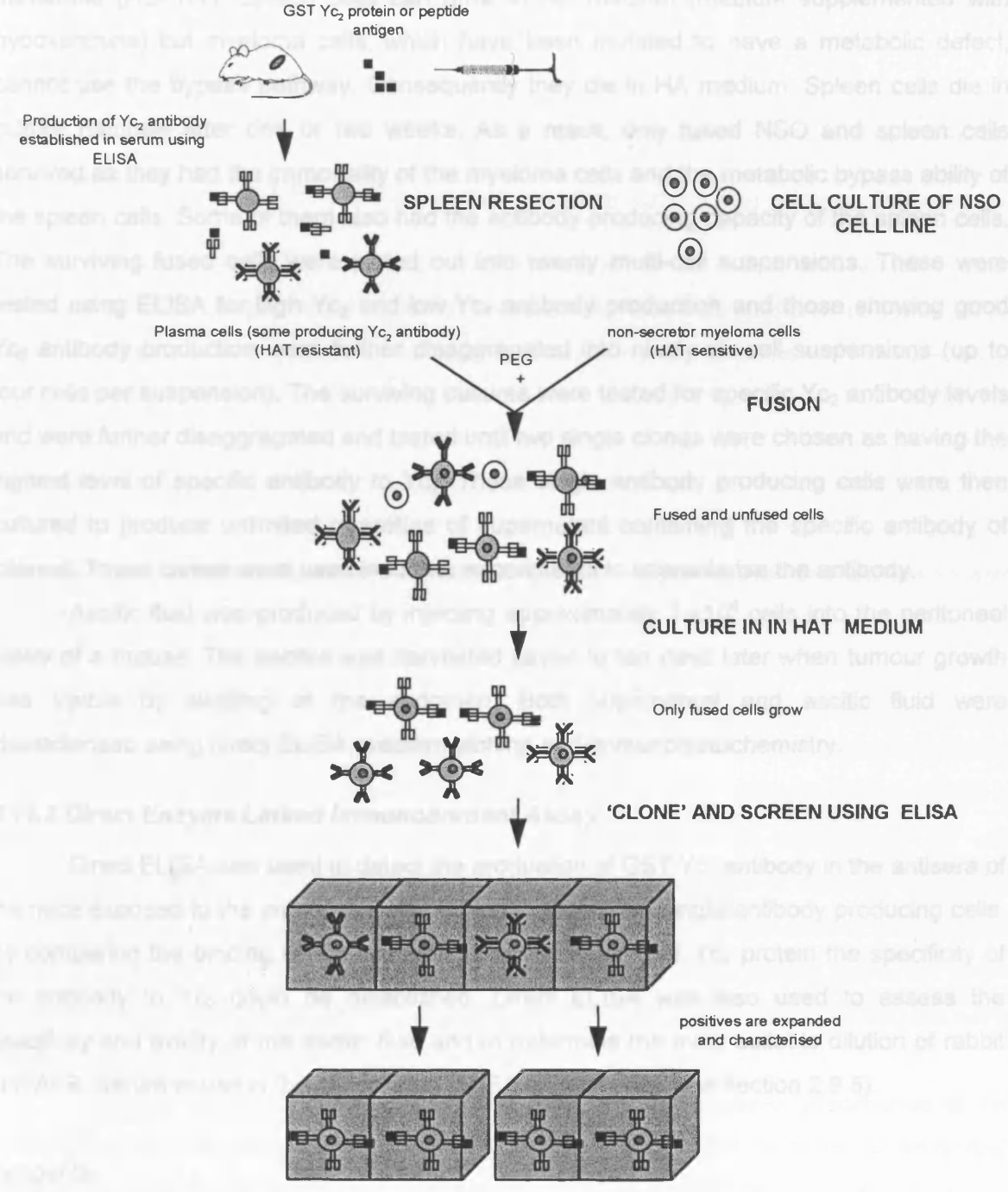


Figure 2.11 Monoclonal antibody production

has the property of making cell membranes fluid and promotes membrane fusion. Any combination of fusion may occur but only spleen cell + NSO cell fusions survive in the presence of hypoxanthine azaserine (HA). Azaserine is a powerful toxin which blocks the *de novo* pathway of purine nucleotide synthesis. This pathway is bypassed if the cell is provided

with the intermediate metabolite, hypoxanthine. The salvage pathway synthesises purine from exogeneous hypoxanthine using the enzyme hypoxanthine-guanine phosphoribosyl tranferase (HGPRT). Spleen cells can grow in HA medium (medium supplemented with hypoxanthine) but myeloma cells, which have been mutated to have a metabolic defect, cannot use the bypass pathway. Consequently they die in HA medium. Spleen cells die in culture naturally after one or two weeks. As a result, only fused NSO and spleen cells survived as they had the immortality of the myeloma cells and the metabolic bypass ability of the spleen cells. Some of them also had the antibody producing capacity of the spleen cells. The surviving fused cells were plated out into twenty multi-cell suspensions. These were tested using ELISA for high Yc_2 and low Yc_1 antibody production and those showing good Yc_2 antibody production were further disaggregated into ninety-six cell suspensions (up to four cells per suspension). The surviving cultures were tested for specific Yc_2 antibody levels and were further disaggregated and tested until two single clones were chosen as having the highest level of specific antibody to Yc_2. These single antibody producing cells were then cultured to produce unlimited quantities of supernatant containing the specific antibody of interest. These clones were used in further experiments to characterise the antibody.

Ascitic fluid was produced by injecting approximately 3 $x10^6$ cells into the peritoneal cavity of a mouse. The ascites was harvested seven to ten days later when tumour growth was visible by swelling of the abdomen. Both supernatant and ascitic fluid were characterised using direct ELISA, western blotting and immunohistochemistry.

2.11.3 Direct Enzyme Linked Immunosorbant Assay

Direct ELISA was used to detect the production of GST Yc_2 antibody in the antisera of the mice exposed to the immunogens and also to determine single antibody producing cells. By comparing the binding of the antibody to recombinant GST Yc_1 protein the specificity of the antibody to Yc_2 could be established. Direct ELISA was also used to assess the specificity and avidity of the ascitic fluid and to determine the most suitable dilution of rabbit anti-AFB_1 serum to use in the competitive ELISA experiments (see section 2.9.5).

Reagents

3,3',5,5'-tetramethylbenzedine (TMB) liquid substrate

Anti-mouse IgG peroxidase conjugated 1/2000 dilution BSA 100 ng/ml

Dilution buffer (K_2HPO_4 10 mM/ NaCl 150 mM/ BSA 1%/ Tween 20 0.1%, pH 7.2)

ELISA microtitre plates

GST Yc_1 recombinant protein

GST Yc_2 recombinant protein

H_2SO_4 0.5 M

PBS containing 3% BSA

Plate coating buffer (Na_2HP0_4/KH_2PO_4 0.01 M, NaCl 0.14 M, pH 8.2)

Washing buffer (PBS pH 7.4, $MgCl_2.6H_20$ 1.5 mM, Tween 20 0.05% v/v)

Method

The antigens GST Yc_1 and GST Yc_2 were diluted 50 ng/ml in plate coating buffer and 50 µl was used to coat one half of ELISA microtitre plates by adding to each well and allowing to dry at 37°C overnight. The other half of the plate was coated with BSA 100 ng/ml. The ELISA plates were then wrapped in foil and stored at -20°C until required. The plates were washed four times in washing buffer using an immersion technique. The ELISA plates were completely submerged in the buffer in a plastic box and the surface of the plate gently rubbed to avoid air locks in the wells. Two minutes soaking time was allowed for the first wash. The liquid was shaken out and the plates dried by vigorously banging the inverted plate on several layers of tissue. The nonspecific sites were blocked by incubating each well with PBS-BSA (200 µl) for 60 min at 37°C. If polyclonal antisera was being tested the sera was diluted using doubling dilutions 1:100 - 1:1600 in dilution buffer and applied to both halves of the plates in duplicate. Tissue culture supernatant was used neat. Ascitic fluid was diluted 1 in 100-10,000 in dilution buffer. A negative control of dilution buffer alone was also applied in duplicate to each side of the plate. The plates were sealed and allowed to incubate at RT for 60 min with continuous shaking. The plates were washed four times in washing buffer as described above. The secondary antibody, anti-mouse IgG was diluted 1:2000 in dilution buffer and 50 µl applied to all the wells. The plates were incubated at RT with continuous shaking for 60 min. The plates were washed four times in washing buffer and once in distilled water as described above before adding 50 µl of the substrate TMB to each well. After incubation at RT for 30 min the reaction was stopped by adding H_2SO_4 0.5 M (50 µl) which changed the blue colour to yellow. The absorbance was measured at 450 nm using a microtitre plate reader (Labsystems Multiskan plus).

The true absorbance was calculated by subtracting the mean absorbance of the antibody on the BSA side of the plate from the mean of the absorbance of the corresponding wells on the GST Yc_1 or GST Yc_2 antigen side of the plate. The specificity of the antibody for either GST Yc_1 or GST Yc_2 protein was then determined by plotting the true absorbance for each antigen.

2.12 Slotblots

Slotblots were carried out to determine the specificity of the monoclonal antibody raised against Yc_2 recombinant protein for the Yc_2 subunit as compared with the Yc_1 subunit and also to establish whether the antibody would exhibit a specific affinity for the synthetic

peptide representing amino acids 206 to 217 present in the Yc_2 sequence (Fig 2.12). The avidity of the antibody was also determined using successive ten-fold dilutions of the antigen. A set of experiments was also carried out using slotblots to examine the binding of the Yc_2 monoclonal antibody to mutant Yc_2 and Yc_1 recombinant proteins prepared by Mr P McDonagh, Dept. of Nuclear Magnetic Resonance, University of Leicester. Three Yc_1 mutated recombinant proteins were used: E208D contained a mutated amino acid at position 208 converting the normal glutamic acid to aspartic acid. H108Y contained a mutation at position 108 converting histidine to tyrosine. The third mutated protein E208D/H108Y contained both mutations.Three Yc_2 mutated proteins were used: Y108L contained a mutated amino acid at position 108 converting tyrosine at this position to leucine. D208M contained a mutated amino acid at position 208 converting aspartic acid to methionine. Y108L/D208M contained both mutations.ECL™ Western blotting (Amersham Life Science), a light emitting non-radioactive method for the detection of immobilized specific antigens was used. The principle of ECL detection lies in the chemiluminescence resulting from a chemical

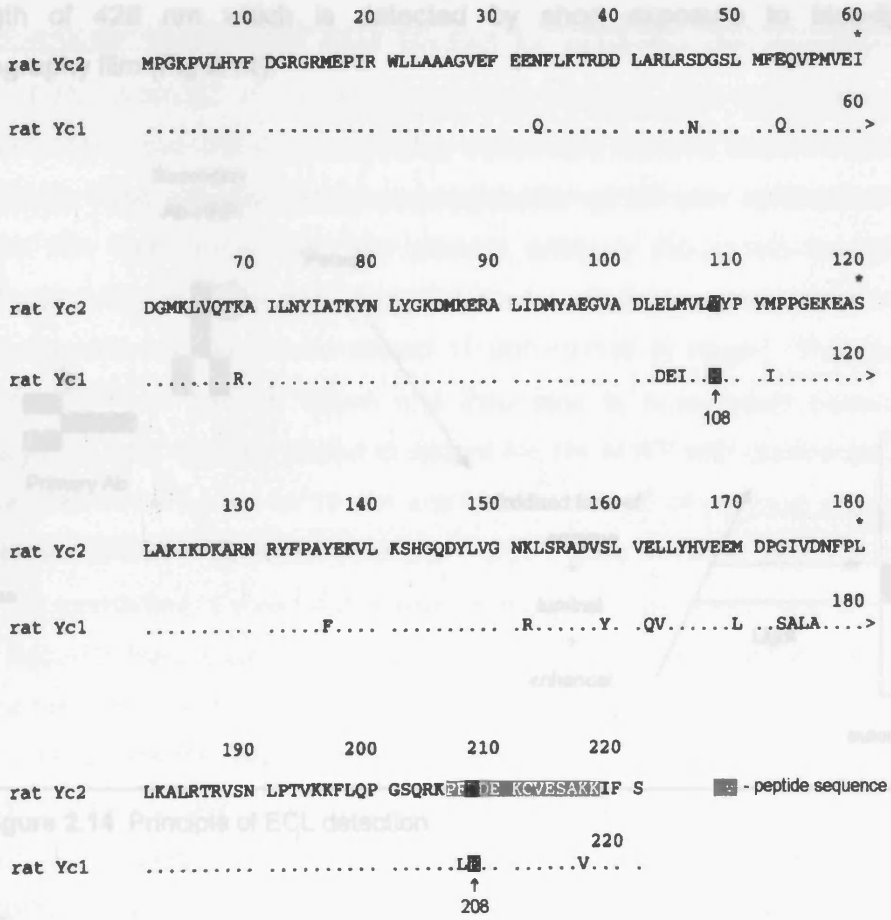


Figure 2.12 GST Yc_1 and Yc_2 amino acid sequences aligned.

reaction. This chemical reaction involves horseradish peroxidase/hydrogen peroxide catalysed oxidation of luminol in alkaline conditions. Oxidised luminol rapidly decays from its excited state to ground state during which it emits light (Fig 2.13). This chemiluminescence is enhanced in the presence of

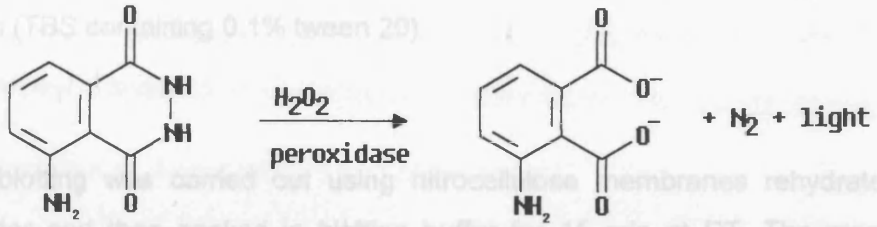


Figure 2.13 Oxidation of luminol

phenol which acts as a chemical enhancer increasing the emission of light up to one thousand fold and extends the emmision time. The maximum light emission is at a wavelength of 428 nm which is detected by short exposure to blue-light sensitive autoradiography film (Fig 2.14).

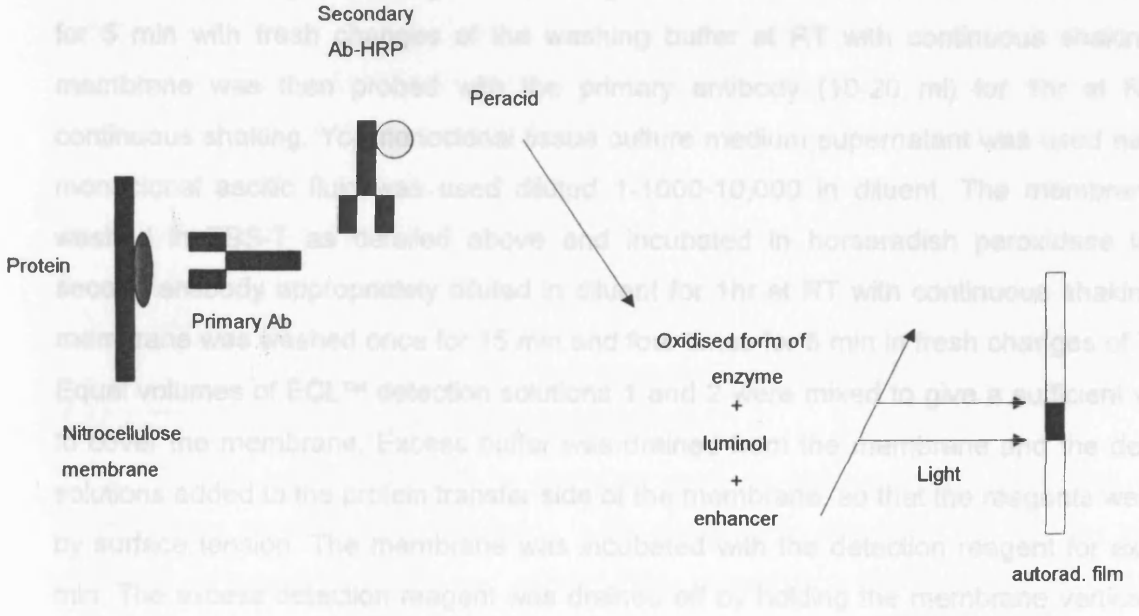


Figure 2.14 Principle of ECL detection

Reagents

Blocking solution (TBS or TBS-T containing 5-10% w/v Marvel)

Blotting buffer (Tris 48 mM, glycine 39 mM, SDS 1.3 mM, 20% methanol)

Diluent (TBS containing 0.1% Marvel)

ECL™ detection reagents (Amersham Life Sciences)

Horseradish peroxidase conjugated anti-mouse IgG diluted 1 in 2000

Hybond™-ECL nitrocellulose membrane

Tris buffered solution (TBS) (Tris HCl 20 mM, sodium chloride 137 mM adjusted to pH 7.6 with hydrochloric acid)

TBS-Tween (TBS containing 0.1% tween 20)

Method

Slotblotting was carried out using nitrocellulose membranes rehydrated briefly in distilled water and then soaked in blotting buffer for 15 min at RT. The membrane was assembled in the slotblotter on top of two similar sized pieces of 3MM Whatman paper presoaked in blotting buffer. A vacuum line from a water pump was connected to the apparatus. The samples were loaded in 100 µl volumes, under vacuum, and washed through with blotting buffer. Following blotting the membrane was then rinsed briefly in blotting buffer.

Nonspecific protein sites were blocked by incubating the membrane in blocking solution (20 ml) overnight at 4°C with continuous shaking. The membrane was rinsed in TBS-T briefly using two changes of washing buffer then washed once for 15 min and twice for 5 min with fresh changes of the washing buffer at RT with continuous shaking. The membrane was then probed with the primary antibody (10-20 ml) for 1hr at RT with continuous shaking. Yc_2 monoclonal tissue culture medium supernatant was used neat. Yc_2 monoclonal ascitic fluid was used diluted 1-1000-10,000 in diluent. The membrane was washed in TBS-T as detailed above and incubated in horseradish peroxidase labelled second antibody appropriately diluted in diluent for 1hr at RT with continuous shaking. The membrane was washed once for 15 min and four times for 5 min in fresh changes of TBS-T. Equal volumes of ECL™ detection solutions 1 and 2 were mixed to give a sufficient volume to cover the membrane. Excess buffer was drained from the membrane and the detection solutions added to the protein transfer side of the membrane, so that the reagents were held by surface tension. The membrane was incubated with the detection reagent for exactly 1 min. The excess detection reagent was drained off by holding the membrane vertically and drawing off the excess with tissue paper. The membrane was placed protein side down on Saranwrap and wrapped to form an envelope. The membrane was then exposed to autoradiography film as quickly as possible. The first exposure was for 15 secs to determine the optimum length of exposure time required, this varied from 1 sec to 30 min.

2.13 Western Blotting

Western blotting was carried out to check the specificity of the antibodies used in the immunohistochemical experiments and to determine the suitability of the Yc_2 monoclonal antibody for use with denatured proteins at levels present in normal adult rat liver samples and in human foetal liver. Human foetal livers of sixteen and nineteen weeks gestation were kindly donated by Dr D Holt, The Karim Centre for Meningitis Research, Queen Charlotte and Chelsea Hospital, London.

2.13.1 Preparation of liver cytosols and microsomes

Reagents

Centrifuge tubes 15 ml and 30 ml

Homogeniser (Omni International 1000)

KCl 150 mM

Method

Two grams of each liver was weighed out into glass universals and chopped finely using a scalpel. The livers were then homogenised in KCl (6 ml) using a hand held homogeniser on high setting. The homogenates were transferred to 30 ml centrifuge tubes and spun at 10,000g for 20 min at 4°C. Equal volumes of supernatant were transferred to 15 ml centrifuge tubes and spun at 100,000g for 1hr at 4°C. The supernatants (*i.e.* the cytosols) were then aliquoted and stored at -70°C. The pellet which represented the microsomal fraction was resuspended in KCl (3.5 ml) by vortexing, aliquoted and stored at -70°C. Quantitation of protein was carried out using the Bradford method (Bradford, 1976) (see section 2.7.1).

2.13.2 SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated by electrophoresis on polyacrylamide gels. The sample proteins were prepared in a denaturing buffer designed to dissociate the proteins into their individual polypeptide subunits and prevent them recombining (Sambrook *et al.*, 1989). The protein mixtures were dissociated by boiling in the presence of excess SDS (a strongly anionic detergent) and mercaptoethanol (reducing agent). SDS bound to the dissociated polypeptides which induced a negative charged. As the amount of SDS bound is almost always proportional to the molecular weight of the polypeptide, independent of its sequence, they migrated in accordance with the size of the polypeptide (Weber and Osborn,1969). A stacking gel of high porosity was used in a discontinuous gel system to 'stack' the proteins

on the surface of the resolving gel. The resolving gel then acted as a sieve at uniform pH and voltage to separate the subunits according to size.

Reagents

Electrode buffer

glycine 1.45%

SDS 0.1%

Tris base 0.3%

Resolving gel

12.5% from stock solution (40% w/v acrylamide/0.8% w/v N,N-methylene bisacrylamide)

Ammonium persulphate 0.01%

N,N,N',N'-tetramethylethylenediamine (TEMED)

Sodium dodecyl sulphate (SDS) 0.1%

Tris HCl 0.75 M pH 8.8

Sample buffer

2-mercaptoethanol 10%

Bromophenol blue 0.002%

SDS 0.1%

Tris HCl 0.75 M pH 6.5

Urea 50%

Stacking gel

acrylamide from stock solution 3%

Ammonium persulphate 0.1%

SDS 0.1%

TEMED 0.1%

Tris HCl 0.75 M pH 6.5

Note: Each gel was deaerated before adding the ammonium persulphate, SDS and TEMED.

Method

Gels were cast in a vertical gel apparatus. The resolving gel was poured initially and overlaid with the aqueous layer of 2-butanol to ensure an even surface on the gel. After 30 min the aqueous sec-butanol was poured off and the surface of the resolving gel washed twice with distilled water. The stacking gel was poured on top of the resolving gel, the comb inserted and the gel allowed to set for 1 hr. Samples were denatured by boiling in sample buffer for 5 min before loading and were then electrophoresed at 30 mA constant current until the bromophenol blue tracking dye reached the bottom of the gel. The gel was then

transferred to nitrocellulose membrane using a semi-dry blotting system. Where the Yc_2 monoclonal antibody was to be used, in detection of Yc_2 antigen, SDS was removed by washing the gel in 20% 2-propanol in 50 mM Tris HCl pH 8.0 for 1 hour at room temperature with continuous shaking. The wash buffer was changed twice. The gel was then incubated for 1 hour in a mixture of 50 mM Tris HCl pH 8.0 and 5 mM DTT with continuous shaking, before blotting.

2.13.3 Electrophoretic Protein Blotting

Due to both the low porosity of and the low diffusion rate into polyacrylamide gels they are not a suitable medium in which to probe the proteins. By transferring the proteins onto nitrocellulose membranes replicas of the original gel separations are achieved and a wide variety of analytical procedures can then be carried out (Towbin *et al.*, 1979).

Reagents

Hybond™-ECL nitrocellulose membrane

Semi-dry transfer unit TE 70 Semiphor™ (Hoefer Scientific Instruments)

Transfer buffer (Tris HCl 48 mM, glycine 39 mM, SDS 1.3 mM, Methanol 20%)

Method

The transfer unit consisted of a platinum coated anode on which was placed three layers of Whatman 3MM paper which had been presoaked in transfer buffer. On top of this was placed the membrane which had been briefly rehydrated in distilled water and soaked for 15 min in transfer buffer. The gel was laid on top of the membrane. The cover containing the cathode was placed on top and the apparatus run at a constant current of 0.8 mA/cm^2 of gel for 1 hour at RT (Fig 2.15).

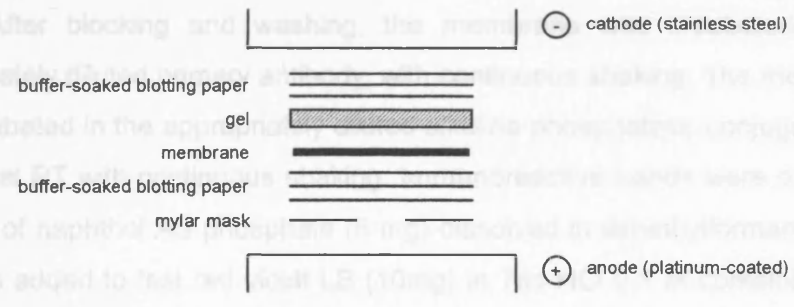


Figure 2.15 Semi-dry blotting system

After blotting, to ensure that complete and even transfer of the proteins had taken place, the gel was stained for protein in Page blue 0.25% in methanol 20%/ acetic acid 10% and destained in the same solution omitting the page blue.

2.13.4 Detection of antigens on protein blots

Once the separated proteins had been electrophoresed and transferred onto nitrocellulose membrane they were probed with antibodies to detect specific antigens, using a modification of a method described by Chu *et al.* 1989.

Reagents

Alkaline Phosphatase conjugated anti-mouse or anti-rabbit IgG diluted 1 in 1000

Diluent (TBS containing 0.05% Tween 20 and 0.1% Marvel)

Fast red violet LB

Hybond™-ECL nitrocellulose membrane

Napthol AS phosphate

Primary antibody

Glutathione-*S*-transferase 7.7, aldehyde reductase and Glutathione-*S*-transferase Yc_1/Yc_2 polyclonal antibodies used at 1 in 1000 dilution in TBS 0.05% Tween 20 and 1% Marvel. Yc_2 monoclonal antibody used as neat supernatant. CK-18 monoclonal antibody used at 1 in 50 dilution of supernatant in TBS containing 0.05% tween 20 and 1% Marvel.

Tris HCl 0.1 M pH 8.5 containing 10 mM magnesium chloride

Method

Note: Blocking and washing steps were carried out as for the ECL™ western detection system.

After blocking and washing, the membrane was incubated for 1 hr at RT in appropriately diluted primary antibody, with continuous shaking. The membrane was washed and incubated in the appropriately diluted alkaline phosphatase conjugated second antibody for 1 hr at RT with continuous shaking. Immunoreactive bands were detected with a filtered solution of naphthol AS phosphate (6 mg) dissolved in dimethylformamide (200 µl) which in turn was added to fast red violet LB (10mg) in Tris HCl 0.1 M containing $MgCl_2$ 10 mM (20 ml). The blot was immersed in the substrate solution until the bands appeared ~5 min. The reaction was stopped by washing in distilled water. The blot was air-dried on filter paper.

Chapter Three

Animal Statistics

3.1 Introduction

The results in this chapter indicate the body and liver weight statistics of both the untreated and treated animals used in the AFB_1 feeding study. These measurements were taken from two weeks before commencing the feeding of AFB_1 in the diet (acclimatisation period) until the end of the study, seventy-nine weeks in total. The animals were weighed twice weekly and the livers were weighed immediately after each animal was culled.

The animals were obtained at three weeks of age and allowed to acclimatise for two weeks on standard RM1 powdered diet. The test animals were then started at five weeks of age on a diet containing AFB_1 at 4ppm in arachis oil mixed with standard RM1 powdered diet. The feeding of this AFB_1 diet to the test animals continued *ad libitum* for twelve weeks, after which it was replaced by standard RM1 pellets for the remainder of the study. The control (untreated) animals received an identical diet to that of the treated animals, but without the AFB_1. The diet consumption of each animal was calculated by taking the difference in the weight of the diet before and after consumption per cage and averaging for the number of animals contained in that cage. The food containers were replenished twice weekly. From the diet consumption data the approximate total amount of AFB_1 ingestion was calculated.

3.2 Results - Analyses of animal statistics

3.2.1 Body and Liver Weights

The body weights were calculated weekly until week 12 and then as monthly averages to indicate the trend of change in body weight, of both the test and control animals.

A. Body Weights

A comparison of the control and test animals body weights indicated a trend of decreased gain in weight in the test animals shortly after starting the AFB_1 diet (Fig 3.1). This difference remained until twenty-four weeks on the study (that is, twelve weeks after ceasing the AFB_1 diet) when the difference became minimal. As can be seen using statistical analysis, however, no significant statistical difference existed between the control and test animals weights overall throughout the initial twelve week period of the study, which showed a close correlation of 0.99 (Table 3.1).

Throughout the remainder of the study, that is, from week twenty-four until week seventy-seven, the weights remained comparable with a few minor fluctuations. These

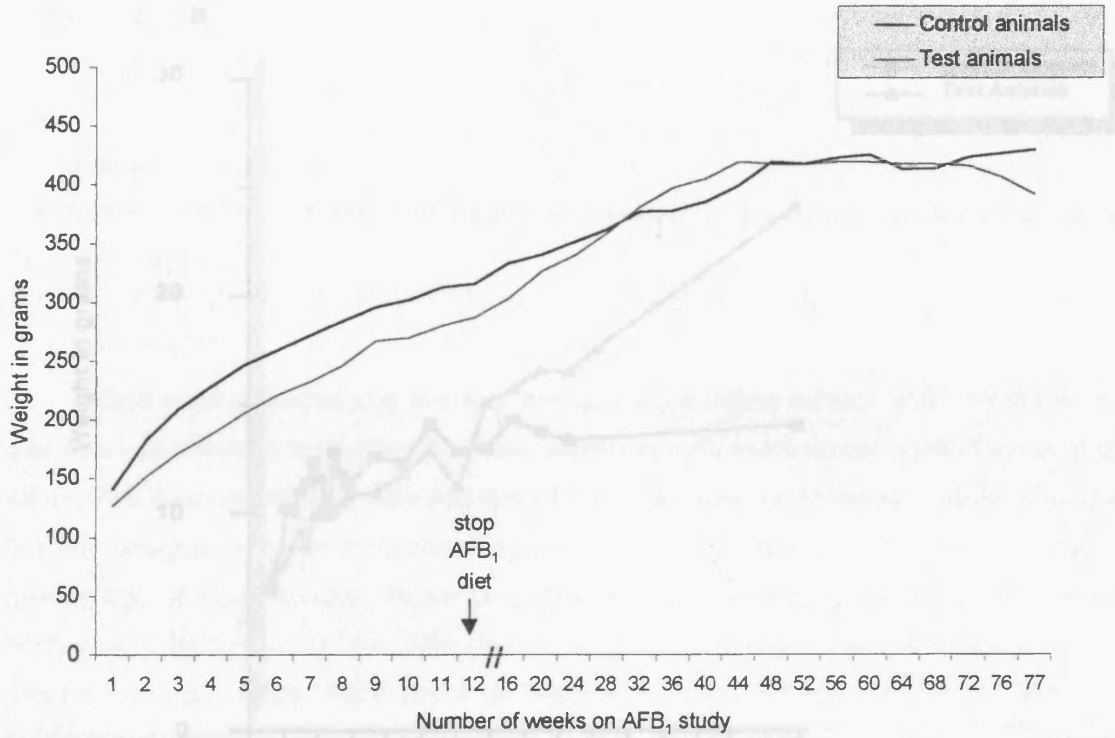


Figure 3.1 Body weight curves for control and test animals on AFB_1 study

Table 3.1 Statistical analysis (appendix I) to determine the statistical difference, if any, between the body weights of the control and test animals during the initial twelve week period of the study.

One-Way Analysis of Variance

Source	DF	SS	MS	F	P
Factor	1	7126.5	7126.5	2.5	0.128
Error	22	62708.04	2850.365		
Total	23	69834.54			

Group	N	Mean Wt.	StDev
Control	12	219.8g	51.73
Test	12	254.3g	54.99

Individual 95% CIs For Mean
Based on Pooled StDev

```
----+---------+---------+---------+--
(---------*---------)
          (---------*---------)
----+---------+---------+---------+--
225       250       275
```

Pooled StDev = 55.1

Correlations (Pearson)

Correlation of Control and Test bodyweights = 0.992

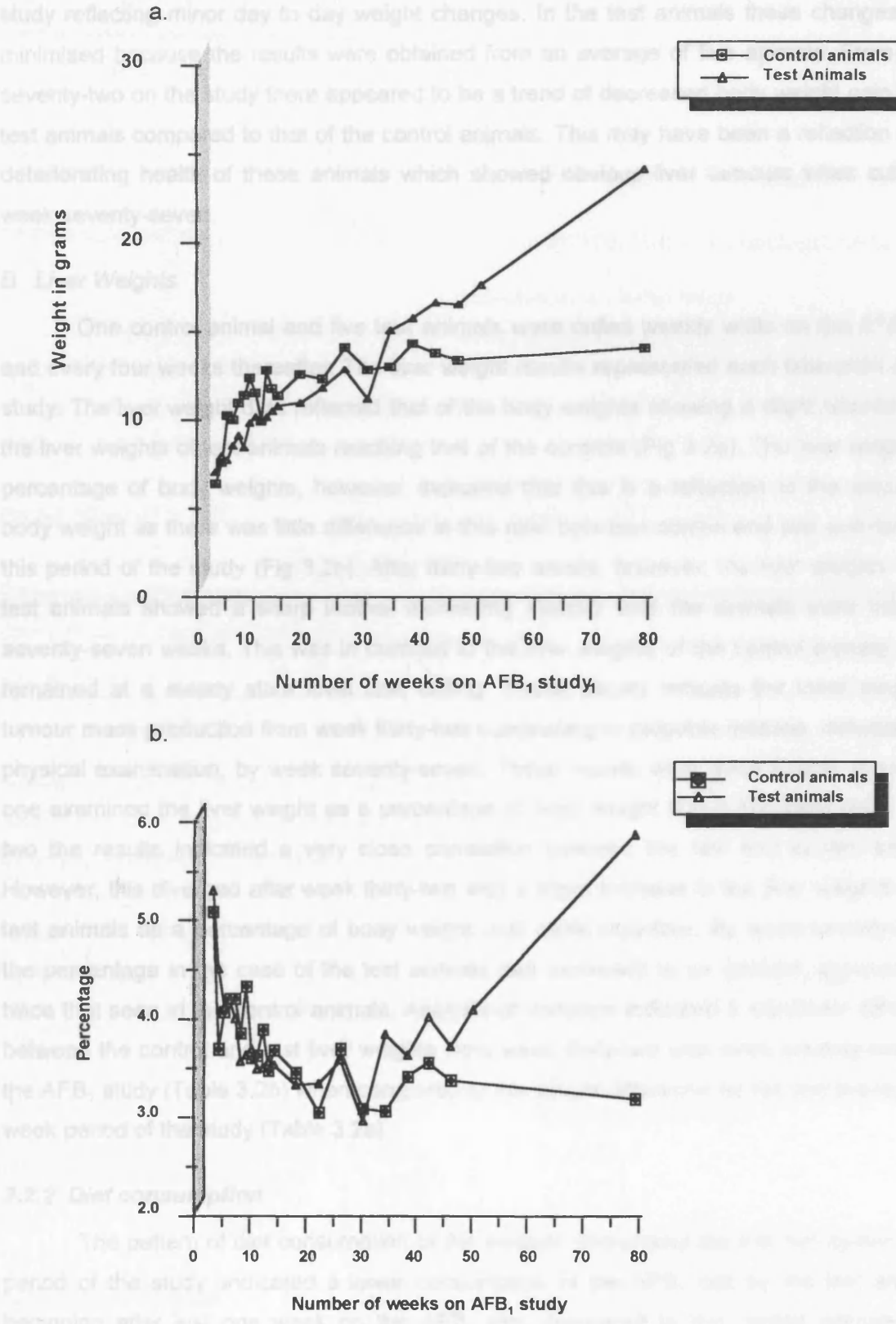


Figure 3.2 a. Liver weights of test and control animals throughout the period of the AFB_1 feeding study. b. Liver weights as a percentage of body weights for both control and test animals throughout the period of the AFB_1 feeding study

fluctuations probably arose due to the decreased number of control animals remaining on the study reflecting minor day to day weight changes. In the test animals these changes were minimised because the results were obtained from an average of five animals. From week seventy-two on the study there appeared to be a trend of decreased body weight gain in the test animals compared to that of the control animals. This may have been a reflection of the deteriorating health of these animals which showed obvious liver tumours when culled at week seventy-seven.

B. Liver Weights

One control animal and five test animals were culled weekly while on the AFB_1 diet and every four weeks thereafter. The liver weight results represented each time-point on the study. The liver weight data reflected that of the body weights showing a slight retardation in the liver weights of test animals reaching that of the controls (Fig 3.2a). The liver weights as percentage of body weights, however, indicated that this is a reflection of the decreased body weight as there was little difference in this ratio between control and test animals over this period of the study (Fig 3.2b). After thirty-two weeks, however, the liver weights of the test animals showed a sharp incline, increasing steadily until the animals were culled at seventy-seven weeks. This was in contrast to the liver weights of the control animals which remained at a steady state level until culling. These results indicate the initial stages of tumour mass production from week thirty-two culminating in palpable masses, detectable by physical examination, by week seventy-seven. These results were even further amplified if one examined the liver weight as a percentage of body weight (Fig 3.3b). Until week thirty-two the results indicated a very close correlation between the test and control animals. However, this diverged after week thirty-two with a slight increase in the liver weights of the test animals as a percentage of body weight until week forty-four. By week seventy-seven the percentage in the case of the test animals had increased to six percent, approximately twice that seen in the control animals. Analysis of variance indicated a significant difference between the control and test liver weights from week thirty-two until week seventy-seven of the AFB_1 study (Table 3.2b) when compared to the weight difference for the first twenty-eight week period of the study (Table 3.2a).

3.2.2 Diet consumption

The pattern of diet consumption of the animals, throughout the first twenty-four week period of the study, indicated a lower consumption of the AFB_1 diet by the test animals, beginning after just one week on the AFB_1 diet, compared to the control animals. This reduced intake of food continued throughout this twenty-four week period, never quite reaching the level of intake consumed by the control animals, despite the removal of the

Table 3.2a

Analysis of variance indicating no significant difference between control and test liver weights during the first twenty-eight weeks on the study.

One-Way Analysis of Variance

Source	DF	SS	MS	F	p
Factor	1	4.71	4.71	0.85	0.363
Error	30	165.62	5.52		
Total	31	170.33			

Group	N	Mean Wt.	StDev
Control	16	9.09g	2.920
Test	16	8.32g	1.585

Pooled StDev = 2.350

Individual 95% CIs For Mean
Based on Pooled StDev

```
--------+---------+---------+---------
        (---------*---------)
(---------*---------)
--------+---------+---------+---------
        8.0       9.0       10.0
```

Correlations (Pearson)

Correlation of Control and Test animal liver weights = 0.521

Table 3.2b

Analysis of variance indicating a significant difference between the control and test animal liver weights from week thirty-two to week seventy-seven of the study.

One-way Analysis of Variance

Source	DF	SS	MS	F	p
Factor	1	38.03	38.03	5.46	0.048
Error	8	55.72	6.96		
Total	9	93.74			

Group	N	Mean Wt.	StDev
Control	5	12.24g	0.531
Test	5	16.14g	3.694

Pooled StDev = 2.639

Individual 95% CIs For Mean
Based on Pooled StDev

```
--------+---------+---------+---------
(-------*-------)
        (-------*-------)
--------+---------+---------+---------
        12.0      15.0      18.0
```

Correlations (Pearson)

Correlation of Control and Test animal liver weights = 0.423

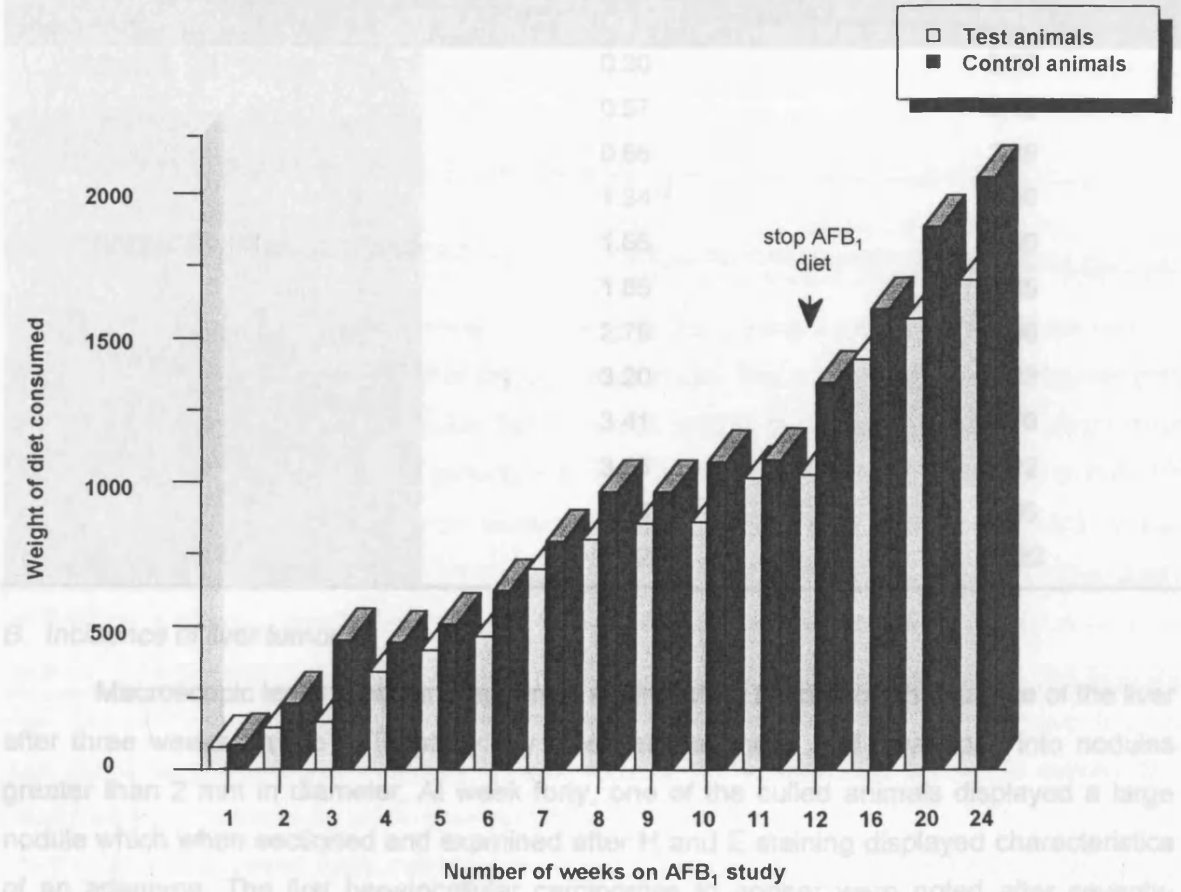


Figure 3.3 Total diet consumption in grams of the test and control animals throughout the twelve week AFB_1 feeding program and the consecutive twelve weeks on control diet

AFB_1 diet at week twelve (Fig 3.3). Two possible explanations for this effect are either a reduction in appetite of the test animals due to a toxic effect, caused by the AFB_1, which affected the well-being of the animals or the test animals disliked the diet, due to an ability to taste or smell it.

A. AFB_1 consumed

Table 3.3 indicates the average amount of AFB_1 consumed per animal, based on the diet consumption data, for each time-point of the study during the administration of the AFB_1 diet. The AFB_1 consumption in milligrams of AFB_1 ingested per kilogram of rat is also indicated. These results indicated (after twelve weeks on the AFB_1 diet) the average amount of AFB_1 ingested by the animals which finally produced liver tumours, sixty-five weeks later.

Table 3.3

Number of weeks on AFB_1 diet	Amount of AFB_1 consumed (milligrams)	AFB_1 consumed mg/kg
1	0.30	2.37
2	0.57	3.12
3	0.66	3.39
4	1.34	4.50
5	1.66	5.40
6	1.85	5.95
7	2.79	7.06
8	3.20	8.03
9	3.41	8.70
10	3.45	9.22
11	4.06	9.86
12	4.12	10.32

B. Incidence of liver tumours

Macroscopic lesions became apparent as tiny white lesions on the surface of the liver after three weeks on the AFB_1 study. By week eleven these had developed into nodules greater than 2 mm in diameter. At week forty, one of the culled animals displayed a large nodule which when sectioned and examined after H and E staining displayed characteristics of an adenoma. The first hepatocellular carcinomas to appear were noted after seventy-seven weeks on the study. These were distributed throughout the liver as multiple nodules varying in size from a few millimeters to several centimeters. The nodules were usually soft and greyish with some appearing haemorrhagic and necrotic.

3.3 Summary and Discussion

The animal study used to examine the carcinogenicity of aflatoxin B_1 was chosen for several reasons. Previous research had indicated species and gender differences in the carcinogenic response to AFB_1 (see review Cullen and Newberne, 1994). Of the possible rodents which could have been used, the male Fischer rat was the most sensitive to these effects. This strain has also been shown to have relatively high sensitivity to the hepatotoxic effects of AFB_1 (Wogan, 1977). Oral administration was chosen as the most effective route to give a chronic low dose of AFB_1 and was most comparable to the route of human exposure. Chronic exposure of humans to low levels of aflatoxin occurs more commonly than acute exposure and evidence indicates that chronic exposure to AFB_1 is more likely to lead to cancer (Wogan, 1976). *In vivo* studies were chosen in preference to *in vitro* studies because *in vitro* systems are not yet available which can satisfactorily express the complex metabolic pathways of AFB_1 metabolism occurring *in vivo* in the liver for example, hepatocytes maintained *in vitro* rapidly lose their ability to metabolise xenobiotics including AFB_1.

Loss of weight or poor growth is one of the first indications of exposure to aflatoxins and the occurrence of a toxic response (Wogan, 1976). Several studies have shown an inhibition of DNA and RNA synthesis and the development of cell necrosis during the initial few weeks of feeding AFB_1 which later recovers by regeneration and reestablishment of the hepatocyte population (Neal, 1973; Godoy *et al.,* 1976). Body weight and liver weight data obtained from the animals on this study indicated an initial deviation from that of the controls as early as the second week on the diet until week 12 during the period of AFB_1 exposure in the diet. Statistical analysis indicated that these differences were not significant. During the next twelve weeks there was a steady increase in body weight gain of the test animals so that the bodyweights reached that of the control animals. The combined data of body weights and diet consumption over the initial twelve week period therefore indicates a slight toxic response in combination with a reduction in diet consumption due to a dislike of the test animals for the AFB_1 diet through an ability to taste or smell it. The presence of macroscopic lesions after three weeks of AFB_1 exposure in the diet verifies the toxic response. The ability of the test animals to reach the weight of the control animals by week twenty-four shows the lack of a permanent toxic effect of the AFB_1 diet on the growth potential of the test animals but the process of hepatocarcinogenesis was irreversible as the macroscopic lesions continued to develop into nodules greater than 2mm in diameter by week eleven and eventually into tumours by week seventy-seven.

The liver weight data indicated the initial signs of tumour mass production occurring after thirty-two weeks on the study, twenty weeks after ceasing the AFB_1 diet. The tumour formation appears subsequently to have progressed slowly but consistently resulting in palpable masses detectable by physical examination at seventy-seven weeks. The average liver weight in these test animals was approximately 10 grams heavier than the liver weight of the age-matched control animals. Statistical analysis revealed a significant difference in the liver weights of the control and test animals after thirty-two weeks on the study. The animals which produced liver tumours ingested a total of approximately 4.12 mg or an equivalent of 10.32 mg/kg of AFB_1 over a twelve week period. This amount therefore was sufficient to induce irreversibly the process of hepatocarcinogenesis in these animals.

Table 3.4 compares the results of three different published AFB_1 feeding studies to produce hepatocellular carcinomas in rats, with the AFB_1 feeding study described in this thesis. The potency of AFB_1 is apparent, as levels as low as 0.015 ppm are effective in producing 100% incidence of liver cancer. However it appears lower doses are only effective if fed for a period in excess of a certain 'threshold' of time (Wogan and Newberne, 1967) (Table 3.4). There also appears to be a dose response effect with the tumour incidence rising with an increased dose as seen in the experiments carried out by Butler and Barnes, (1968) (Table 3.4).

Table 3.4

Summary of incidence of hepatic carcinomas in rats (a. Data obtained from Wogan and Newberne, 1967. b. Data unpublished obtained from the AFB_1 study. c. Data obtained from Butler and Barnes, 1968. d. Data obtained from Godoy *et al.*, 1976)

Sex and strain of animals	AFB_1 (ppm in diet)	Duration of feeding toxic diet (weeks)	Duration of feeding normal diet (weeks)	Percentage of liver tumours
Purified AFB_1				
a.				
Male	1	41		82
Fischer	0.3	52		30
	0.015	68		100
b.				
Male	4	12	65	100
Fischer	4	12	48	0
–				
c.				
Rossetti meal[1]				
Male	5	9	54	100
Wistar	5	6	83	63
	5	3	93	15
	5	1	97	0
d.				
MP meal[2]				
Male	4	6	40	100
Fischer				

[1] Rossetti meal was peanut meal contaminated with aflatoxins. The aflatoxin levels were determined by chemical assay of the aflatoxin source and levels were adjusted by dilution with uncontaminated meals. Although there were minor variations in the kinds of aflatoxins present, AFB_1 was the chief component.

[2] MP meal was peanut meal naturally contaminated with AFB_1. Examination revealed the presence of other mycotoxins for example, Cyclopiazonic acid (Neal GE, personal communication). The peanut meal was diluted 50:50 with normal diet to attain the correct level of AFB_1.

Interestingly the study carried out by Godoy *et al.,* (1976) feeding a meal naturally contaminated with aflatoxins and containing 4 ppm AFB_1 to male Fischer rats for six weeks resulted in 100% incidence of liver tumours after only forty weeks on a normal diet. There is a marked difference between this study and the one carried out here in the time delay before the appearance of tumours despite the similarity in dose of AFB_1 and the decreased length of time on the AFB_1 diet in the study by Godoy *et al.* It would appear apart from seasonal differences in the sensitivity of Fischer rats to aflatoxin as suggested by Godoy *et al.,* (1976) there may be a more inherent variation between the study carried out by Godoy *et al.,* and the AFB_1 feeding study reported here which accounts for this difference. One significant difference between these two studies is the age of the animals which were fed the AFB_1 diet. Godoy *et al.,* (1976) indicates the weight of the animals beginning the AFB_1 diet to be 280 grams, the weight of the animals beginning the AFB_1 diet in this study was 127 grams. This indicates the age of the animals in this study to be much lower than that in the study by Godoy *et al.,* (1976) during the feeding of AFB_1. However the most likely explanation for the difference in tumour formation between the two studies is the presence of other fungal contaminants in the MP diet used by Godoy *et al.*, which included Cyclopiazonic acid (Neal GE, personal communication). This finding has relevance to human exposure as it is extremely likely that diets 'naturally' contaminated with aflatoxins will also contain other mycotoxins.

In the study described in this thesis the early signs indicated a carcinogenic response as expected with treatment with AFB_1 for example, the appearance of small white nodules on the surface of the liver and the weight difference between the control and test animals. By week eleven these small nodules had progressed to more substantial lesions. These results in conjunction with the immunohistochemistry suggested the initial progression of carcinogenesis which would continue after removal of the carcinogen. However it appears, from the long delay between treatment and appearance of the tumours, the process of tumorigenesis, although irreversible, progressed slowly due to the lack of promotional effects by the AFB_1. If the diet had been continued for a longer period of time it appears probable the progression of carcinogenesis would have been more advanced resulting in the production of tumours at a much earlier stage.

Chapter Four

Quantitation and determination of AFB_1 adducts

4.1 Introduction

Previous studies have indicated an almost linear relationship between the dose of aflatoxin and the total number of DNA adducts formed in animals exposed chronically to AFB_1. In liver a highly linear relationship between DNA adduct formation and tumour occurrence is also obtained *i.e.* chronic AFB_1 dose → AFB_1-adducts → risk of tumour formation (see reviews Bailey, 1994 and Eaton and Gallagher, 1994; Croy and Wogan, 1981). In rat liver the 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-aflatoxin B_1 (N^7-guanyl) adduct forms very quickly upon exposure to AFB_1, but has a half life of only seven and a half hours, as it is either converted to the fapy adduct, repaired or lost by depurination from the body. Enzyme repair of the N^7-guanyl adducts appears to play a small role in the removal of these adducts and the major loss is due to spontaneous depurination and conversion to the fapy adduct (Leadon *et al.,* 1981). The fapy (8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydro-pyrimid-5-yl formamido) 9-hydroxy AFB_1) adduct being a more stable AFB_1 DNA adduct is relatively resistant to DNA repair processes.

The fapy adduct represents twenty percent of the total DNA adducts formed and is the most frequently detected adduct in tissues due to its persistence in the body (Croy and Wogan, 1981). Leadon *et al.,* (1981) examined the kinetics of the disappearance of the fapy adduct in human fibroblasts and observed no decrease within 48 hours of formation. Excision of AFB_1-fapy from DNA is reported to occur through the action of fapy-DNA glycosylase. Chetsanga and Frenette (1983) studied the action of the *Escherichia coli* enzyme and confirmed that AFB_1-fapy lesions accumulate in rat liver with time and are repaired at a very slow rate. The importance of the persistence of the fapy adduct in tumour initiation is unclear since work done by Bailey *et al.,* 1988 indicates that initial adduct formation rather than persistent adduct level may correlate with tumour formation in rainbow trout and salmon. It is likely the importance of this adduct depends on its preferential binding sites in DNA and RNA and its ability to be repaired correctly which varies among species. Muench *et al.,* (1983) examined the sites on dsDNA targeted by AFB_1 and found certain guanine sites to be preferentially involved depending on the surrounding nucleotide sequence.

It has been well established that chronic low dose feeding of AFB_1 results in the induction of phase II enzymes in the liver resulting in resistance to further toxic insult by AFB_1 (Neal *et al.,* 1981). In the presence of this enzymatic profile the level of covalent binding of AFB_1 to DNA has been shown to be reduced (Neal, 1973; Mandel *et al.,* 1987).

The level and pattern of AFB_1 adduct formation/removal was examined in the animals on the AFB_1 feeding study. Some of these animals had been injected with 100 μCi/ 100 g (17.4 μg/kg) tritiated AFB_1 two hours before cull. The rationale behind [^{3}H]AFB_1 exposure in

this way was to determine the level of new AFB_1 adducts forming in animals chronically exposed to AFB_1. The absolute amount of [^{3}H]AFB_1 injected per animal is shown in Table A2.1 in Appendix II. A major aim of this part of the thesis was also to quantify the total number of AFB_1 adducts present in the livers of fed animals throughout the twelve week AFB_1 feeding period of the study. This was carried out by immunoassay (competitive ELISA) using a polyclonal antibody recognising AFB_1. Included in these samples were some test and control animals which had been injected with [^{3}H]AFB_1 before cull. These samples were also used to determine the type of AFB_1 adduct present using HPLC fractionation and scintillation counting. Although the amount of AFB_1 injected was minimal (17.4 μg /kg) several controls were included to determine the number of adducts resulting from injection of this amount of AFB_1, in order to correct the ELISA estimations of total accumulated adducts since these animals also received the [^{3}H]AFB_1. The control samples also determined the baseline level which is expected using such a potentially sensitive assay.

The number of [^{3}H]AFB_1 adducts present in the control and test animals which received [^{3}H]AFB_1 by injection was quantified by measuring the level of tritium present. In the presence of a biochemical profile indicating the induction of a mechanism of resistance one would expect to see a reduction in the capacity to form new [^{3}H]AFB_1 adducts in the AFB_1-fed animals. Age-matched controls were also injected with [^{3}H]AFB_1 and the level of adducts present in these animals was compared to that of the AFB_1 fed animals. In addition the high DNA specificity of AFB_1 adduct formation was demonstrated by comparing the level of [^{3}H]AFB_1 adducts formed on total nucleic acids with that of DNA alone.

It had initially been intended to quantitate the level of adducts both by ELISA and using ultraviolet detection at 365 nm following separation by HPLC. It was anticipated the results from the UV detection could have then been compared to the total level of adducts present as indicated using competitive ELISA. However, difficulties were encountered due to the low level of sensitivity of U.V. detection and this method was abandoned and measurements made by ELISA alone.

Autoradiography was carried out in an attempt to determine the sites of the [^{3}H]AFB_1 incorporation. Fixed liver sections prepared from the test and control animals exposed to [^{3}H]AFB_1 were used, from week three to week twelve of the study, during the feeding of AFB_1. In the early weeks of the study and in the control animals areas (if any) of preferential binding of AFB_1 could be determined by comparing the number of 'grains' present in specified areas of the sections. In the animals chronically exposed to AFB_1 one would expect to find tritium incorporation reduced in the foci of altered hepatocytes displaying the phenotype indicating a mechanism of resistance.

4.2 Results

4.2.1 Method Development

A. Identification of the major AFB_1 adduct present

Preparation of the nucleic acid extracts for use in detection and quantitation of AFB_1 adducts by UV detection and scintillation counting was by SDS/proteinase K digestion. Preparation of the nucleic acid extracts, for use in the competitive ELISA experiments was performed, using the Genepure nucleic acid extraction system (Applied Biosystems). This method was chosen in preference to the conventional SDS/proteinase K digestion method as it provided larger quantities of purer nucleic acids more conveniently. The results indicated that the major type of adduct present in the livers of animals injected with [^{3}H]AFB_1 was the fapy AFB_1 adduct. As stated earlier the initial adducts formed following exposure to AFB_1 are the N^7-guanyl adducts, with a maximum level being reached two hours after exposure. Therefore it was expected to see mainly N^7-guanyl [^{3}H]AFB_1 adducts present in the livers of the animals two hours after injection with [^{3}H]AFB_1. The detection of solely fapy adducts therefore, indicates a conversion of the N^7-guanyl adducts to the ring opened form had taken place during the processing of the genomic extractions, due to the process being carried out at alkaline pH. The Genepure system was thought to be less likely to induce this transformation as the pH levels did not exceed 8.0. Analysis revealed this was not the case and conversion of the N^7-guanyl adduct to the fapy had occurred in these samples also. Therefore the adducts detected in both the ELISA experiments and scintillation counting refer to the fapy adduct, this is not a true reflection, however, of the *in vivo* state as there will have been some loss of adducts during the conversion process and is an artefact of the extraction procedures.

Reverse phase HPLC was used to fractionate the hydrolysates of genomic DNA from liver samples taken from animals given a [^{3}H]AFB_1 injection i.p. two hours before cull. Reference standards for AFB_1-fapy and AFB_1-N^7 guanyl adducts (kindly donated by Mr D Judah, MRC, Leics) were run every time a preparative reverse phase HPLC was carried out to determine the retention time for each of the adducts (Fig 4.1). The eluted samples were collected at 30 sec fractions for 5 mins, into minivials, using the method described in section 2.9.3. The radioactive count of each sample was then measured in a liquid scintillation analyser. The identity of [^{3}H]AFB_1 adduct present was determined by comparing the elution time which gave the peak value of tritium counts with the retention times of the reference standards (Fig 4.2). In all of the samples measured the elution time of the fractions was 30 secs after the retention time of the AFB_1-fapy adduct as determined by the ultraviolet detector. An elapsed elution time of 30 secs from that of the retention time reflected the

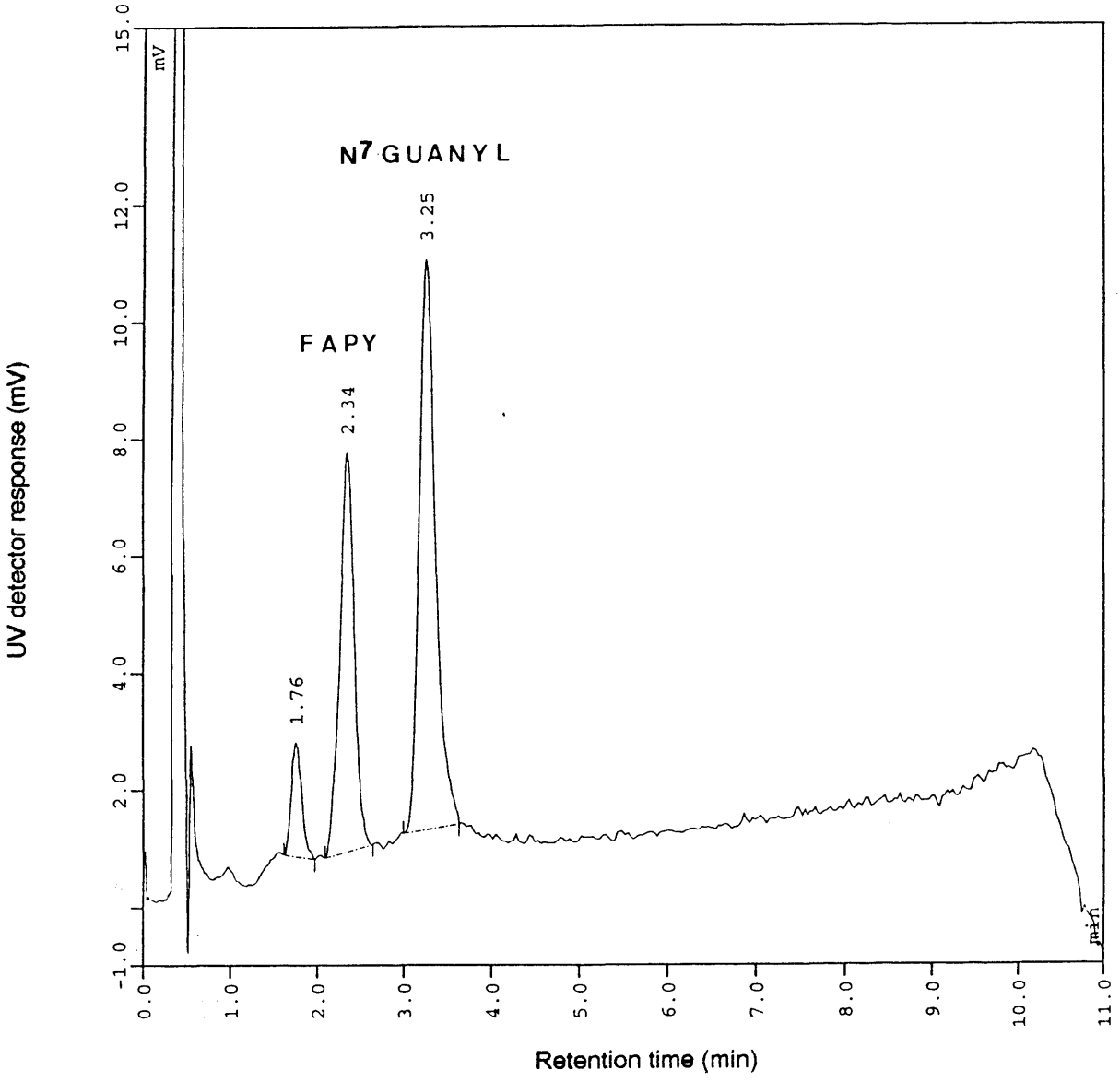


Figure 4.1 Retention times of N^7-guanyl and fapy adducts of aflatoxin B_1-epoxide measured using HPLC

expected time-delay in eluate passing from detector to the fractionator. This indicated the major AFB_1 adduct present in the samples to be the AFB_1-fapy adduct.

B. Method of hydrolysis and depurination

Hydrochloric acid (0.1 M) hydrolysis at 75°C for 15 min, resulting in depurination, was used to release the adducts for quantitation by HPLC and scintillation counting. However, as the AFB_1 adducts were not at a sufficient level to be detected by the ultraviolet (UV) detector at 365 nm, a more efficient method of hydrolysis and depurination was sought. Depurination with Potassium acetate (pH 5.2) at 95°C for 15 mins was found to release more adducts from the nucleic acid extracts, as determined by scintillation counting and therefore was used to release the adducts (see section 2.9.2) for quantitation by ELISA. Unfortunately the UV detector was still unable to detect sufficient level of adducts for reliable quantitation of the level of adducts present. As a result only the measurement of radioactivity was used for quantitation of the $[^3H]AFB_1$ adducts.

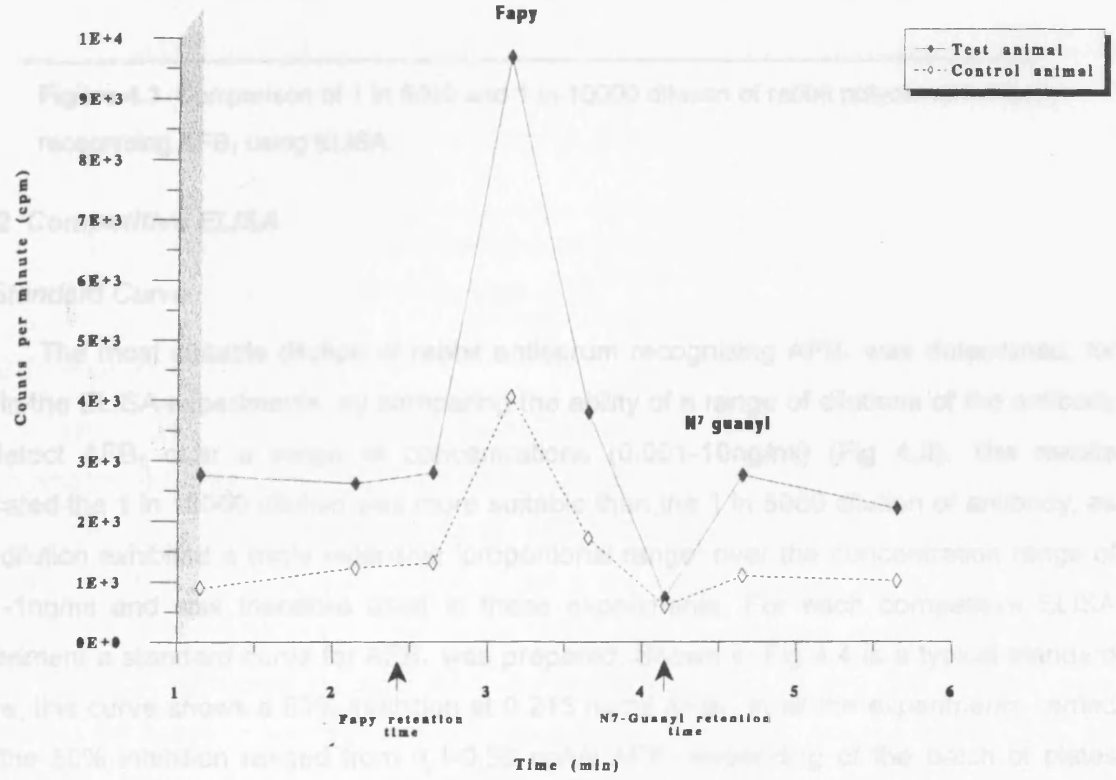


Figure 4.2 Liquid scintillation counts of nucleic acid hydrolysates collected following fractionation using HPLC, from test and control animals injected with $[^3H]AFB_1$ at 12 weeks on the study

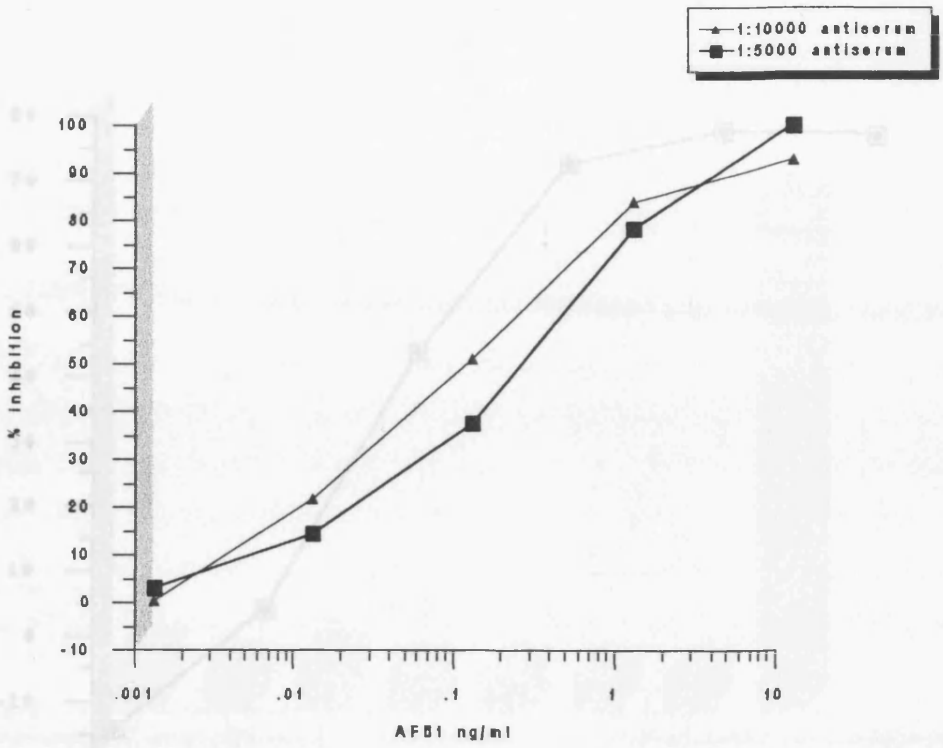


Figure 4.3 Comparison of 1 in 5000 and 1 in 10000 dilution of rabbit polyclonal antibody recognising AFB_1 using ELISA

4.2.2 Competitive ELISA

A. Standard Curve

The most suitable dilution of rabbit antiserum recognising AFB_1 was determined, for use in the ELISA experiments, by comparing the ability of a range of dilutions of the antibody to detect AFB_1 over a range of concentrations (0.001-10ng/ml) (Fig 4.3). The results indicated the 1 in 10000 dilution was more suitable than the 1 in 5000 dilution of antibody, as this dilution exhibited a more extensive 'proportional range' over the concentration range of 0.01-1ng/ml and was therefore used in these experiments. For each competitive ELISA experiment a standard curve for AFB_1 was prepared. Shown in Fig 4.4 is a typical standard curve, this curve shows a 50% inhibition at 0.215 ng/ml AFB_1. In all the experiments carried out the 50% inhibition ranged from 0.1-0.38 ng/ml AFB_1 depending of the batch of plates prepared with the AFB_1-BSA conjugate. Each result was normalised using a positive control sample for which the AFB_1 concentration was known to eliminate curve to curve variation. The detection limit was 0.01 ng/ml.

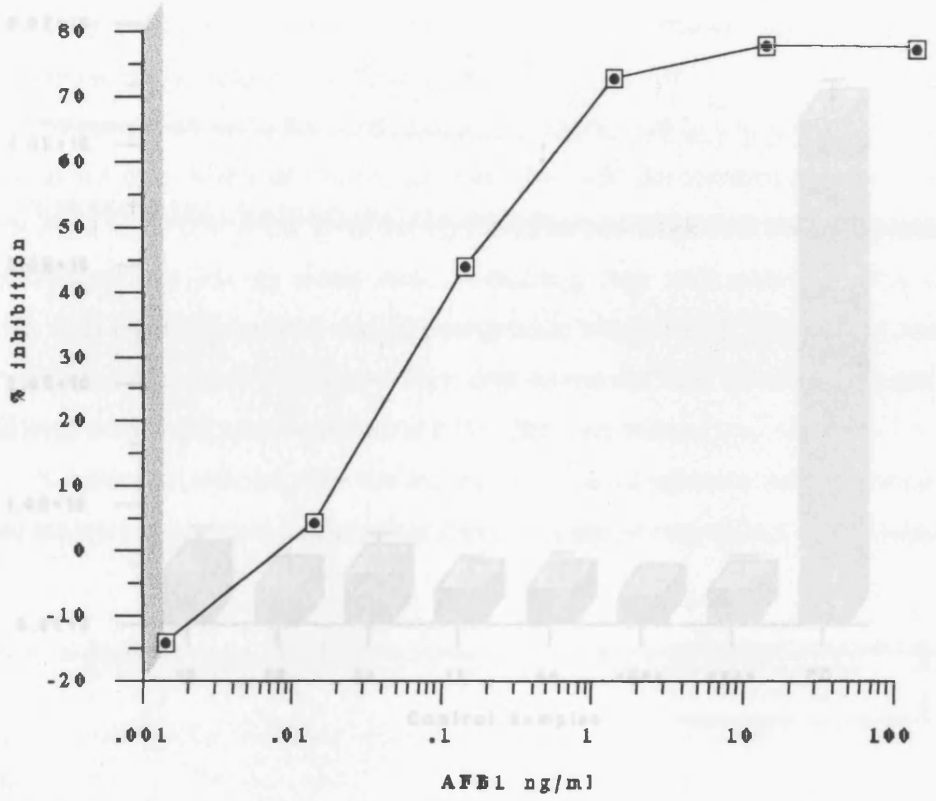


Figure 4.4 Typical ELISA competition curve for AFB_1 using rabbit polyclonal antibody. Individual values are the mean of duplicate wells.

B. AFB_1 adducts

Several preliminary competitive ELISA experiments were run with each sample of DNA hydrolysate to establish the correct dilution required to bring the percentage inhibition of each sample within the proportionate (steep) part of the curve. When this dilution had been established the experiment was repeated using the same sample up to five times to eliminate spurious results which may occur using such a sensitive assay. Raw data for ELISA results is contained in Table A2.2 in Appendix II. Several negative controls were included in the assay to establish the AFB_1-specificity of the assay; herring sperm DNA, rat kidney DNA, ethoxyquin treated rat liver DNA, guanosine and adenosine nucleotides. Two animals which had been fed AFB_1 (4 ppm) for twelve weeks, then returned to a normal diet for four weeks and eight weeks, respectively, were also included to determine the persistence of the AFB_1 adducts after removal of the AFB_1 diet (Fig 4.5). No significantly high reactivity of the AFB_1 antibody with any of the negative control samples was observed. These results indicated a baseline in the method equivalent to approximately 152 pg AFB_1

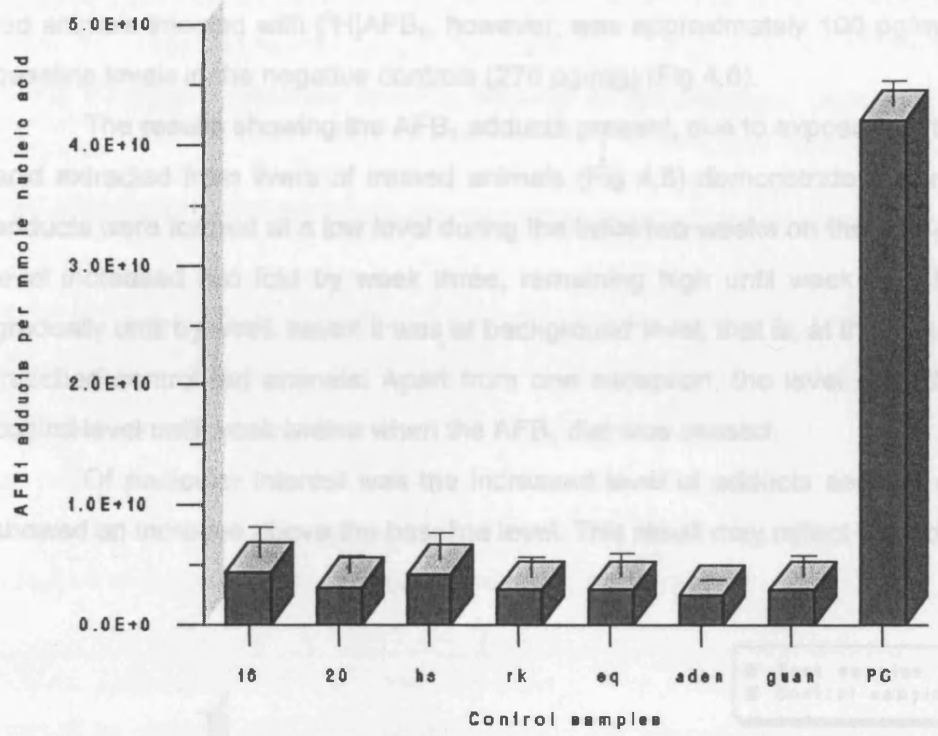


Figure 4.5 AFB_1 adduct levels of control samples (16 and 20 - animals fed AFB_1 (4 ppm) for 12 weeks and returned to control diet for 4 and 8 weeks, respectively; HS - herring sperm DNA, RK - rat kidney DNA, EQ - ethoxyquin treated rat liver DNA; aden - adenosine; guan - guanosine; PC - positive control animal injected with 1 mg/kg [^{3}H]-AFB_1 two hours prior to cull)

adducts per mg nucleic acid. The animals which had been fed AFB_1 and returned to the normal diet showed baseline levels of the AFB_1 adducts (159 pg/mg) after eight weeks following removal of the AFB_1 diet. The level of AFB_1 adducts present in the animals four weeks following removal of the AFB_1 diet showed approximately 40% more adducts present than the negative controls. This reflects the nature of repair of the fapy adducts which is reported to occur very slowly. Extraction of genomic DNA from the liver of an animal injected i.p. with 1 mg/ kg [^{3}H]AFB_1 two hours before cull was also included as a positive control for comparison. This animal showed 2065 pg AFB_1 adducts per mg nucleic acid, twelve times the baseline levels observed in the negative controls.

DNA extracted from an age-matched control fed animal for each time-point was assayed. Some of these controls had been injected with [^{3}H]AFB_1 (17.4 μg/kg) that is, weeks 4,6,9 and 12. These results thus indicated the levels of AFB_1 adducts present due to the

[^{3}H]AFB_1. The results of these assays using the samples from the control fed animals which had not received [^{3}H]AFB_1 were in the order of the baseline levels observed in the other non-AFB_1 injected negative controls (161 pg/mg). The level of adducts measured in the control fed animals injected with [^{3}H]AFB_1, however, was approximately 100 pg/mg higher than the baseline levels in the negative controls (270 pg/mg) (Fig 4.6).

The results showing the AFB_1 adducts present, due to exposure in the diet, in nucleic acid extracted from livers of treated animals (Fig 4.6) demonstrate a trend whereby AFB_1 adducts were formed at a low level during the initial two weeks on the AFB_1 diet. This adduct level increased two fold by week three, remaining high until week five, the level then fell gradually until by week seven it was at background level, that is, at the level seen in the age-matched control fed animals. Apart from one exception, the level of adducts remained at control level until week twelve when the AFB_1 diet was ceased.

Of particular interest was the increased level of adducts seen at week eight which showed an increase above the baseline level. This result may reflect the individual nature of

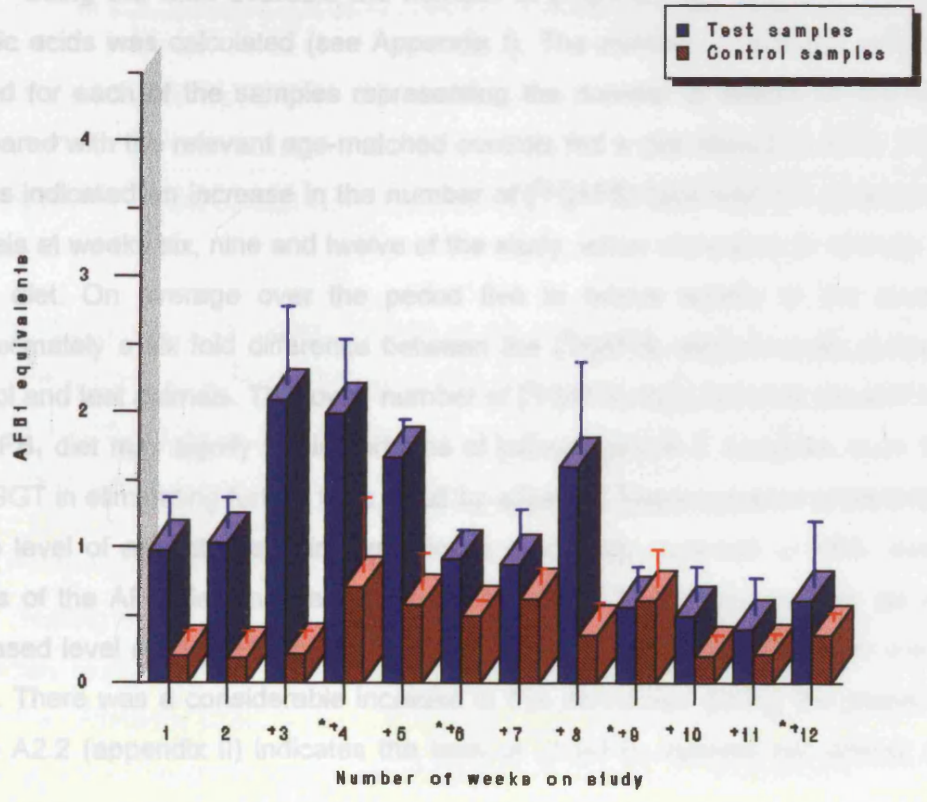


Figure 4.6 AFB_1 adduct levels present in control and test animals (*- control and $^+$ - test animals injected with [^{3}H]AFB_1 - the level of [^{3}H]AFB_1 injected into these animals was 17.4 μg/kg and accounts for not more than 100 pg/mg [^{3}H]AFB_1 adducts per animal)

that particular animal and also reflects a concurrent decrease in the numbers of FAH displaying the resistance phenotype present in the animals at this time-point of the study (Fig 5.10). This result supports inversely the presence of a mechanism of resistance by resulting in the increased formation of AFB_1 adducts in the presence of lower levels of phase II enzymes.

4.2.3 HPLC separation and Radioactivity measurements

A. Number of AFB_1-fapy adducts present in samples containing [^{3}H]-AFB_1

NOTE: Due to the difference between the methods used in the preparation and hydrolysis of the DNA samples assayed in the ELISA and scintillation counting experiments these cannot be compared quantitatively. However, the results obtained from liquid scintillation counting clearly indicate the relative changes in levels of [^{3}H]AFB_1 adduct formation, following i.p. injection of [^{3}H]AFB_1 , throughout this period of the study.

Using the data available the number of [^{3}H]AFB_1-fapy adducts /mmole of DNA or nucleic acids was calculated (see Appendix I). The number of adducts obtained was then plotted for each of the samples representing the number of weeks on the AFB_1 diet and compared with the relevant age-matched controls fed a diet absent in AFB_1 (Fig 4.7). These results indicated an increase in the number of [^{3}H]AFB_1-fapy adducts present in the control animals at weeks six, nine and twelve of the study, when compared to animals receiving the AFB_1 diet. On average over the period five to twelve weeks of the study there was approximately a six fold difference between the [^{3}H]AFB_1 adduct levels present in DNA of control and test animals. The lower number of [^{3}H]AFB_1-fapy adducts present in animals fed the AFB_1 diet may signify the importance of induced phase II enzymes such as GST's, AR and GGT in eliminating further toxic insult by aflatoxin. There appears to be a slight increase in the level of adducts seen in the animals chronically exposed to AFB_1 during the latter weeks of the AFB_1 feeding period. These increased levels can possibly be related to the increased level of [^{3}H]AFB_1 injected as the animals were dosed on a per unit body weight basis. There was a considerable increase in this parameter during the period of the study. Table A2.2 (appendix II) indicates the level of [^{3}H]AFB_1 injected per animal at each time-point.

Week four indicated that a similar level of [^{3}H]AFB_1 adducts were formed in the control and test animals. This result was presumably due to the high level of retention of

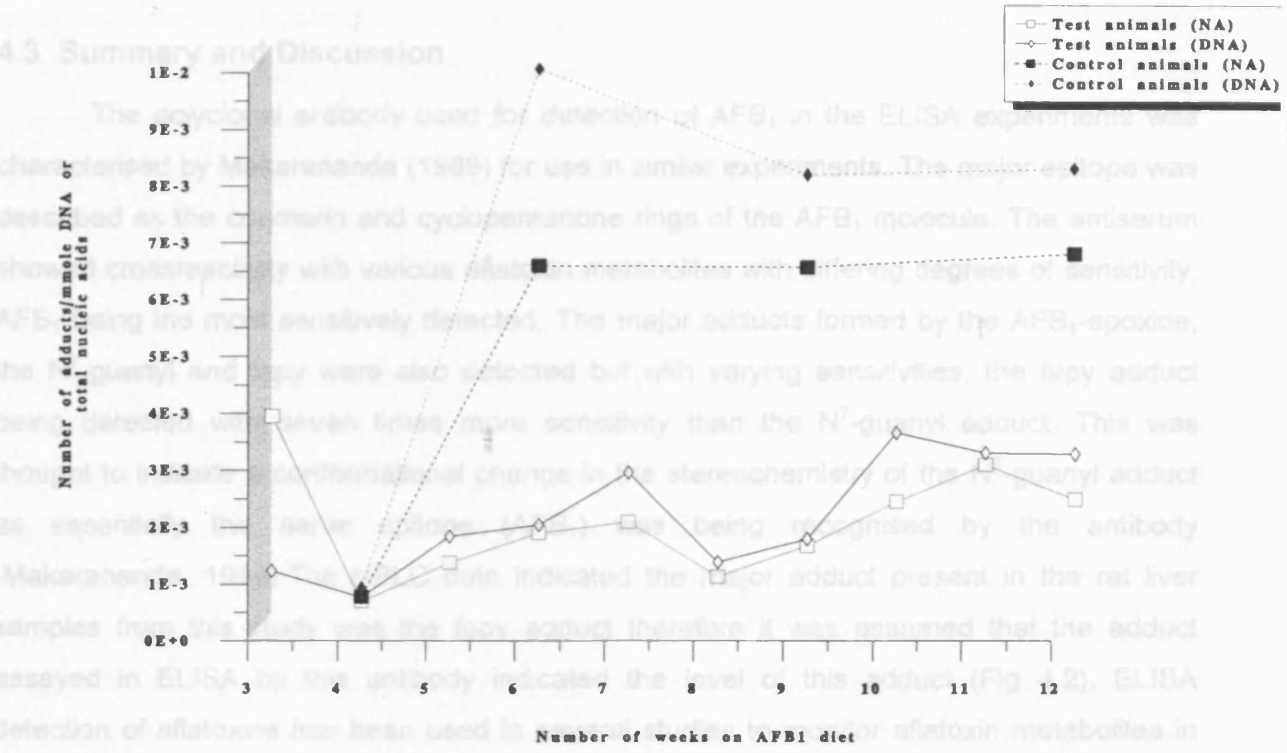


Figure 4.7 [^{3}H]AFB_1 -fapy adducts/mmole DNA or total nucleic acids (NA) in test and control animals

neonatal detoxifying metabolism evident at this stage in both control and AFB_1-fed animals. When the number of adducts/mmole of total nucleic acid were compared to that of the DNA samples a higher level of adducts per unit nucleic acid was noted in the samples containing DNA only (Fig 4.7), showing the higher specificity of AFB_1 toward DNA than RNA.

4.2.4 Autoradiography of fixed tissue

Having established that a lower number of [^{3}H]AFB_1 adducts were present in animals chronically exposed to AFB_1 after increasing time it was hypothesised that this effect was due to the resistance phenotype present in the animals after four weeks on the AFB_1 diet. An experiment was therefore carried out to determine if the reduction in adduct formation seen was located in the focal areas expressing the resistant phenotype. It was anticipated that these areas of altered enzyme expression would also be revealed as areas lacking in or having lower radioactive incorporation. Unfortunately it appeared that the method was not sensitive enough to detect accurately such small levels of radioactivity, and only a general background level of 'grains' was observed with no difference between control and test liver sections from week three to week twelve on the AFB_1 study. Further studies would therefore

be necessary to demonstrate this relationship. Limited amounts of [^{3}H]AFB_1 available and time precluded this from the present work.

4.3 Summary and Discussion

The polyclonal antibody used for detection of AFB_1 in the ELISA experiments was characterised by Makarananda (1989) for use in similar experiments. The major epitope was described as the coumarin and cyclopentanone rings of the AFB_1 molecule. The antiserum showed crossreactivity with various aflatoxin metabolites with differing degrees of sensitivity, AFB_1 being the most sensitively detected. The major adducts formed by the AFB_1-epoxide, the N^7-guanyl and fapy were also detected but with varying sensitivities, the fapy adduct being detected with seven times more sensitivity than the N^7-guanyl adduct. This was thought to indicate a conformational change in the stereochemistry of the N^7-guanyl adduct as essentially the same epitope (AFB_1) was being recognised by the antibody (Makarananda, 1989).The HPLC data indicated the major adduct present in the rat liver samples from this study was the fapy adduct therefore it was assumed that the adduct assayed in ELISA by this antibody indicated the level of this adduct (Fig 4.2). ELISA detection of aflatoxins has been used in several studies to monitor aflatoxin metabolites in urine, serum and milk in an attempt to provide direct evidence for exposure to aflatoxins (Ross *et al.,* 1992; Autrup *et al.,* 1990; Sheaber *et al.,* 1993). ELISA was used in this study to monitor the total level of aflatoxin nucleic acid adducts present in rat liver samples chronically exposed to AFB_1 (4 ppm) for 12 weeks. An animal fed a control diet but which received an i.p. injection of 1mg/kg AFB_1 two hours before culling was used as a positive control sample for this technique. This was considered a dose which would result in a large number of adducts and produced approximately twelve fold more adducts compared to the baseline levels observed in the negative controls. This sample was also used to eliminate variation between each experiment by normalising each curve to its known concentration. Comparison of the baseline level, that is the apparent level of adducts present in control fed animals which did not receive [^{3}H]AFB_1 with those in animals which were fed AFB_1, revealed the level of adducts resulting from [^{3}H]AFB_1 injection present to be less than 100 adducts pg/ mg nucleic acids. The liquid scintillation counts indicated the maximum level of adducts resulting from the injection of [^{3}H]AFB_1, occurred in the control animals from weeks 6,9 and 12 (Fig 4.5).

In the AFB_1 fed animals the number of adducts rose steeply in the first three weeks on the diet from approximately 485 to 1043 AFB_1 adducts per mg nucleic acid, this level then fell progressively from 1043 to 455 AFB_1 adducts per mg nucleic acid after six weeks on the diet (Fig 4.6). This pattern would appear to indicate a positive dose response of AFB_1 feeding to adduct formation early in the study (weeks 1-3) followed by slow repair of the existing

adducts (weeks 3-5) where the level of adducts remains roughly at a steady state level, repair being balanced by the formation of new adducts. The decrease in adduct level seen at week six continuing throughout the following weeks (except for the anomalous result at week eight) however cannot be explained by repair alone, especially as the repair of fapy adducts is reported to be slower than the formation of new adducts (see review Bailey, 1994). The level of adducts present in the animal four weeks after chronic exposure to AFB_1 showed a level 40% higher than that in the baseline level recorded for the negative controls. Only after eight weeks following removal of the AFB_1 diet did the level drop to the baseline observed for the non-exposed control animals. It is therefore likely that the adduct levels seen from weeks 6-12 signifies the development of a resistance mechanism resulting in lower adduct formation during this period of the study in combination with a slow repair of the existing adducts. The histochemical profile supports this hypothesis with induction of glutathione conjugating enzymes GST's and GGT and also AR enzyme noted in the liver from this time-point. This is the pattern reported in Fischer rats after chronic exposure to AFB_1.

Of interest is the delay in accumulation of adducts during the early weeks of the study, with a steep increase in the level of adducts only being noted after two weeks on the AFB_1 diet (Fig 4.6). The maximum number of N^7-guanyl adducts are reported to be present in the liver two hours after exposure of rats to AFB_1 with twenty percent of these converting to the fapy adduct within twenty-four hours. Therefore it would have been anticipated that the level of adducts would have been at the highest level in the control fed animals at the early stages of the study (Croy and Wogan, 1981). The histochemical profile of the control animals during the first two weeks of the study (six and seven weeks old, respectively) however, showed generally high levels of GST's including 7.7 and Yc_2 and AR in the liver parenchyma (Figs 5.2, 3 and 4). Similarly the levels of GGT were seen to be higher in these animals when compared to those present subsequently in control animals eight to twelve weeks old (Fig 5.11). This demonstrated a longer retention time of the neonatal expression of phase II detoxifying enzymes in the animals in this study compared to that previously observed in this laboratory. These results therefore reflect a mechanism of resistance present in the animals during the first two weeks on the AFB_1 diet which resulted in a high conjugating ability of the liver towards AFB_1 and low adduct formation with nucleic acids. Only after three weeks on the control diet when the levels of AFB_1 conjugating enzymes in the liver had dropped to the levels normal in mature rats was there a significant number of adducts formed. Figure 4.8 compares the level of adducts with the resistance profile at each time-point during the feeding of AFB_1. A close correlation is seen between the increase in resistance and the decrease in adduct levels and vice versa. Note in particular week eight, which showed both a decrease in the level of enzymes conferring resistance and an increase in the level of AFB_1 adducts.

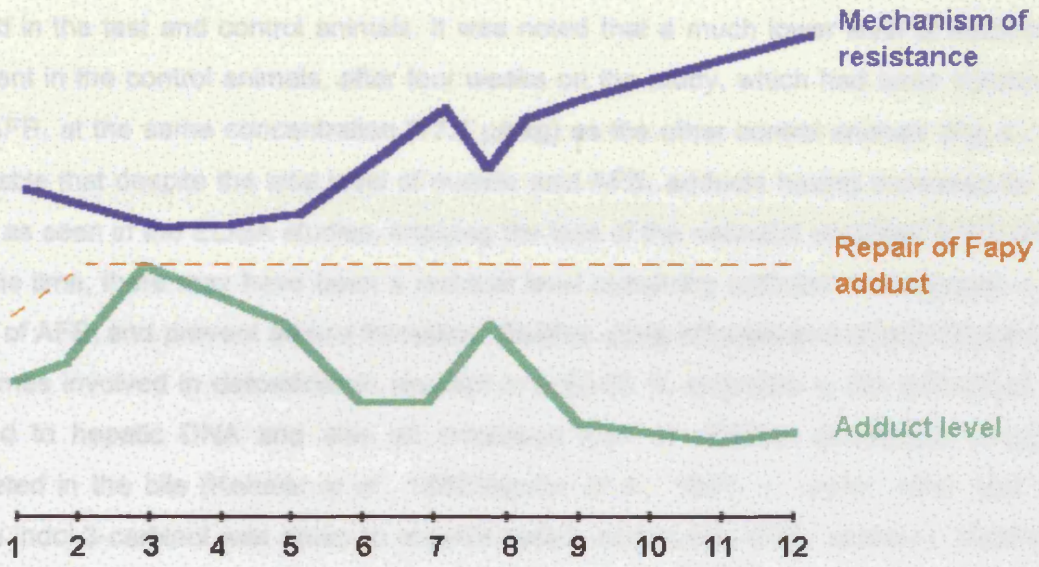


Figure 4.8 Diagram incorporating possible mechanisms interfering with AFB_1 adduct formation/removal. Adduct level reflects the pattern of AFB_1 adducts seen when measured using ELISA. Mechanism of resistance was plotted according to the number of foci of altered hepatocytes displaying the resistance biochemical phenotype or levels of γ-glutamyltransferase activity. Repair of fapy adducts was not examined in this study and therefore only represents the possible level of repair which may be occurring throughout the period of the study. The initial low level of repair occurring shortly after exposure to AFB_1 before the significant conversion of the N^7-guanyl adduct to the fapy adduct.

HPLC fractionation and radioactivity measurements of the hydrolysed rat liver DNA samples were carried out to determine the levels of AFB_1 adducts formed in the liver after an injection of 100μCi [^{3}H]AFB_1 two hours before cull at various time-points of the study. The level of adducts measured therefore represented only the additional adducts formed from the [^{3}H]AFB_1 injected. HPLC analyses showed that the major adduct present in these samples was that of the fapy (Fig 4.7). The level of adducts in the animals fed the AFB_1 diet from 3-12 weeks varied from 0.5-3.5x 10^{-3} adducts per mmole DNA, whereas in contrast, the level of adducts present in the control fed animals was at a much higher level varying from 0.5-8.5 x 10^{-3} adducts per mmole DNA (Fig 4.7). This difference in level of adducts can be accounted for by the resistance mechanism discussed previously as determined by the high levels of phase II enzymes in the animals chronically fed AFB_1 (Neal *et al.*, 1981;Tsuda *et al.*, 1988).

The control animals, after loss of the neonatal enzyme profile, are lacking this mechanism whereby the AFB_1 is efficiently eliminated by detoxification pathways and therefore a higher level of adducts occur. At only one time-point that is, week four were similar adduct levels found in the test and control animals. It was noted that a much lower level of adducts was present in the control animals, after four weeks on the study, which had been injected with [^{3}H]AFB_1 at the same concentration (17.4 μg/kg) as the other control animals (Fig 4.7). It is probable that despite the total level of nucleic acid AFB_1 adducts having increased by week four, as seen in the ELISA studies, implying the loss of the neonatal enzymes in the animals by this time, there may have been a residual level remaining sufficient to conjugate a small level of AFB_1 and prevent adduct formation. Studies using ethoxyquin a potent inducer of the enzymes involved in detoxification resulted in a 60-90 % reduction in the amount of AFB_1 bound to hepatic DNA and also an increased level of aflatoxin glutathione conjugates excreted in the bile (Kensler *et al.,* 1986;Mandel *et al.,* 1987). A similar effect was noted when indol-3-carbinol was given to rodents before and during AFB_1 exposure (Dashwood, 1989).

If it was not a mechanism of resistance which accounted for the higher levels of adducts present in control fed compared to AFB_1 fed animals, an alternative explanation of these results could be that the level of [^{3}H]AFB_1 adduct formation was decreased because there was already a level of adducts present which did not permit the addition of further adducts, that is, a 'saturating level'. Studies examining the total level of adducts present in these animals carried out using ELISA, however, showed that this was not the case. The number of adducts detected by ELISA in the liver DNA of a positive control animal injected with [^{3}H]AFB_1 1 mg/kg was approximately 4 fold greater than the average number of adducts present in the AFB_1 fed animals and 7.5 fold greater than those present in the animals fed the control diet which had been injected with [^{3}H]AFB_1 two hours before culling (Fig 4.5 and Fig 4.6). This demonstrated the relatively low level of adducts present following the dietary exposure to AFB_1 compared to the levels which could be achieved.

A comparison of the level of adducts present in DNA and total nucleic acids shows a higher specificity of the AFB_1-epoxide for DNA than RNA, approximately 20% more adducts were present per unit DNA from the control fed animals following injection of [^{3}H]AFB_1 than on the total nucleic acids when similar quantities of each were used (Fig 4.7). Swenson *et al.,* (1974) showed a 1.5 fold higher specificity of AFB_1 for DNA compared with RNA. Formation of DNA adducts with the AFB_1-epoxide is well characterised and results in a decrease in the synthesis of DNA, nuclear RNA and protein (see review Cullen and Newberne, 1994; Neal, 1973). Formation of RNA adducts is less well characterised although it is known that the AFB_1-epoxide can also form adducts with guanine nucleotides of RNA. These adducts are

thought to interfere with cellular protein synthesis and inhibit protein translation (see review Cullen and Newberne, 1994; Eaton and Gallagher, 1994).

Chapter Five

Protein Expression and Foci of Altered Hepatocytes

5.1 Introduction

Biotransformation of aflatoxin plays a central role in the toxic and carcinogenic effects of this mycotoxin in many species. Metabolic activation is necessary for mutagenic activity and the toxic effects of AFB_1 are due to the ability of its metabolites to react with cellular macromolecules. Activation of AFB_1 to AFB_1-8,9-epoxide is mediated by several cytochrome P450's in humans. Two P450 isozymes have been indicated as being important in this role. CYP1A2 is more active at low substrate concentration, whereas CYP3A4 is more active at higher substrate concentrations (Gallagher *et al.,* 1994). In rats, also, several P450's have been shown to have this role, in particular P450 2C11 a male specific cytochrome P450 has been shown to be important in the sensitivity of the male rat compared to the female (Shimada *et al.,* 1987).

In conjunction with bioactivation of AFB_1, metabolic detoxification of AFB_1 and the reactive metabolite occurs. The ability to produce metabolites less toxic than the epoxide plays a significant role in the effects of AFB_1. One of the important detoxification mechanisms involves conjugation of the reactive epoxide with glutathione (GSH). The glutathione *S*-transferases catalyse the conjugation of the electrophilic AFB_1-epoxide to glutathione forming the first step in the mercapturic acid pathway. GST's are frequently used as early markers of carcinogenesis, in particular GST 7.7 has been most useful because of its increased expression in single cells at a very early stage. The appearance of these positive single cells has been noted following treatment with various carcinogens and with time they progress through clonal expansion to form foci and finally lesions which may have neoplastic potential. A significant direct correlation between numbers of GST 7.7 positive cells and large foci in livers of the same rats was noted after treatment with AAF (Sato, 1988). The GST isozyme subunit, Yc_2, induced in rats by AFB_1 has also recently been shown to display high specificity for the AFB_1-epoxide and is expressed at high levels in preneoplastic and neoplastic lesions (Hayes *et al.*, 1992).

GGT has long been used as a phenotypic marker for malignant cells. It has been especially useful because in the liver, hepatocytes show a high GGT activity in the foetus but very low activity in the adult. Therefore, foci of phenotypically altered cells stand out clearly against the negative background of the adult liver parenchyma. GGT activity in the normal male rat liver is only seen in the bile duct epithelial cells. GGT activity in rat livers treated with AFB_1 has been reported to show a biphasic response, bile duct proliferation occurring early as a toxic response followed by the appearance of foci positive for GGT (Manson, 1983). The elevation in GGT levels in the foci increases the availability of intracellular glutathione through the γ-glutamyl cycle (Fig 1.3) which may serve to supply the increased requirements for glutathione for conjugation with reactive metabolites in the detoxification pathways, in

addition GGT is involved in breakdown of AFB_1-GSH conjugates for excretion via the mercapturic acid pathway (Hanigan and Pitot, 1985; Moss *et al.,* 1984).

While much work has been done examining the detoxification pathway of AFB_1 epoxide mediated by GST's resulting in lowered DNA and RNA adduct formation, little has been done to look at the pathways by which products of the AFB_1-epoxide which bind to protein are detoxified. These account for 50% of the macromolecular binding by AFB_1 (Bailey, 1994). Hayes *et al.* (1993) described a novel aldehyde reductase (AR) which metabolises AFB_1-dihydrodiol. The aldehyde reductase reduces the dialdehydic phenolate form of AFB_1-dihydrodiol, which forms Schiff bases with primary amine groups in proteins, to an AFB_1-dialcohol. Normal levels of expression of this AR as shown by Hayes *et al.* (1993) indicate low levels in foetal liver and in young male rats on control diets, moderately increased AR in rat liver containing preneoplastic nodules and high levels in HCC induced by AFB_1. The potential for AFB_1 dialcohol formation is increased in rat livers showing preneoplastic nodules as well as in HCC, in rats fed a diet of AFB_1.

Of the many cytokeratin polypeptides identified so far various combinations are found to be expressed in almost all epithelial cell types. Cytokeratin 19 (CK-19) in normal rat liver is located in the bile duct epithelium. It's expression has been used to show bile duct proliferation following treatment with carcinogens such as AFB_1. Work carried out by Green *et al.,* (1990) has demonstrated an increase in expression of cytokeratin 19 during the progression of HCC in rats treated with AFB_1. The lower level of cytokeratin 19 positive foci compared with the presence of other markers as seen during this study was reported by Green *et al.,* (1990) to indicate the possibility of CK-19 being a more useful marker of carcinogenesis, since this expression may not occur in those cells which are remodelled following removal of the carcinogen.

Biochemical changes which result in the development of a mechanism of resistance against the toxin, induced by chronic low level exposure, could involve a repression of the cytochrome P450's involved in the metabolism of AFB_1 and/or an induction of the enzymes involved in the detoxification pathways, for example, GST's, aldehyde reductase and γ-glutamyltransferase. Neal *et al.,* (1981) in their study of a mechanism of resistance to cytotoxicity preceding hepatocarcinogenesis concluded that chronic low level feeding of AFB_1 resulted in increased levels of detoxifying enzymes, but the level of activation of AFB_1 remained unchanged. The reduced level of binding of AFB_1 to DNA reported previously, therefore, is most likely due to the increase in detoxification enzymes and not, at least initially, a decrease in the bioactivating enzymes. The large increase in detoxifying enzymes accompanied by increased in vivo production of AFB_1-GSH conjugate is consistent with this scenario. If there were very low levels of the activating P450 enzymes little epoxide and

hence AFB_1-GSH would be formed. This resistance response presumably arises as an antagonistic response to the cytotoxic properties of chemical carcinogens in an attempt to maintain the cell population. It does, however, presumably also allow expression of the carcinogenic potential by permitting initiated cells to replicate.

5.2 Results

5.2.1 Western blotting

Each of the antibodies was used to probe suitable cytosolic or microsomal extracts, which were blotted onto nitro-cellulose membrane following gel electrophoresis, to determine its specificity before use in immunohistochemistry. As these experiments were carried out essentially as a qualitative screening procedure, optimisation of each individual blot was not carried out.

Each blotting assay included four cytosolic extracts prepared from the livers of two control-fed and two AFB_1-fed animals on the AFB_1 study, a protein molecular weight marker to allow approximate molecular weight determination of the proteins detected, BSA as a non-specific protein control, two cytosolic extracts prepared from human foetal livers (kindly donated by Dr Daphne Holt, Queen Charlotte and Chelsea hospital, London) and when available, a pure sample of the protein antigen appropriate to the antibody being examined.

A. Glutathione S-transferase 7-7 polyclonal antibody

This polyclonal antibody detected GST 7-7 protein (M_r 26000) in two of the samples present on the blot (Fig 5.1), that is, in lane 6 containing the pure protein used as a positive reference standard and in lane 3 containing the cytosolic fraction prepared from an AFB_1-fed animal after twenty weeks on the AFB_1 diet. The protein band present in lane 3 was faint and is difficult to visualise following scanning see Fig 5.1. These results indicate the inability of this antibody to detect the very low levels of GST 7-7 present in normal adult rat liver and after one week on the AFB_1 study. No crossreactivity was observed against human GST isoforms.

B. Aldehyde reductase polyclonal antibody

This polyclonal antibody specifically detected the aldehyde reductase protein (M_r 36600) in the rat liver samples which appeared to be the same size as the pure AR protein (Fig 5.1, blot 2). No crossreactivity was observed in the cytosols prepared from human foetal livers or with BSA. Visually it appears there is more AR protein present in the sample prepared from the AFB_1-animal 20 weeks on the AFB_1 study than in the AFB_1-fed animal 1

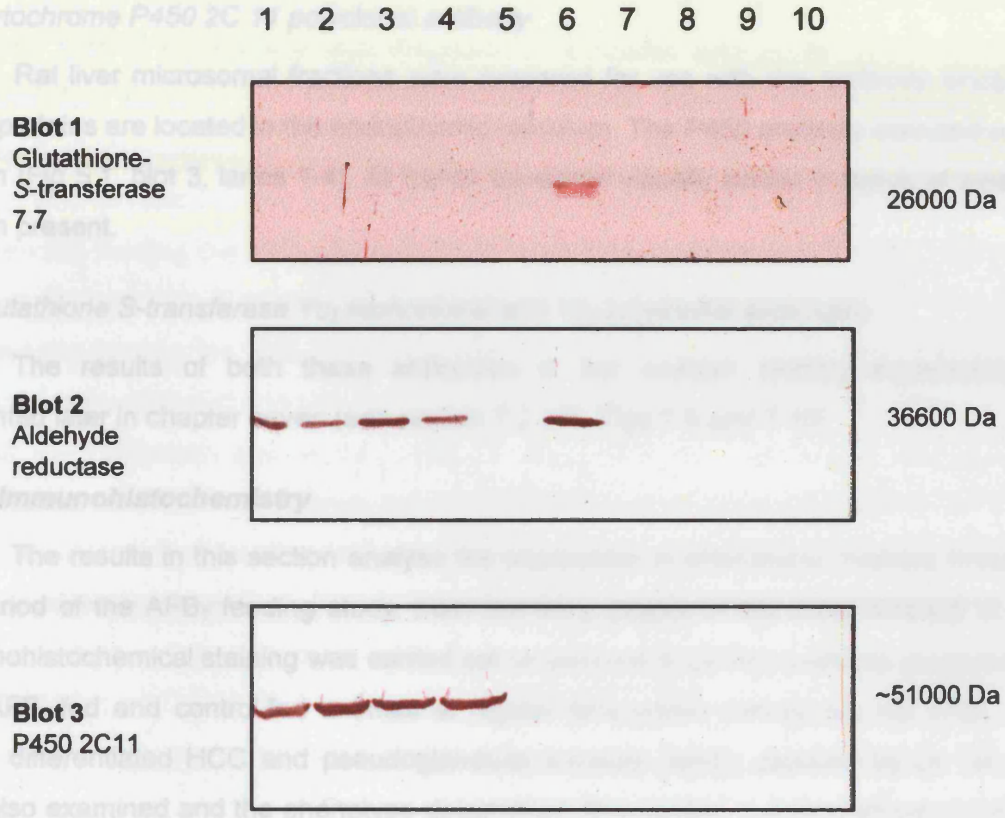


Figure 5.1 Western blotting analysis of antibodies for use in immunohistochemistry. Blot 1: (lanes 1-4 rat liver cytosol, 10μg) lane 1 AFB1-treated animal 1 week on study, lane 2 age-matched control animal, lane 3 AFB1-treated animal 20 weeks study, lane 4 age-matched control animal, lane 5 molecular weight marker, lane 6 glutathione S-transferase 7-7 protein, lane 7 BSA protein, lanes 8 and 9 human foetal cytosols, lane 10 molecular weight marker. Blot 2: (lanes 1-4 rat liver cytosol, 10μg) lanes 1-5 as for blot 1, lane 6 aldehyde reductase protein, lanes 7-10 as for blot 1. Blot 3: (lanes 1-4 rat liver microsomes, 10μg) lanes 1-5 as for blot 1.

week on the AFB_1 study. Both control-fed animals appear to contain less protein than either of the AFB_1-fed animals. Due to the method employed to visualise the protein in each of the samples it was not possible to quantify the exact amount present.

C. Cytochrome P450 2C 11 polyclonal antibody

Rat liver microsomal fractions were prepared for use with this antibody since these P450 proteins are located in the endoplasmic reticulum. The P450 antibody detected a single protein (Fig 5.1, blot 3, lanes 1-4). All bands appeared visually similar in terms of amount of protein present.

D. Glutathione S-transferase Yc_2 monoclonal and Yc_2 polyclonal antibodies

The results of both these antibodies in the western blotting experiments are presented later in chapter seven (see section 7.2.3.B, Figs 7.9 and 7.10)

5.2.2 Immunohistochemistry

The results in this section analyse the expression of phenotypic markers throughout the period of the AFB_1 feeding study, from the early stages of the study through to HCC. Immunohistochemical staining was carried out on acetone fixed liver sections prepared from both AFB_1-fed and control-fed animals at regular time-points throughout the AFB_1 study. Poorly differentiated HCC and pseudoglandular tumours (kindly donated by Dr GE Neal) were also examined and the phenotype determined. See section 2.4 for method of induction of these tumour types.

Difficulties were encountered when sections obtained after nine weeks on the study failed to give reproducible results following staining with the various antibodies. In particular, the P450 and cytokeratin antibodies and the histochemical stain for GGT were involved. The sections failed to show the expected staining patterns. GGT activity was not detected in the bile duct epithelial cells as would be expected and was only faintly visible in advanced preneoplastic lesions. Some of the polyclonal antibodies which had demonstrated very strong expression of antigens previously, maintained some level of activity for example, anti-GST 7-7, AR and GST Yc_2/Yc_1 which allowed analyses of the expression of these antigens in the sections. These results could indicate the loss of the antigenic site due to protein denaturation caused by either heat or chemicals and the problem was eventually traced to the long periods of time the sections were left in IMS before processing. As the processing of these samples was not carried out in-house it took several weeks to locate the reason for the problem. It was solved by leaving the sections in acetone at -20°C until immediately prior to processing.

A. Glutathione S-transferases (7.7, Yc_2)

A1. GST 7-7

Using a polyclonal antibody to detect the presence of GST 7-7 the protein was observed both in the nucleus and cytoplasm of epithelial cells in the bile duct areas of untreated liver sections. In some sections cells located between the hepatocytes that is, sinusoidal macrophages or kupffer cells also stained positively using this antibody. No staining was detected in the normal liver parenchyma (Fig 5.2 a and b). One week after commencing feeding the AFB_1 diet individual hepatocytes showed positive staining with this antibody both in the nucleus and cytoplasm of the cells (Fig 5.2b). Four weeks after commencing feeding the AFB_1 diet strong GST 7-7 staining was found localised in clusters of hepatocytes forming recognisable foci (Fig 5.2d). Of note is the presence of GST 7-7 protein both in the cytoplasm and in the nuclei of single initiated cells and in foci of altered hepatocytes. By seven weeks large areas of the liver were covered with strongly stained foci (Fig 5.2e). By ten weeks on the AFB_1 study it was very difficult to distinguish individual foci.

The antibody against GST 7-7 was also used to analyse the expression of this enzyme in both primary tumours induced in male Fischer rat liver by AFB_1 followed by transplantation into nude mice and in the tumours induced in the present AFB_1 study. All tumours were strongly positive after probing with this antibody (Figs 5.8b and 5.9b).

A2. GST Yc_2

A polyclonal antibody raised in rabbits using GST Yc_2 as the antigen demonstrated positive staining throughout untreated liver during the entire period of the study (Fig 5.3a). Previous western blotting studies by Hayes *et al.*, 1992 indicated the presence of Yc_2 in foetal liver, in liver containing foci and in HCC induced by AFB_1, but not in normal rat hepatic cytosol. However, this antibody showed positive staining as a general background level in the liver indicating crossreactivity (see also chapter seven). This was consistent with the report of Hayes *et al.*, 1992 who demonstrated, using immunoblotting, the antiserum from which this antibody was prepared showed crossreactivity with both GST Yc_1 and Ya subunits. Therefore any results obtained using the Yc_1 /Yc_2 antibody in attempts to examine Yc_2 expression would be compromised by reaction with Ya and Yc_1 expression.

Following the production of the specific monoclonal antibody against GST Yc_2 probing of a selection of sections was repeated. Using the monoclonal antibody against GST Yc_2 the protein was detected strongly in bile duct epithelia and weakly in the parenchyma located mainly in the centrilobular region in untreated liver (Fig 5.3b) from rats six weeks of age. By seven or eight weeks of age the protein was detected solely in the

Figure 5.2 Immunohistochemical staining for Glutathione S-transferase 7-7 in liver section from control and AFB_1-treated rats

a) Background level of GST 7-7 staining on liver section from control animal 6 weeks of age showing staining normally in bile duct epithelium. Magnification x4.

b) Individual hepatocyte showing strong GST 7-7 staining, one week after feeding AFB_1 (4 ppm). Note also staining in sinusoidal macrophages or kupffer cells in the liver of control and AFB_1 fed animals. Magnification x20.

c) Many individual cells showing strong GST 7-7 staining three weeks after feeding AFB_1 (4 ppm). Staining also localised in bile duct epithelium demonstrating bile duct proliferation. Magnification x4.

d) Strong GST 7-7 staining localised in focus of altered hepatocytes, four weeks after feeding AFB_1 (4 ppm). Magnification x40.

e) Many foci of altered hepatocytes staining with GST 7-7 covering large areas of the liver, six weeks after feeding AFB_1 (4 ppm). Magnification x4.

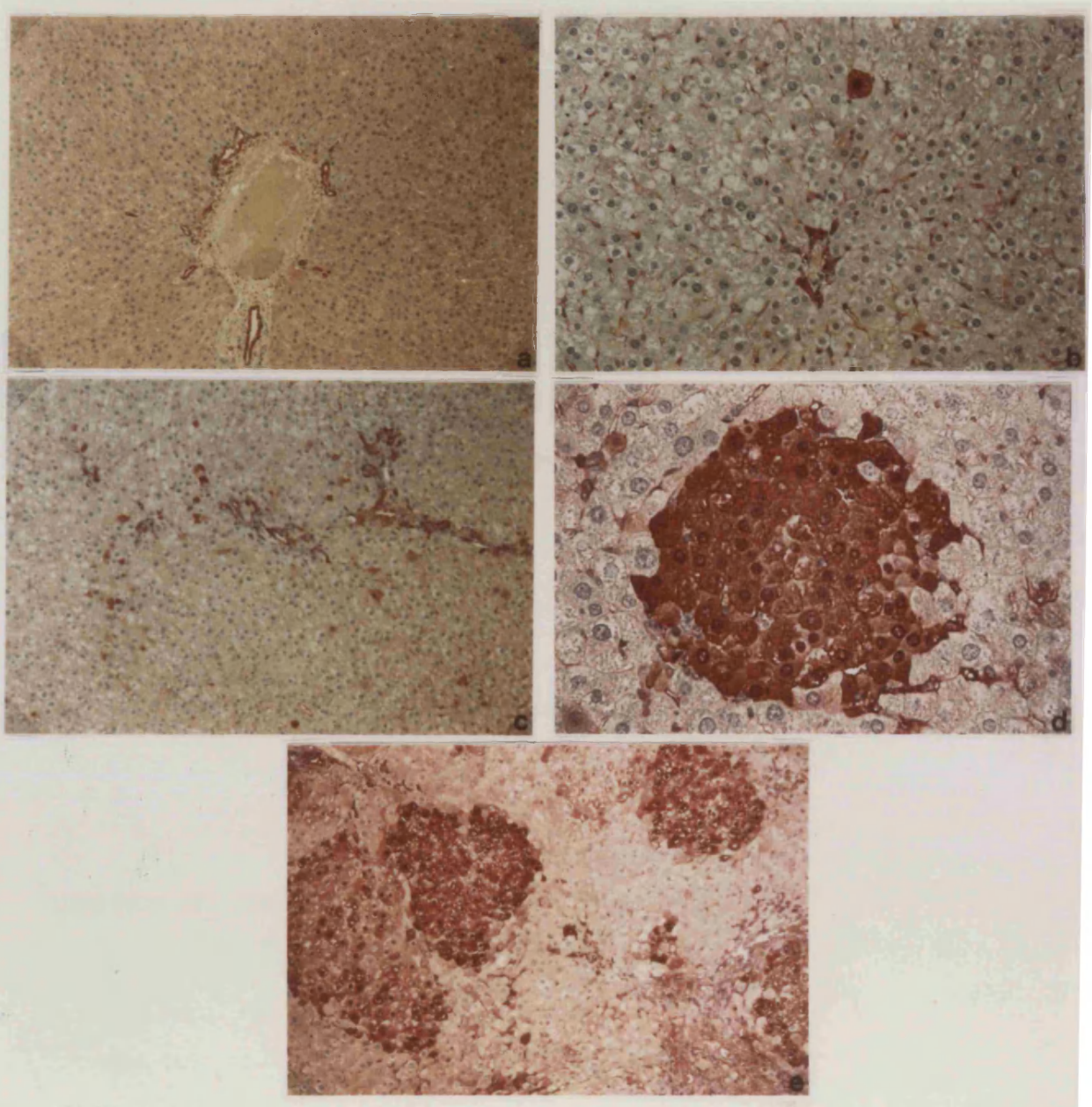

a
b
c
d
e

Figure 5.3 Immunohistochemical staining for Glutathione S-transferase Yc_2 in liver sections from control and AFB_1-treated rats

a) Background level of GST Yc_1/ Yc_2/ Ya[a] staining on liver section from control animal, six weeks of age showing staining in parenchyma. Magnification x4.

b) Background level of GST Yc_2[b] staining on liver section from control animal six weeks of age showing weak staining in parenchyma in a centrilobular pattern. Magnification x4.

c) Background level of GST Yc_2 staining on liver section from control animal eleven weeks of age showing localisation in bile ducts within portal tracts. Magnification x4.

d) Strong GST Yc_2 staining localised in focus of altered hepatocytes, eight weeks after feeding AFB_1 (4 ppm). Magnification x20.

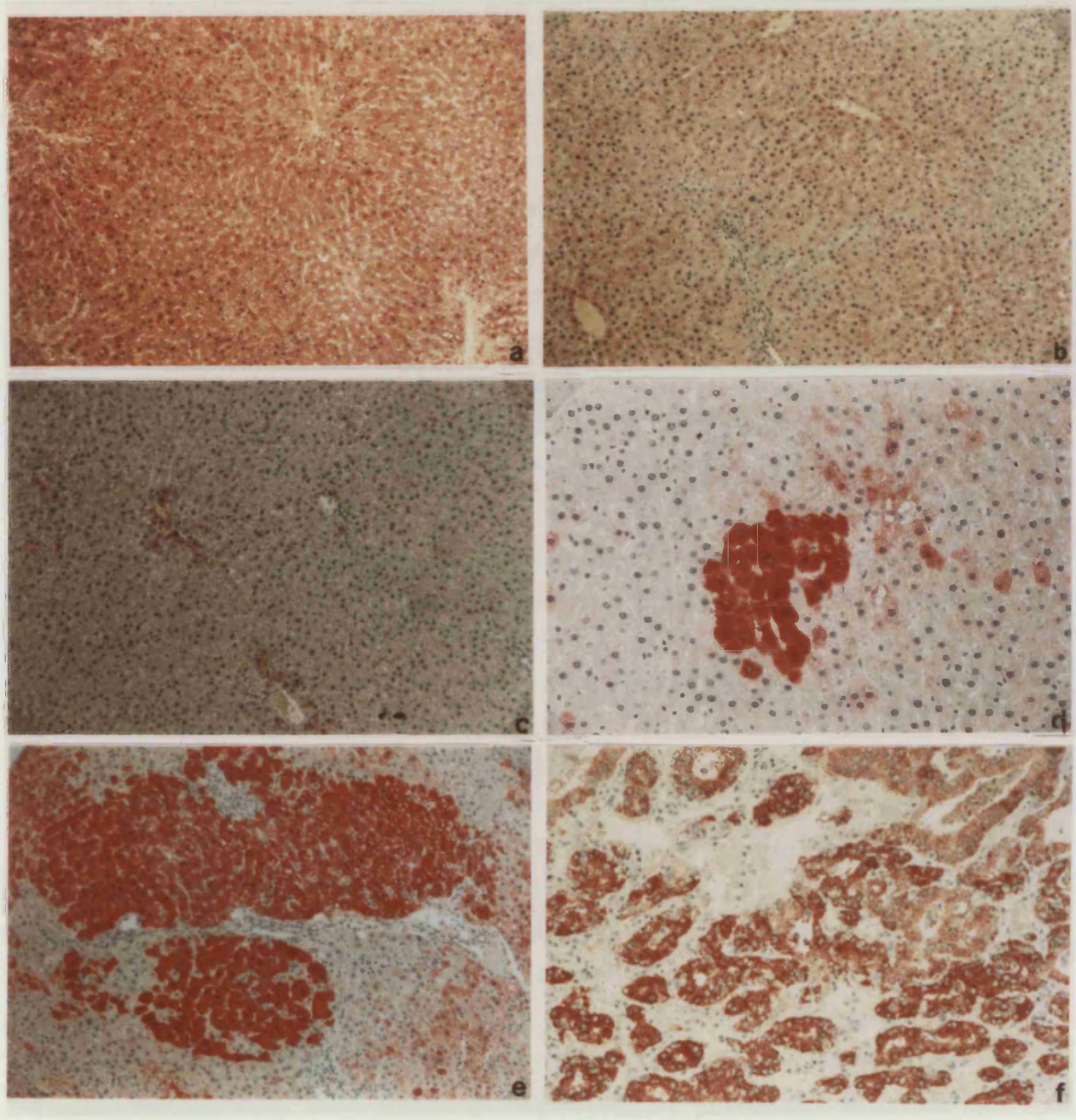
e) Many foci of altered hepatocytes showing strong staining with GST YC_2 covering large areas of the liver, nine weeks after feeding AFB_1 (4 ppm). Magnification x4.

f) Strong GST Yc_2 staining localised in hepatocellular carcinoma, seventy-seven weeks after feeding AFB_1. Magnification x20.

---

[a] Colour plate a) displays the expression of GST Yc_2 following binding with GST Yc_2 polyclonal antibody

[b] Colour plates b) - f) display the expression of GST Yc_2 following binding with GST Yc_2 monoclonal antibody


a
b
c
d
e
f

bile duct epithelium of untreated animals (Fig 5.3c). Areas of hepatocytes, recognisable as foci, in the livers of AFB_1-treated animals stained strongly with this antibody after week eight of the study (Fig 5.3d). By nine weeks on the study large areas of the liver were occupied by foci and by ten weeks it was difficult to distinguish individual foci (Fig 5.3e).

The pseudoglandular and poorly differentiated HCC resulting from the tumour transplantation studies and induced in the AFB_1 study showed very strong expression using both the polyclonal and monoclonal anti-Yc_2 antibodies (Figs 5.8, 5.9 and 5.3f).

B. Aldehyde reductase

Using a polyclonal antibody against aldehyde reductase the protein was detected in liver parenchyma and bile duct epithelium of untreated animals in the early weeks of the AFB_1 study (Fig 5.4a). By the third week of feeding AFB_1, when the animals were eight weeks old, staining indicated a predominantly centrilobular expression of the AR protein (Fig 5.4b). This was only observed in livers from untreated controls. The livers of AFB_1-fed rats continued to demonstrate a general expression throughout the liver. After one week on the AFB_1 study individual hepatocytes were observed as staining more positively than the surrounding hepatocytes (Fig 5.4c). By four weeks strong staining was localised in recognisable foci (Fig 5.4d). After seven weeks on the AFB_1 study large areas of the liver were staining positive for AR (Fig 5.4e) and by ten weeks it was impossible to distinguish individual foci.

Similar to the expression of GST 7-7 all tumours examined using the AR antibody demonstrated very strong expression of AR (Figs 5.8 and 5.9).

C. Cytochrome P450 (2C11)

Liver sections from untreated animals stained with a polyclonal antibody against cytochrome P450 2C11 detected the protein in the parenchymal cells (Fig 5.5a). In the livers of untreated animals eight weeks old, positive staining indicated a predominant periportal location (Fig 5.5b). In treated liver, after six weeks on the AFB_1 study, areas recognisable as foci were observed as negative compared to surrounding liver when stained with this antibody (Fig 5.5c). The expression of cytochrome P450 2C11 remained variable throughout the remainder of the study. Large areas negatively staining with this antibody were observed. However, the nature of the cells, in that they appeared to contain large vacuoles, made absolute identification of negative foci difficult in the later stages of the study (Fig 5.5d). Hence, following the progression of hepatocarcinogenesis in this study after seven weeks on the AFB_1 diet using this antibody was not possible.

Pseudoglandular tumours resulting from the transplantation studies showed no staining with this antibody. Poorly differentiated HCC induced in nude mice and in the AFB_1

Figure 5.4 Immunohistochemical staining for Aldehyde reductase in liver section from control and AFB_1–treated rats

a) Background level of AR staining on liver section from control animal six weeks of age showing staining throughout the parenchyma. Magnification x4.

b) Background level of AR staining on liver section from control animal eleven weeks of age showing centrilobular localisation of AR stain. Magnification x4.

c) Individual hepatocyte showing strong AR staining, one week after feeding AFB_1 (4 ppm). Magnification x20.

d) Strong AR staining localised in focus of altered hepatocytes, four weeks after feeding AFB_1 (4 ppm). Magnification x40.

e) Many foci of altered hepatocytes staining with AR covering large areas of the liver, six weeks after feeding AFB_1 (4 ppm). Magnification x4.

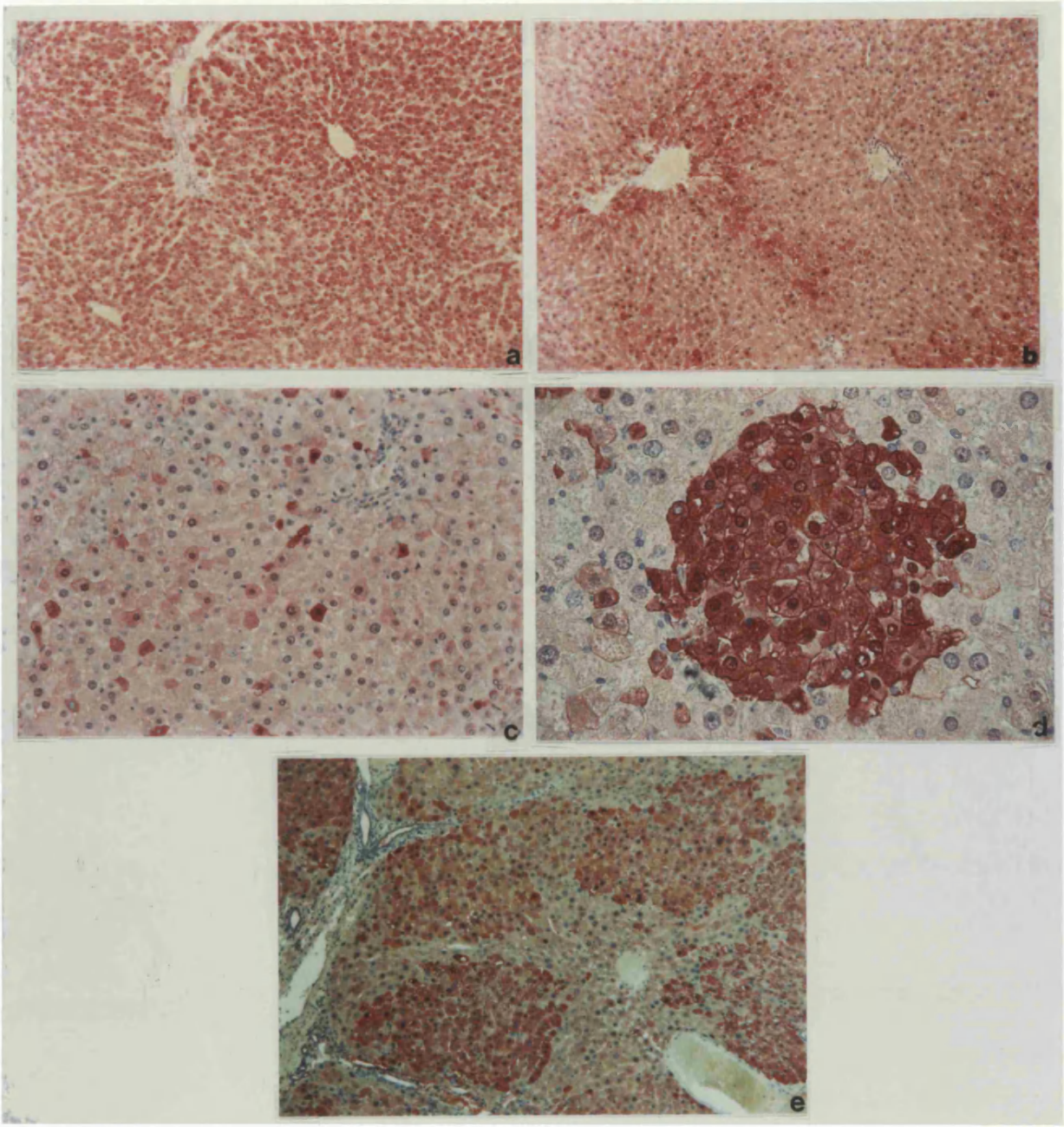

a
b
c
d
e

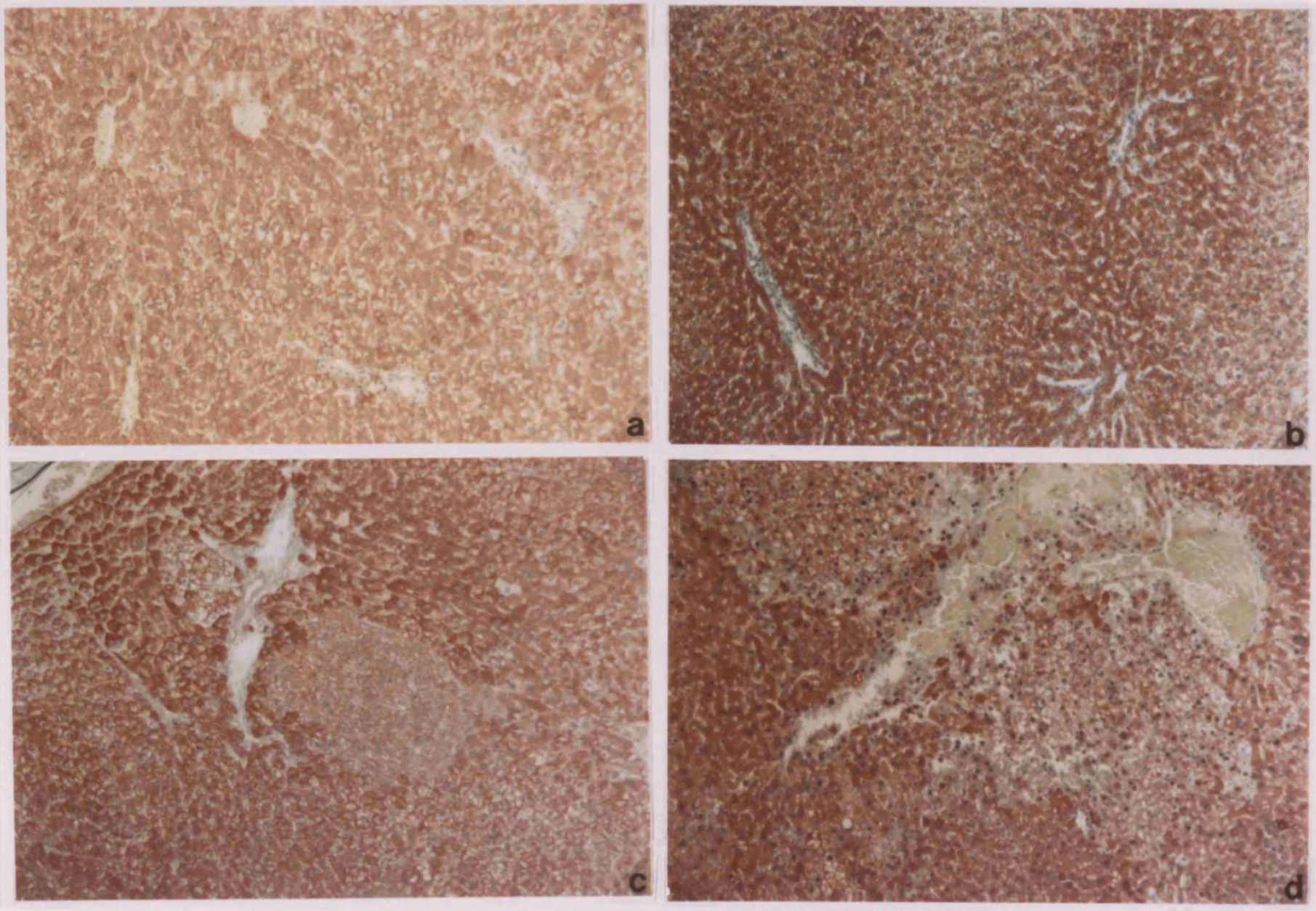
Figure 5.5 Immunohistochemical staining for P450 2C11 in liver section from control and AFB_1–treated rats

a) Background level of P450 2C11 staining on liver section from control animal six weeks of age showing staining in parenchyma. Magnification x4.

b) Background level of staining on liver section from control animal eleven weeks of age showing periportal location of P450 2C11 stain. Magnification x4.

c) Decrease in P450 2C11 staining in focus of altered hepatocytes, six weeks after feeding AFB_1 (4 ppm). Magnification x4.

d) Several foci of altered hepatocytes showing decreased staining with P450 2C11 covering large areas of the liver, seven weeks after feeding AFB_1 (4 ppm). Magnification x4.


a
b
c
d

study showed variable expression of P450 2C11, some areas appeared negatively stained whilst others were positively stained. This variability in pattern of staining reflects the nature of the tumour which appears to have a composition heterogeneous in cell type (Figs 5.8 and 5.9).

D. Cytokeratins (18 and 19)

In normal rat liver CK-19 is only expressed in the bile duct epithelium. Using a commercially prepared monoclonal antibody to CK-19 untreated livers showed only bile duct localisation (Fig 5.6a). In the AFB_1 feeding study bile duct proliferation was pronounced by three weeks on the study indicated migration away from the portal tracts (Fig 5.6b). A few controls also showed very slight bile duct proliferation. By week six on the study the proliferating bile duct areas had extended to fuse together and form a network throughout the section. No foci were observed staining positively with this antibody before sixty-nine weeks on the study. Staining within these foci appeared strongest in the ductular areas of the focus (Fig 5.6c).

The pseudoglandular tumours produced in the transplantation studies appeared strongly positive for CK-19, strongest expression occurring in ducts and glandular parts of the tumour (Fig 5.6d). In the poorly differentiated HCC produced in the transplantation studies and the HCC induced in the present AFB_1 study weakly positive expression of CK-19 was also observed (Figs 5.8 and 5.9). Staining using a monoclonal antibody against CK-18 was also carried out on the tumours induced in the AFB_1 study. The pattern of staining was found to mirror that observed using the CK-19 antibody .

Of the eight human sections examined, only one section contained a bile duct area and this was positive for CK-19 expression. None of the other sections, which displayed HCC, were positive for this expression.

E. Gamma-glutamyltransferase

Histochemical staining for GGT activity using acetone fixed sections from untreated rats showed only bile duct localisation (Fig 5.7b). However, as with CK-19 expression, bile duct proliferation indicated by GGT activity was noted in treated livers after three weeks on the AFB_1 study (Fig 5.7c). Some bile duct proliferation was also noted in some of the untreated animals, but was slight compared to that observed in treated livers. By week four on the study small numbers of foci were seen in the parenchyma which stained positively for GGT (Fig 5.7d).

Pseudoglandular tumours induced using the transplanted primary tumours and HCC induced in the AFB_1 study were positive for GGT, showing strongest activity in ducts and

Figure 5.6 Immunohistochemical staining for Cytokeratin 19 in liver sections from control and AFB_1-treated rats.

a) Normal staining pattern of CK-19 in adult rat liver showing strong CK-19 stain in bile ducts within portal tracts, six weeks of age. Magnification x20.

b) Staining of CK-19 localised in bile ducts of rat liver demonstrating bile duct proliferation, two weeks after feeding AFB_1 (4 ppm). Magnification x20.

c) Strong CK-19 staining localised in focus of altered hepatocytes, sixty-nine weeks after feeding AFB_1 (4 ppm). Magnification x20

d) Tumour showing staining of CK-19, seventy-seven weeks after feeding AFB_1(4 ppm). Magnification x4.

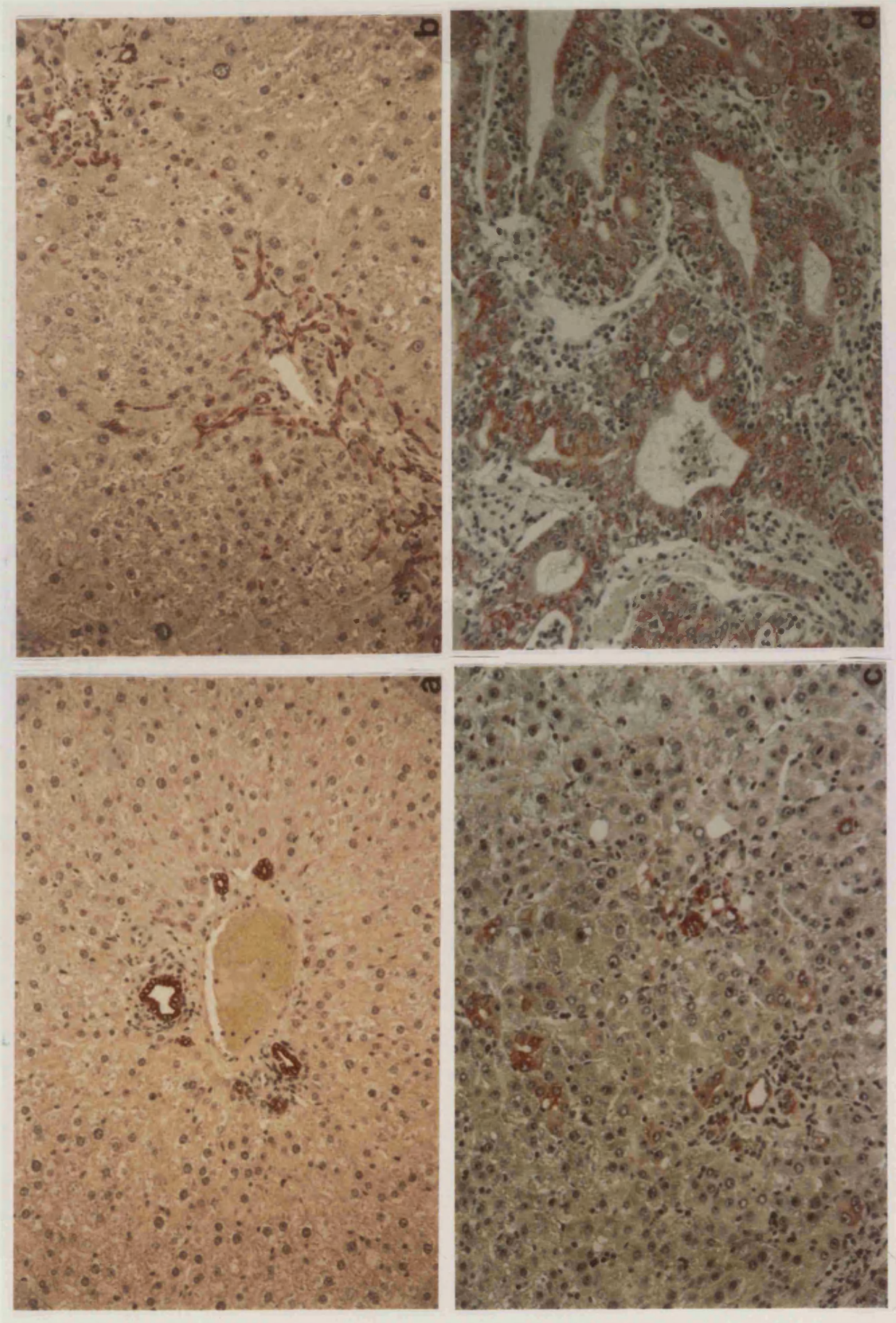

b
d
a
c

Figure 5.7 Immunohistochemical staining for γ-glutamyltransferase in liver and kidney sections from control and AFB_1-treated rats.

a) Staining of GGT located in cortex of rat kidney where it is expressed constitutively. Positive control slide. Magnification x4.

b) Normal staining pattern of GGT in adult rat liver showing strong GGT stain in bile ducts within portal tracts, six weeks of age. Magnification x20.

c) Staining of GGT localised in bile ducts of rat liver demonstrating bile duct proliferation, two weeks after feeding AFB_1 (4 ppm). Magnification x20.

d) GGT staining localised in focus of altered hepatocytes, six weeks after feeding AFB_1 (4 ppm). Magnification x40.

e) Tumour showing staining of GGT, seventy-seven weeks after feeding AFB_1(4 ppm). Magnification x20.

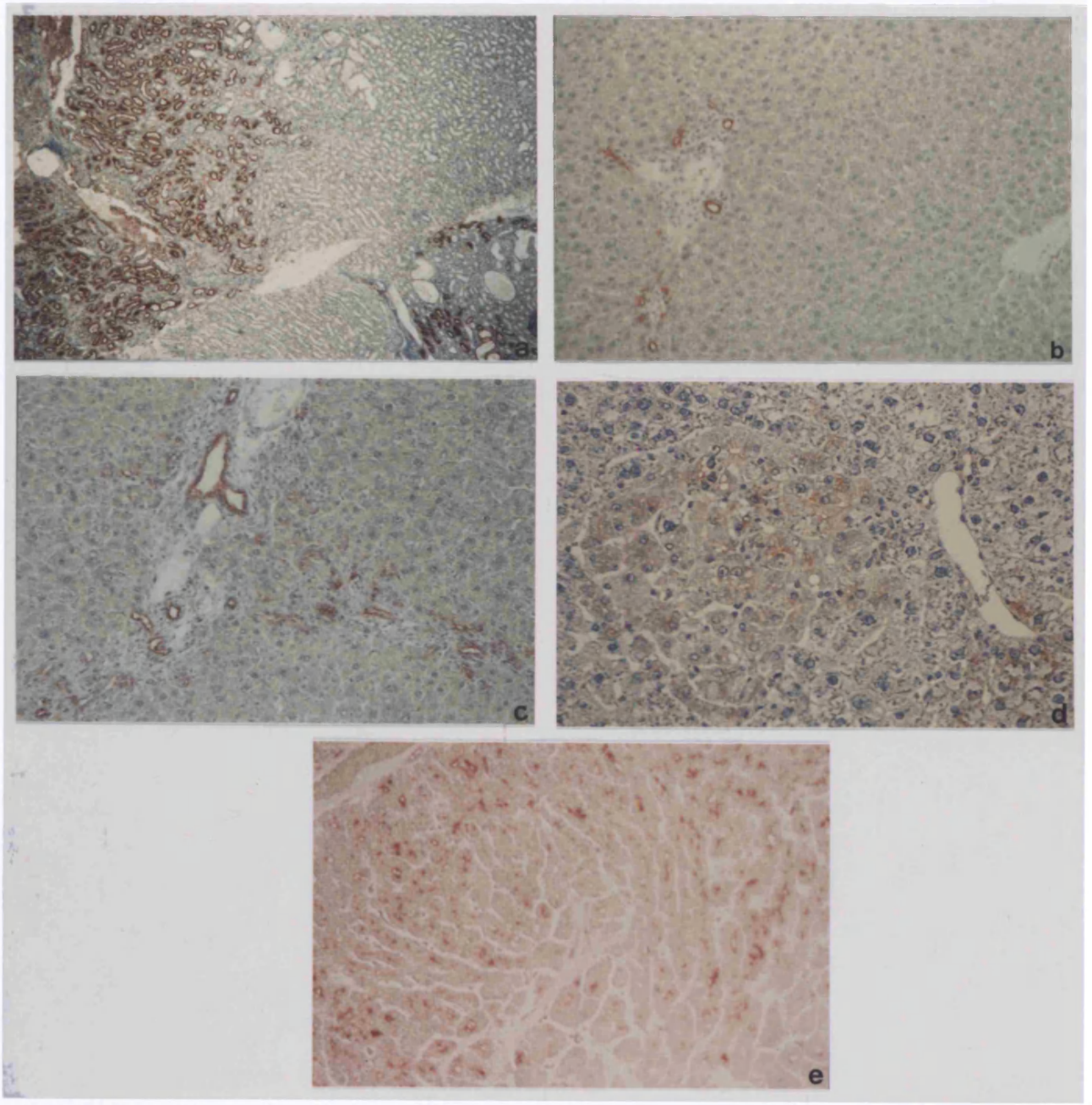

a
b
c
d
e

Figure 5.8 Immunohistochemical staining of serial sections prepared from pseudoglandular tumour induced in nude mouse using JB1 cell-line. The symbol >> indicates the same point on each of the sections. All sections shown at x4 magnification.

a) Haemotoxylin and eosin stain.

b) GST 7-7 stain demonstrating positive staining.

c) AR stain demonstrating positive staining.

d) P450 2C11 stain demonstrating negative staining.

e) GGT stain demonstrating positive staining, located mainly in ductular areas.

f) GST Yc_1/ Yc_2/ Ya stain demonstrating positive staining.

g) CK-19 stain showing positive staining localised in the ductular areas.

Figure 5.9 Immunohistochemical staining of sections prepared from poorly differentiated hepatocellular carcinoma induced in nude mouse using BL10 cell-line. The symbol >> indicates the same point on each of the sections. There is a focus of altered hepatocytes located above and to the right of this point. All sections shown at x4 magnification.

a) Haemotoxylin and eosin stain.

b) GST Yc_1/ Yc_2/ Ya stain demonstrating positive staining.

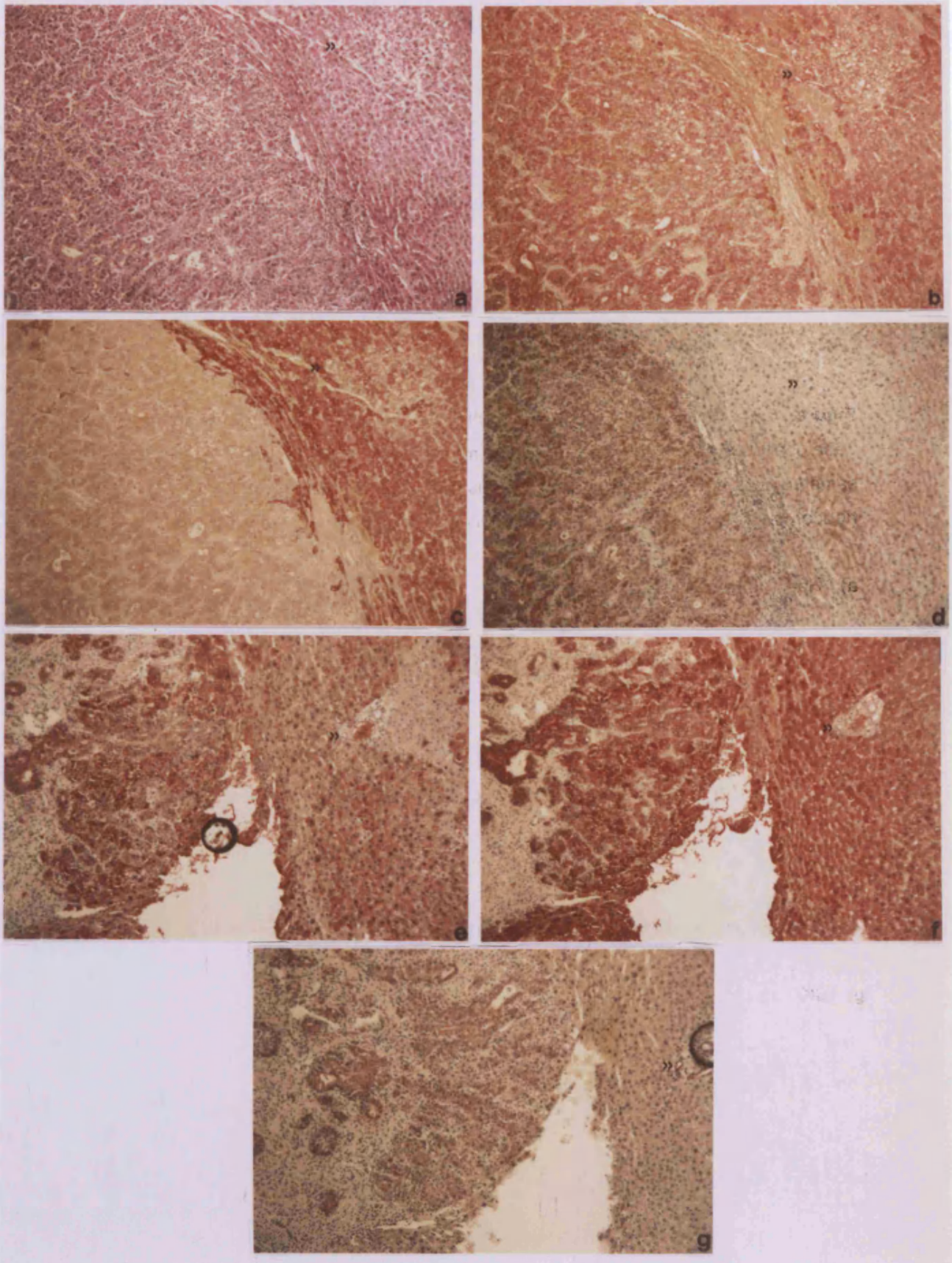
c) P450 2C11 stain demonstrating negative staining.

d) GGT stain demonstrating very weakly positive staining.

e) GST 7-7 stain demonstrating positive staining.

f) AR stain demonstrating positive staining.

g) CK-19 stain showing weakly positive staining.


a
b
c
d
e
f
g

glandular parts of the tumour (Figs 5.8 and 5.7e). Poorly differentiated HCC induced in the same manner displayed only weak expression of GGT (Fig 5.9).

5.2.3 Numbers of foci of altered hepatocytes

Note: The GST Yc_2 antibody referred to in this section is the GST Yc_2 polyclonal antibody.

In order to demonstrate more clearly the relationship between the different markers discussed above, the number of altered foci were counted in serial sections stained with each of the markers. The area of each liver section was calculated and the number of foci greater than ten cells in diameter, positively or negatively stained for each marker, expressed per mm^2 of liver. [c]Disregarding the single focus observed after two weeks on the study the first significant foci were visualised after four weeks. Throughout the study GST 7-7 was expressed by the greatest number of foci and by week ten of the study the foci were so numerous it was impossible to distinguish individual foci to obtain an accurate count. Aldehyde reductase expression followed a very similar pattern to GST 7-7 with only slightly fewer foci showing altered expression (Fig 5.10). GGT activity was present from week four of the study, the numbers increasing dramatically by week six when altered P450 phenotype

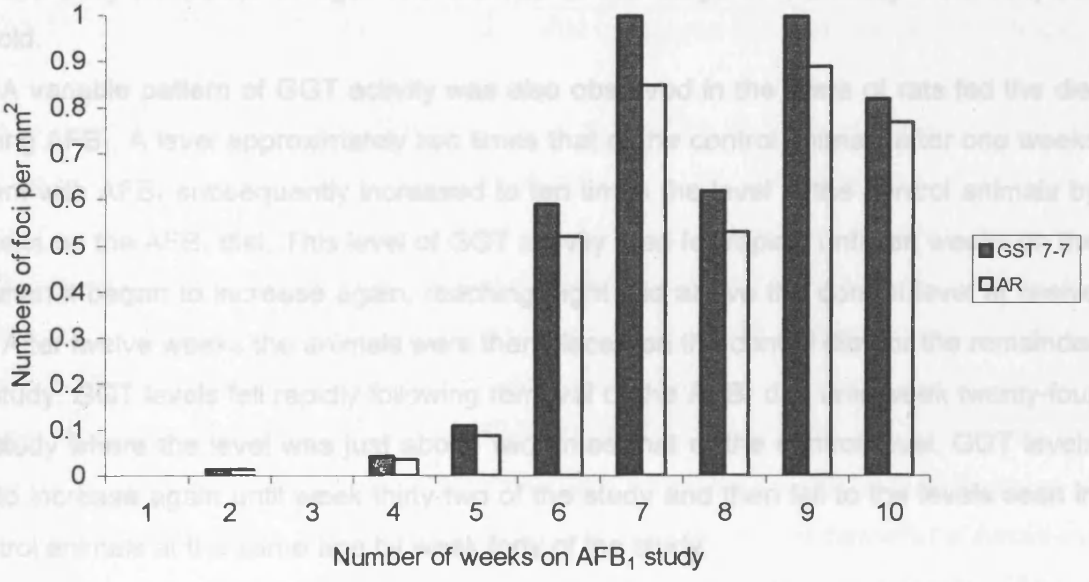


Figure 5.10 Numbers of foci/mm^2 in rat liver sections taken from animals on AFB_1 diet (4 ppm).

[a]One focus of significant size was observed after two weeks on the AFB_1 diet which showed altered phenotype for GST 7-7, AR and P450. However it was disregarded because of its premature appearance. It is likely this focus was already present in the animal before feeding and was rapidly induced by AFB_1.

was also noted. However, the numbers of foci displaying altered GGT phenotype were very low in comparison to GST 7-7 or AR. At week six of the study the highest number of foci displaying altered GST 7-7 phenotype were recorded, only a sixth of these displayed altered GGT expression. GST Yc_2 polyclonal antibody expression did not identify foci of altered hepatocytes in animals fed AFB_1 until week eight on the diet. No cytokeratin 19 positive foci were observed in the sections throughout the period of these analyses.

5.2.4 GGT activity

In an attempt to circumvent the problem of poor histochemical staining for GGT activity in the sections after week nine of the study and to quantify the level of expression seen in the tissue sections GGT activity was measured in homogenised livers (Fig 5.11). These results indicated a level of GGT activity in the untreated rat livers after one week of treatment almost two fold higher than that observed in the treated rat livers at that stage. The level of GGT activity was observed to decrease rapidly in the control animals until a constant level of activity was reached, after seven weeks on the study. This level remained until thirty-six weeks into the study when GGT level in the control animals began to increase. By forty weeks of the study the GGT level was three times the level seen in the control livers between seven and thirty-six weeks of age. The animals at this stage in the study were forty-five weeks old.

A variable pattern of GGT activity was also observed in the livers of rats fed the diet containing AFB_1. A level approximately two times that of the control animals after one weeks treatment with AFB_1 subsequently increased to ten times the level in the control animals by four weeks on the AFB_1 diet. This level of GGT activity then fell rapidly until ten weeks on the study when it began to increase again, reaching eight fold above the control level at twelve weeks. After twelve weeks the animals were then placed on the control diet for the remainder of the study. GGT levels fell rapidly following removal of the AFB_1 diet until week twenty-four of the study where the level was just above two times that of the control level. GGT levels began to increase again until week thirty-two of the study and then fell to the levels seen in the control animals at the same age by week forty of the study.

5.3 Summary and Discussion

Each of the antibodies screened for specificity using the Western blotting and detection assay proved to be specific for the protein which they were used to detect.

Table 5.1 summarises the altered phenotype in foci induced by continuous feeding of AFB_1 (4 ppm) for twelve weeks. Expression of GST 7-7 was noted in single positive cells and small groups of cells one week following exposure to AFB_1. Foci of altered hepatocytes also stained strongly for GST 7-7 although there was heterogeneity within and between lesions.

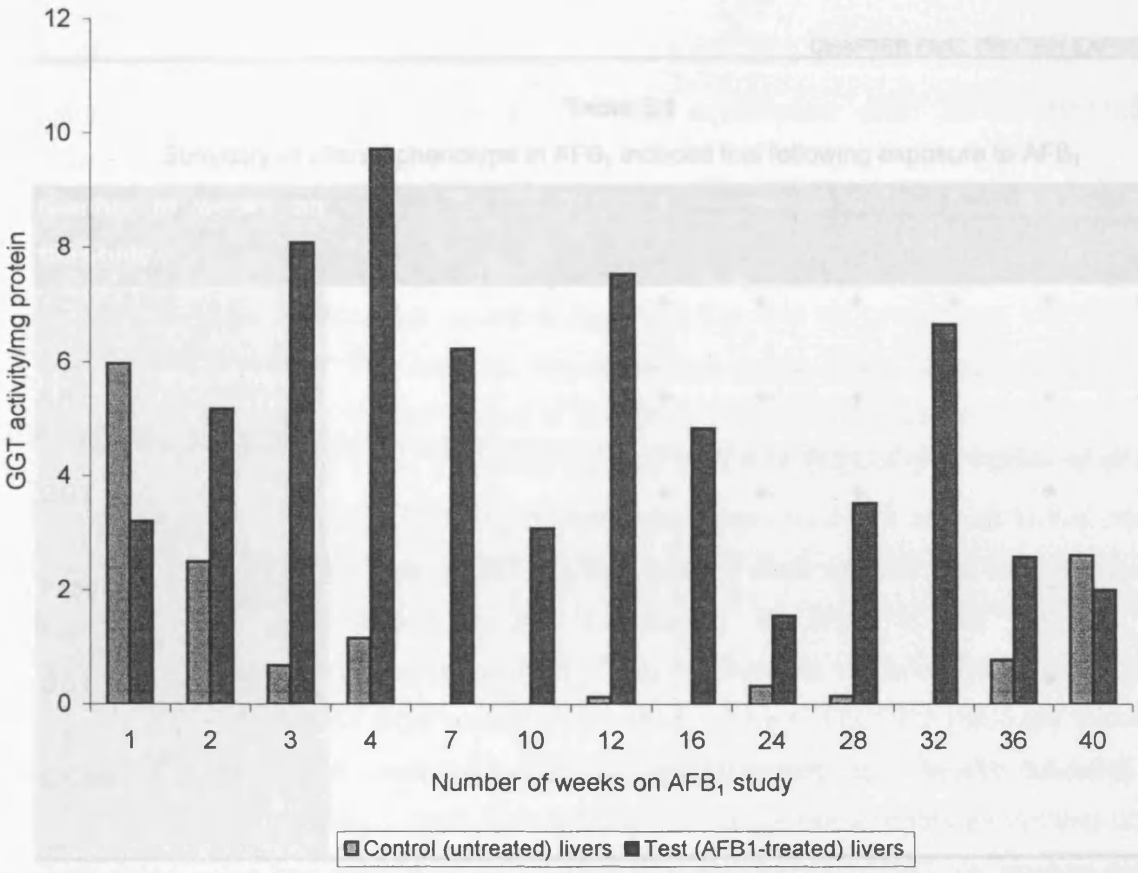


Figure 5.11 GGT activity in F344 rats fed AFB_1 diet compared to F344 rats fed control diet

This is in accordance with several other studies examining the effects of many different carcinogens (Harrison *et al.,* 1990; Sato, 1988; Moore *et al.,* 1987). In view of the lack of conjugation activity of GST 7-7 towards AFB_1 as a substrate and the apparent similarity in response to a variety of carcinogens, it would appear the induced expression of GST 7-7 is a generalised response in the process of hepatocarcinogenesis, largely unrelated to the chemical nature of the inducer. It has been hypothesised that the overexpression of GST 7-7 represents a stress-response phenomenon which also occurs in processes, such as liver disease as a result of alcoholism, not regarded as preneoplastic states (Harrison *et al.,* 1990). The promoter region of the GST 7-7 gene in rat and in man contains a consensus sequence for a *ras*-responsive element, 12-O-tetradecanoyl-phorbal-13-acetate (TPA). In view of the frequent occurrence of mutations in the *ras* genes both in AFB_1 induced rodent carcinogenesis and in several other models of liver carcinogenesis it has been speculated that there is link between the specific expression of GST 7-7 during hepatocarcinogenesis and the activation of the *ras* signal transduction pathway in the early stages of the carcinogenic process (Sato, 1988). Conclusive evidence is not present for either theory. However, it is possible there may be a link between both processes which results in the switching on of the GST 7-7 gene in a general response to toxic insult.

Table 5.1

Summary of altered phenotype in AFB_1 induced foci following exposure to AFB_1

Number of weeks on the study	1	2[a]	3	4	5	6	7	8-12	69
GST 7.7				+	+	+	+	+	+
AR				+	+	+	+	+	+
GGT				+	+	+	+	+	+
P450							-	-	-
GST Yc_2								+	+
CK-19									+

+ -presence of altered phenotype of these antibodies indicating foci of altered hepatocytes greater than 10 cells in diameter.

+ve or -ve indicates whether the protein is increased or decreased or both.

Staining of the sinusoidal macrophages or kupffer cells was noted in some of the sections on which the antibody recognising GST 7-7 was used. These cells are located between the hepatocytes. Expression of GST 7-7 in kupffer has been noted in human liver sections displaying mild alcoholism where the liver appeared quite normal but displayed signs of sublethal injury and fatty change (Harrison *et al.*, 1990). The significance of expression of GST 7-7 in the kupffer cells is unknown but a report by Steinberg *et al.*, (1989) examining the expression of GST's in kupffer cells found differential regulation of GST's in parenchymal and non-parenchymal cells suggesting that this varied expression may be of importance in enabling the liver to deal with a wide variety of hepatoxins. Of note also is the presence of detectable GST 7-7 levels in the nucleus following immunohistochemical staining, especially in the early stages of carcinogenesis in the rat liver following treatment with AFB_1. This phenomenon appears to be associated with several of the GST isoforms and has been reported frequently in studies examining chemical carcinogenesis in rat liver (Harrison *et al.*, 1990; Sato, 1988). It has been suggested that GST in the nuclei either acts to detoxify genotoxic substances translocating from the cytosol or else functions as a carrier

protein of some exogenous and endogenous substances such as carcinogens and hormones (Sato, 1988).

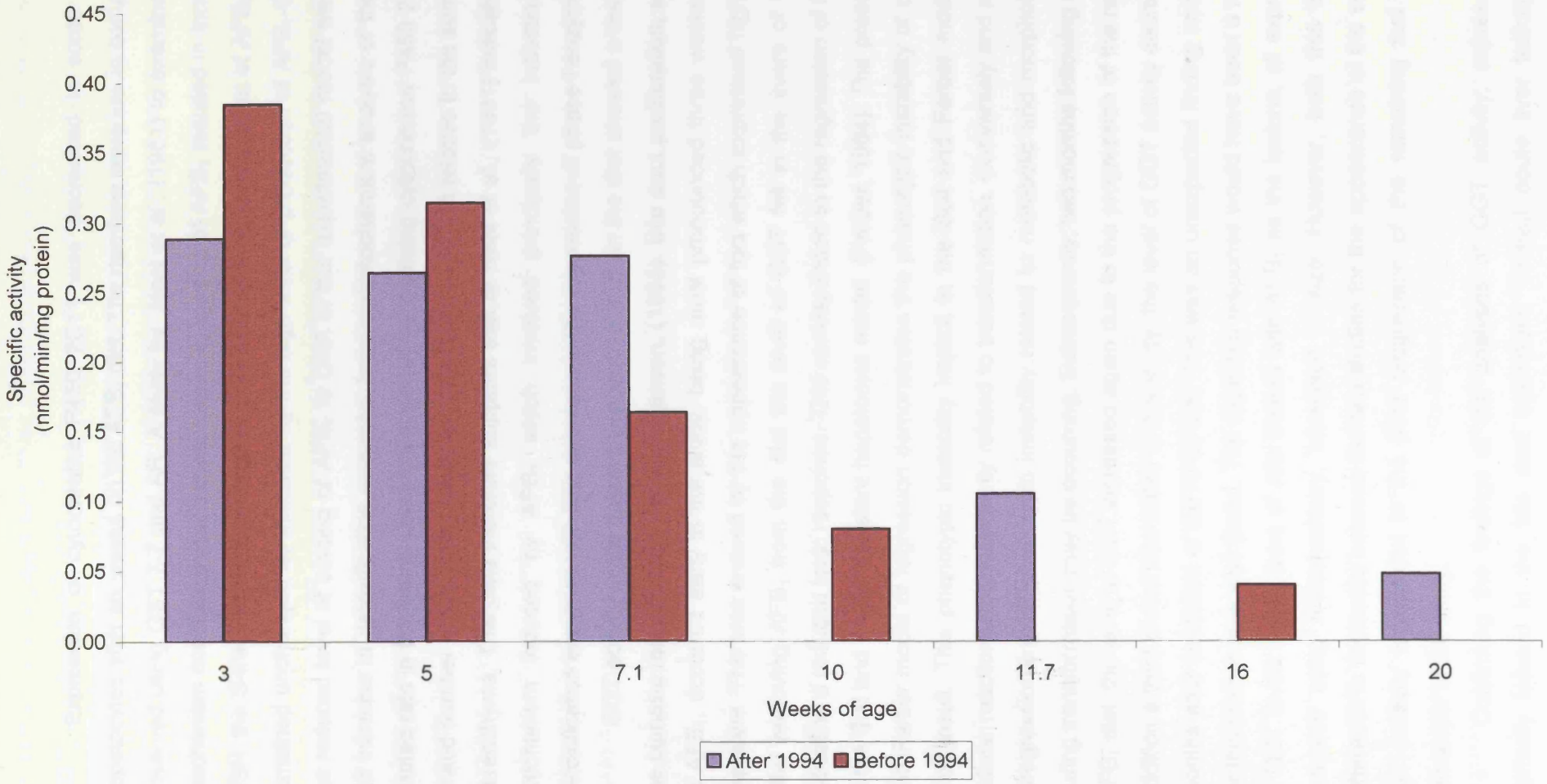
Immunohistochemical staining using the GST Yc_2 monoclonal antibody on untreated liver sections indicated the expression of GST Yc_2 both in the parenchyma and bile duct epithelia of animals up to seven weeks of age. After this time the protein was only detected in the bile duct epithelia. The level of Yc_2 expression found in this study was generally in accordance with the findings of Hayes *et al.* (1994), although the level of GST Yc_2 remained higher for a longer time in the untreated animals than that observed by Hayes *at al.*,(1994) who reported high levels of GST Yc_2 in foetal and young male rats only up to five weeks of age. Beyond this age the level of GST Yc_2 fell rapidly in male animals and was not visualised by their detection method beyond ten weeks of age. In the present study, immunohistochemical staining revealed GST Yc_2 positive foci of altered hepatocytes in AFB_1 treated sections following eight weeks of treatment whereas GST 7-7 positively stained foci of altered hepatocytes became evident at approximately four weeks following AFB_1 treatment. Also in contrast to GST 7-7 expression no single hepatocytes expressing GST Yc_2 were noted in the four week period preceding the appearance of GST Yc_2 positive foci. This would imply an early response resulting from exposure to a chemical carcinogen switching on genes such as GST 7-7 perhaps involved in a general protection mechanism against the carcinogen. This initial stage would then appear to be followed by the induction of GST Yc_2 expression which displays high specific activity for the AFB_1-epoxide. Hence, there may be several stages involved in the induction of detoxification mechanisms resulting from exposure to AFB_1. This is in contrast to a co-ordinate increase in expression of phase II drug metabolising enzymes following exposure to certain chemoprotectors with a concurrent decrease following removal of the inducing agent (Manson *et al.,* In Press). It appears there are different regulatory mechanisms controlling the expression of these genes depending on the inducing agent. In the case of exposure to chemoprotectors regulatory control may be through the antioxidant response element resulting in an concomitant increase in expression of a broad range of phase II drug metabolising enzymes. In the case of a chemical carcinogen such as AFB_1 different control mechanisms are activated which are not switched off following removal of the chemical.

Another enzyme which would appear to be induced as an early response following exposure to a chemical carcinogen is aldehyde reductase. Normal levels of expression of aldehyde reductase as shown by Hayes *et al.* (1993) indicate low levels in foetal liver and in young male rats on control diets, moderately increased aldehyde reductase in rat liver containing preneoplastic lesions and high levels in HCC induced by AFB_1. In contrast, antibody staining of the liver sections prepared from the untreated animals in the AFB_1 feeding study showed uniform moderate expression of the aldehyde reductase protein

throughout the various cell types constituting the liver until eight weeks of age, centrilobular localisation was then noted. There are two possible explanations for this difference. Firstly, it could be due to the difference in methods used to detect the expression of protein in the samples. Hayes *et al.,* (1993) used western blotting analysis compared to immunohistochemistry used in the AFB_1 feeding study. The immunohistochemical analysis is more sensitive in detecting low levels of expression of protein because the low level is concentrated in small areas (cells) whereas western blot analysis uses total extracts of liver protein so each protein is much more diluted. Alternatively there could be a more fundamental difference between the two studies. As in the case of the expression of GST Yc_2 it is probable that the level of aldehyde reductase remained at a higher level for a longer time in the untreated animals in the present study than in that reported by Hayes *et al.* (1994). Although the strain and sex of animals, male Fischer 344 rats, were similar in each study; the source of the animals, diet and conditions of husbandry of the animals were different. Some in-house studies were carried out to compare the levels of various enzymes over time in the animals used for this study and in those used for previous studies including those carried out by Hayes *et al.,* (1993). The results indicated a slower reduction in the level of both Yc_2 and AR during and after the weaning period in the animals used in the AFB_1 feeding study presented here compared to the animals used in the previous studies (unpublished data kindly provided by Mr D Judah, MRC Toxicology Unit, Leics) (Fig 5.12).

Similar to the expression of GST 7-7, AR was expressed in single positive cells which progressed to minifoci and eventually foci of altered hepatocytes. The time-scale for each of these changes were similar for both enzymes. The numbers of foci/mm_2 induced in AFB_1 fed animals showed very close correlation when examined using the antibodies recognising GST 7-7 and AR, both in the number of foci formed and in their chronological order of their appearance (Fig 5.10).. This similarity between GST 7-7 and AR is also reflected in studies done by Hayes *et al.,* (1993) in ethoxyquin treated rats. Fischer 344 rats are highly sensitive to AFB_1, but can resist its carcinogenic effects when fed a diet containing ethoxyquin or other antioxidants, mainly due to their ability to increase the efficiency of the various detoxification pathways through induction of the enzymes involved. Treatment of Fischer rats with ethoxyquin resulted in overexpression in both liver and kidney of AR and GST 7-7 but only in liver of GST Yc_2. This may indicate a similar mechanism of control for both of these proteins, for example deregulation of the *ras* tyrosine kinase pathway. Further studies identifying the structure of the AR gene and examining the regulation of expression of AR in alternative models of hepatocarcinogenesis will further reveal whether transcriptional control similarities exist between GST 7-7 and AR.

Figure 5.12 Comparison of AFB_1-GSH conjugate present in male F344 rats used in the study carried out by Hayes *et al.*, (1994) and in the AFB_1 study presented in this thesis



Expression of cytochrome P450 2C11 was decreased in some foci of altered hepatocytes after six weeks on the AFB_1 diet. The numbers were low in comparison to those observed using GST 7-7 and AR. A study by Neal *et al.,* (1981) to examine the resistance mechanism concluded that chronic low level feeding of AFB_1 resulted in increased levels of GSH, the *S*-transferase enzyme and GGT but the level of activation of AFB_1 to the epoxide remained unchanged as indicated by the high level of production of AFB_1-GSH. Therefore, the reduced level of binding of AFB_1 to DNA in this experimental model was due mainly to the increase in detoxification enzymes. Immunohistochemical analysis of the livers of AFB_1 treated rats in this study using the antibody recognising cytochrome P450 2C11 detected a limited number of foci which displayed reduced expression relative to the surrounding normal parenchyma. This data therefore supports that of Neal *et al.,* (1981) indicating a resistance mechanism induced by AFB_1 which involves principally the induction of phase II detoxification enzymes with little decrease in the expression of phase I enzymes.

GGT activity in the animals exposed to AFB_1 in the diet showed a response similar to the biphasic response reported by Manson, (1983). Bile duct proliferation, a toxic response to AFB_1, occurred early in the study, being most pronounced three weeks following initial exposure. This was ensued by the appearance of foci which expressed high levels of GGT. After removing AFB_1 from the diet the level of GGT fell in the livers of treated animals indicating a reduced toxic response. This corresponded to the regression of foci as indicated in the Solt and Farber resistant hepatocyte model (Farber, 1984). The presence of the Solt and Farber model of regression demonstrates the phenotypic instability of cells undergoing toxic insult. This phenotypic instability implicit in the Solt and Farber model may "mimic" cellular reactions not necessarily related to transformation. Conversely and more probably a "progression-linked" phenotypic instability related to metabolic and morphological changes during transformation may be occurring. Subsequently, two months following cessation of the AFB_1 diet, the level of GGT increased again due to the progression of the remaining foci. In addition a third phase was noted in this study, the level of GGT activity decreased again five months after cessation of the AFB_1 diet. This was an unexpected finding and no explanation for this phenomena was found. The expected response would have been a relative increase in GGT levels in the livers of rats treated with AFB_1 as the lesions, all expressing GGT as indicated using histochemistry, increased in size. However, both this finding and the difference in timescale between the two studies for the appearance of the second phase of GGT activity is reflected in the poor progression of the remaining foci throughout the remainder of the study.

Confirming the findings of the analyses of GGT activity, expression of CK-19, normally located in the bile duct epithelium, verified acute liver toxicity, indicated by

prominent bile duct proliferation three weeks following treatment with AFB_1. In contrast to the pattern observed by GGT expression however, no foci of altered hepatocytes displaying positive cytokeratin expression were noted until week sixty-nine of the study. A previous study carried out by Green *et al.,* (1990) reported foci of altered hepatocytes expressing CK-19 after fourteen weeks of treatment with AFB_1 (2 ppm) in the diet. In view of the absence of CK-19 positive foci in this study it is possible the treatment was not continued for a long enough period of time for this phenotypic alteration to be manifested. Conversely, the concentration of AFB_1 used in the feeding study presented in this thesis was double that used in the study by Green *et al.,* (1990). It is more likely another factor was responsible for the lack of expression of CK-19 earlier in the study. The retention of high levels of phase II enzymes AR, GGT and especially GST Yc_2 until at least eight weeks of age, most probably conferred a resistance to the carcinogenic effects of AFB_1. The acutely toxic effects of AFB_1 appear to have proceeded unhindered as demonstrated by both GGT and CK-19 expression which indicated extensive bile duct proliferation, although this was only a qualitative assessment and therefore could not be accurately compared to previous studies.

The presence of CK-19 in foci of altered hepatocytes poses various questions as to the origin of cells undergoing transformation. Two schools of thought exist at present as to the cells from which HCC arises. One concept states that HCC arises by dedifferentiation of mature hepatocytes. This concept is supported by the formation of foci and nodules from altered hepatocytes. The second concept states that HCC arise from arrested maturation of stem cells as in the case of proliferating oval cells with the potential to differentiate into either biliary ducts or hepatocytes (Sell, 1993). The second concept is generally supported by the expression of CK-19 in foci of altered hepatocytes which may indicate an origin of these cells from oval cells, especially as the strongest expression of CK-19 is noted in the lumen-like areas of the tumours. However, the low numbers of foci expressing CK-19 and the late appearance of CK-19 expression suggests that not all foci have been derived from differentiation of oval cells. It is possible transformation can occur by both routes, that is, differentiation of oval cells and dedifferentiation of hepatocytes which acquire the expression of CK-19 as a feature of phenotypic instability (Green *et al.,* 1990). Either way CK-19 expression is a good indicator of transformation as late and low level of expression of CK-19 in the AFB_1 feeding study was indicative of low numbers of HCC finally occurring.

Immunohistochemistry of the tumour samples provided by Dr GE Neal revealed two distinct phenotypic patterns. The pseudoglandular tumour type was well structured with differentiated tumour cells placed about lumina in an acinar or tubular pattern. The other, poorly differentiated form was characterised by its anarchic appearance with areas of necrosis separating the parenchymal cells. The immunohistochemical profile summarised in Table 5.2 indicates the differing immunohistochemical profile. Of interest are the proteins

displaying different expression patterns in each of the tumours, that is, GGT, CK-19 and cytochrome P450 2C11. The pseudoglandular tumour, so called because of its architecture of glandular-like structures, displayed strong expression of CK-19 and GGT proteins but lacked expression of P450 2C11. The expression of the latter two enzymes are indicative of an hepatocyte-associated resistance phenotype, whereas the expression of CK-19 in conjunction with the architecture of the tumour suggest differentiation of oval cells as the cell of origin of this tumour type. In contrast the poorly differentiated HCC displayed positive expression of P450 2C11 and very weak if any expression of GGT and CK-19. Two possibilities exist for these differences. Either the cell of origin of this type of tumour is different to the pseudoglandular type and therefore expresses CK-19 only as a result of phenotypic instability of the cell, not related to metabolic or morphological changes. Or the tumour is of an advanced type characterised by extensive necrosis which in its progression has dedifferentiated to the extent that it has lost its ability to continue expressing normally expressed proteins.

Table 5.2

Immunohistochemical pattern of markers in various liver cell types in rat

	GST 7.7	AR	GST Yc_2/Yc_1	P450	CK19	GGT
Normal cell type						
hepatocyte	-	-	-	+	-	-
bile duct	+	+	+	+	+	+
AFB_1 induced cell type						
hepatocyte	+	+	+	+	-	-
bile duct	+	+	+	+	+	+
oval cell	+	+	nd	+	+	-
altered foci	+	+/-	+/-	-	+/-	+/-
HCC						
pseudoglandular	+	+	+	-	+	+
poorly differentiated	+	+	+	+	-/+	-/+
cholangiocarcinoma	+	nd	nd	nd	+	+

nd - no data could be found using these markers

+/- indicates heterozygous population

Other data obtained from personal observation, JA Green, (1991) and Harrison, (1990).

Chapter Six

Ras and *p53* mutation analysis

6.1 Introduction

6.1.1 Ras genes and AFB_1

Several studies have indicated the role of *c-ras* gene mutations in the multistep progression of AFB_1 induced hepatocarcinogenesis in rats (McMahon *et al.*,1986; Soman and Wogan, 1993; McMahon *et al.*, 1990; Sinha *et al.*, 1988). There appears to be a predominance of a transition mutation, in particular G:C → A:T, in the second base of codon 12 in the *c-Ki-ras* gene. This type of mutation is thought to be indicative of depurination at adducted guanine residues. The resulting apurinic sites may encourage the insertion of an adenine into the site which becomes fixed during replication. There is also another hypothesis that purines are more readily inserted opposite apurinic sites in particular dATP which is more efficient than dGTP in promoting elongation during DNA synthesis. This hypothesis is more suited to the mutation type noted in the *p53* gene following exposure to AFB_1 (Sagher and Strauss, 1983), see section 6.1.2. However the mutation occurs the resultant mutant P21 protein formed cannot function correctly in signal transduction pathways, remaining switched on constantly and continually releasing growth stimulating signals into the cell (Satoh *et al.,* 1992). As the N^7-guanyl adduct occurs shortly after exposure to aflatoxin and has a relatively short half-life one would expect this type of mutation, therefore, to occur early in the process of hepatocarcinogenesis. All studies to date have concentrated on examination of the more advanced lesions or tumours for induction of mutations in *c-ras* following exposure to AFB_1. For further details on *ras* genes and their activation by AFB_1 see chapter one, section 1.3.1.

The study presented in this thesis examines lesions, induced by AFB_1, at a number of different stages of progression towards hepatocarcinogenesis, as defined by their biochemical and histopathological profile, for mutations in codons 12 and 61 in both c-Ki-*ras* and N-*ras* genes.

In addition, slides of tumour sections obtained from nude mice, which had been injected subcutaneously with cells from lines derived from liver tumours induced in male Fischer rats treated with AFB_1, were also analysed. Two cell lines were used in these studies which represented two different tumour types that is, pseudoglandular and poorly differentiated hepatocellular carcinoma. These sections acted as positive control samples. Several sections from untreated tissue or apparently normal tissue adjacent to abnormal lesions were analysed and used as negative control samples. The level of RNA expression of both N-*ras* and c-Ki-*ras* genes in the more advanced lesions was also examined.

6.1.2 P53 genes and AFB_1

In contrast to the high incidence of mutational effects of AFB_1 on *c-ras* genes in rodents, very few p53 mutations have been found in nonhuman primates or rats exposed to

aflatoxin. This is despite a frequency of *p53* mutation in humans, which is highly correlated with the exposure to AFB_1. This *p53* mutation appears to target the third base of codon 249 resulting in, most commonly, a G → T transversion (Unsal *et al.,* 1994). Sagher and Strauss, (1983) in an *in vitro* system describes an inherent specificity for the incorporation of adenine nucleotides opposite apurinic sites. This hypothesis fits well with the predominance of G → T transversions noted in the *p53* gene in livers of humans with high risk of exposure to Aflatoxins. No mutations of this type in the *p53* gene have been reported in rodents exposed to AFB_1.

The presence of mutated P53 protein appears to play an important role in the progression of human hepatocarcinogenesis by allowing progression of the cell cycle in the face of DNA damage. The normal role of P53 depends on the ability to bind specifically to target genes and transactivate them. When mutated, the DNA binding ability of P53 is reduced preventing this function. This role and that in the processes of apoptosis and transcriptional regulation are further discussed in chapter one in section 1.3.2.

To determine whether *p53* mutations occurred in the rat hepatocarcinogenic model used in this study, examination of mutations, in the region corresponding to codon 249 of the *p53* gene in humans, codon 243, was carried out in the liver tumours induced by exposure to AFB_1. PCR was performed using small fragments of acetone fixed liver tumour tissue to produce a 247 bp fragment in exon 6 of the rat *p53* gene which was subsequently sequenced. Human liver biopsy samples from the Gambia, an area reported to have high exposure to aflatoxins, were also examined for the presence of abnormalities in the P53 protein. Immunohistochemistry was performed using a suitable antibody recognising both wildtype and mutant forms of the P53 protein.

6.2 Results

6.2.1 Ras gene mutations

A. Ras gene mutations in archival tumour and control tissue

Six tumours induced by subcutaneous injection in nude mice of a cell line derived from tumours induced in male F344 rats by AFB_1 were examined for *c-ras* gene mutations. Each tumour was analysed for mutations in c-Ki-*ras* and N-*ras* in both exons 1 and 2. When reading the sequences particular attention was given to codons 12/13 and codon 61 in both *c-ras* genes. Four of the six tumours were classified as pseudoglandular type tumours and were induced using a cell line known as JB1 (JB11-4). Two of the six tumours were classified as poorly differentiated HCC and were induced using a cell line known as BL10 (BL10 1-2). Of the four pseudoglandular tumours all showed a mutation in the second base of codon 12 of the c-Ki-*ras* gene in exon 1. All of these mutations were a G → A transition changing the sequencing code for c-Ki-*ras* codon 12 from GGT to GAT and which changes the amino acid

Table 6.1

Presence or absence of mutations in c-Ki-*ras* and N-*ras* oncogenes in pseudoglandular and poorly differentiated HCC induced in nude mice by cell lines, JB1 and BL10, respectively (derived from AFB_1 induced liver tumours in F344 rats), in AFB_1 induced liver or lung tumours and control liver tissue from F344 rats.

Tissue	c-Ki-*ras* codon 12 (ggt)	c-Ki-*ras* codon 61(caa)	N-*ras* codon 12/13 (ggt/ggc)	N-*ras* codon 61 (caa)
Cell line derived AFB_1 induced tumour tissue				
JB1 1	g(g/a)t	caa	ggt/ggc	caa
JB1 2	g(g/a)t	caa	ggt/ggc	caa
JB1 3	g(g/a)t	caa	ggt/ggc	caa
JB1 4	g(g/a)t	caa	ggt/ggc	caa
BL10 1	ggt	caa	ggt/ggc	caa
BL10 2	g(g/a)t	caa	ggt/ggc	caa
AFB_1 induced liver tumours in F344 rats				
HTT1	ggt	caa	ggt/ggc	caa
HTT2	ggt	caa	ggt/ggc	caa
[1]HTT3	ggt	caa	ggt/ggc	caa
AFB_1 induced secondary lung tumour in F344 rat				
[1]HTT4	ggt	caa	ggt/ggc	caa
Control liver tissue				
HCT1	ggt	caa	ggt/ggc	caa
[1]HCT2	ggt	caa	ggt/ggc	caa

[1] These tissues were taken from the same animal fed AFB_1 (4 ppm) in the diet for 90 days.

in the P21 protein formed from glycine to aspartic acid. Of the two poorly differentiated HCC one also showed a mutation in codon 12 of the c-Ki-*ras* gene, also a G → A transition in the second base of this codon. All mutations were heterozygous with the wild-type species. No mutations were found in any of the other *c-ras* exons studied using PCR and sequencing (Table 6.1). These results demonstrate that in both the cell lines, BL10 and JB1, prepared from different types of tumours induced by aflatoxin dietary, there is a c-Ki-*ras* mutation, a G → A transition in codon 12 (Figure 6.1).

Four other archival tissues were examined, in this case primary tumours and not cell lines, for mutations in both c-Ki-*ras* and N-*ras* genes. All tumours had been induced in male F344 rats by AFB_1 exposure in the diet, three were liver tumours (HTT1-3) and one was a secondary tumour in the lung (HTT4). None of these tumour samples displayed any mutation in codons 12/13 or 61 of either c-Ki-*ras* or N-*ras* genes (Table 6.1).

Two archival control liver tissue samples were also included in this analysis, one

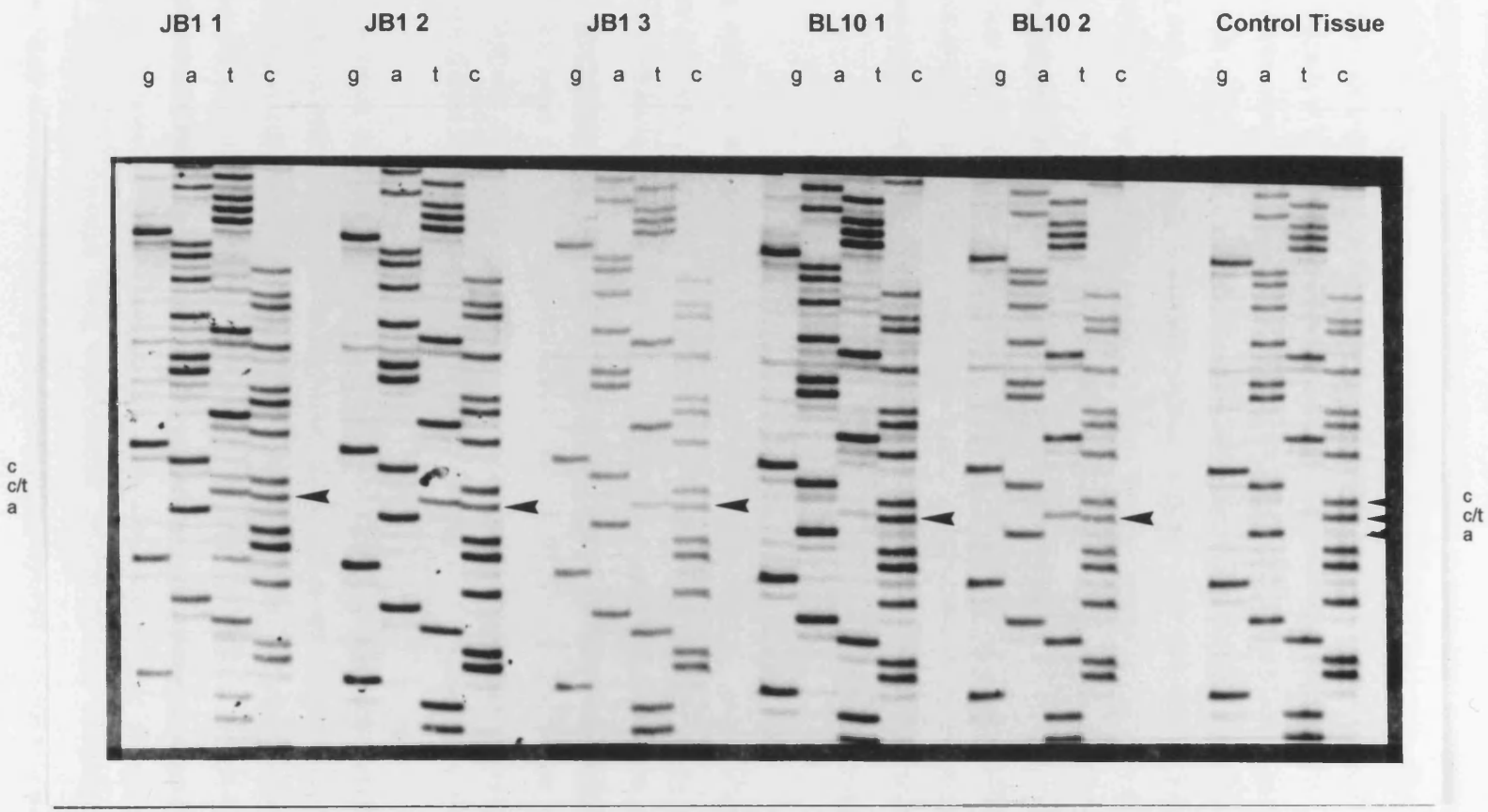


Figure 6.1 Ki-ras exon 1 codon 12 sequence code of pseudoglandular and poorly differentiated HCC induced in nude mice by cell lines, BL10 and JB1, respectively, derived from AFB_1 induced liver tumours in F344 rats. Archival control tissue was prepared from the liver of an untreated F344 rat.

sample was from an untreated control animal (HCT1) and the other was from apparently normal liver tissue adjacent to a tumour following AFB_1 treatment (HCT2). Neither displayed any mutations in codons 12/13 or 61 of c-Ki-*ras* or N-*ras* genes (Table 6.1).

B. Ras gene mutations in preneoplastic lesions and tumours induced in AFB_1 study

Twenty-three foci of altered hepatocytes from different time-points of the study displaying a varied biochemical phenotype and all six tumours induced by AFB_1 at week 77 of the study were analysed for c-Ki and c-N-*ras* gene mutations. As in the case of the JB1 and BL10 xenograft rat tumours produced in nude mice, exons 1 and 2 of the c-Ki-*ras* and N-*ras* genes were analysed. The method involved removal of tissue from unstained sections for PCR, using stained sections to locate the relevant focus/area, followed by sequencing. None of the samples examined displayed mutations in either c-Ki-*ras* or N-*ras* at either codons 12/13 or 61.

Three age-matched liver tissues taken from control animals which were not treated with AFB_1 were also included in the analysis. One of these control samples included liver tissue from an animal 82 weeks of age which contained some small spontaneous foci defined by their biochemical profile. All of the control samples examined displayed the normal genotype for both codons 12/13 and 61 of the c-Ki-*ras* and N-*ras* genes.

See Table A3.1 in Appendix 3 for biochemical profile of foci and tumours using immunohistochemical staining and *c-ras* and *p53* gene mutation analysis of all tissues examined.

C. Ras gene expression

Because mutations in codons 12/13 and 61 of the c-Ki-*ras* and N-*ras* genes were not found in any of the AFB_1 tumours induced in this study, in contrast to the presence of *c-ras* mutations reported in other studies, it was decided to examine c-Ki and N-*ras* gene expression in the tumour samples induced in this study to determine if perhaps another event had occurred which was affecting *c-ras* expression.

Ki-*ras* and N-*ras* cDNA probes were prepared using RT-PCR and used to probe Northern blots loaded with either total or messenger RNA. Both c-Ki-*ras* and N-*ras* probes spanned both exon 1 and exon 2 and gave products of 241 bps and 251 bps, respectively. Sequencing of these probes demonstrated a high degree of homology between them and subsequent experiments indicated high crossreactivity of the probes in binding to both c-Ki-*ras* and N-*ras* RNA resulting in the presence of two bands at 2.1 and 3 Kb, respectively. This was not unexpected from the known sequences of Ki-*ras* and N-*ras*, exons 1 and 2. In addition there was a high level of background which when attempts were made to remove it by stringent washing conditions resulted in a loss of much of the specific signal. As a result it was not possible to make meaningful comparisons of the signals to determine the level of mRNA expression of each gene in the individual samples (Figure 6.2).

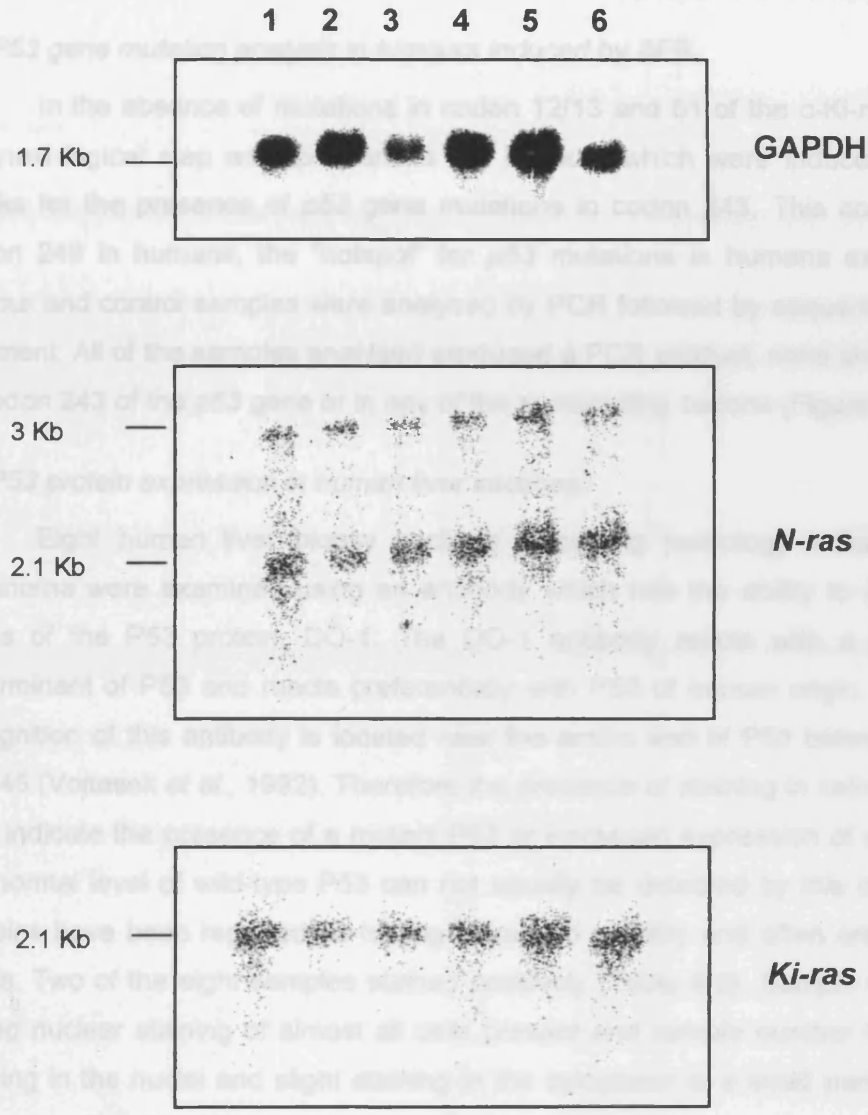


Figure 6.2 Expression of [1]Ki-*ras* and N-*ras* in mRNA prepared from normal rat liver, HCC, normal rat lung or HCC metastases in the lung. Lane 1: Control fed animal, forty-nine weeks old. Lane 2: HCC induced in male F344 rat following treatment with AFB_1. Lane 3: Lung containing HCC metastases from animal in lane 2. Lane 4: HCC induced in male F344 rat seventy-seven weeks following exposure to AFB_1 (4 ppm). Lane 5: Lung from animal in lane 4. Lane 6: Control fed animal from AFB_1 feeding study forty-five weeks old. (The tissues from which the mRNA was prepared and probed in lanes 2 and 3 were kindly donated by Dr MM Manson, MRC Toxicology Unit, University of Leicester).

[1] Only one signal at 2.1 Kb for Ki-*ras* is displayed in this figure as it was possible to remove the crossreacting signal through stringent washing, however much of the original signal was also removed.

6.2.2 P53 gene mutations

A. P53 gene mutation analysis in tumours induced by AFB_1

In the absence of mutations in codon 12/13 and 61 of the c-Ki-*ras and* N-*ras genes* the next logical step was to examine the tumours which were induced by AFB_1 after 77 weeks for the presence of *p53* gene mutations in codon 243. This codon corresponds to codon 249 in humans, the "hotspot" for *p53* mutations in humans exposed to AFB_1. All tumour and control samples were analysed by PCR followed by sequencing of the amplified fragment. All of the samples analysed produced a PCR product, none showed any mutations in codon 243 of the *p53* gene or in any of the surrounding codons (Figure 6.3).

B. P53 protein expression in human liver sections

Eight human liver biopsy sections displaying pathology indicating hepatocellular carcinoma were examined using an antibody which has the ability to recognise all known forms of the P53 protein, DO-1. The DO-1 antibody reacts with a denaturation stable determinant of P53 and reacts preferentially with P53 of human origin. The epitope site of recognition of this antibody is located near the amino end of P53 between amino acids 37 and 45 (Vojtesek *et al.,* 1992). Therefore the presence of staining in cells using this antibody may indicate the presence of a mutant P53 or increased expression of wild-type P53, since the normal level of wild-type P53 can not usually be detected by this method. Mutant P53 proteins have been reported as having increased stability and often are expressed at high levels. Two of the eight samples stained positively (Table 6.2). Sample number 2 displayed strong nuclear staining of almost all cells present and sample number 8 displayed positive staining in the nuclei and slight staining in the cytoplasm in a small percentage of the cells present on the slide (Figure 6.4).

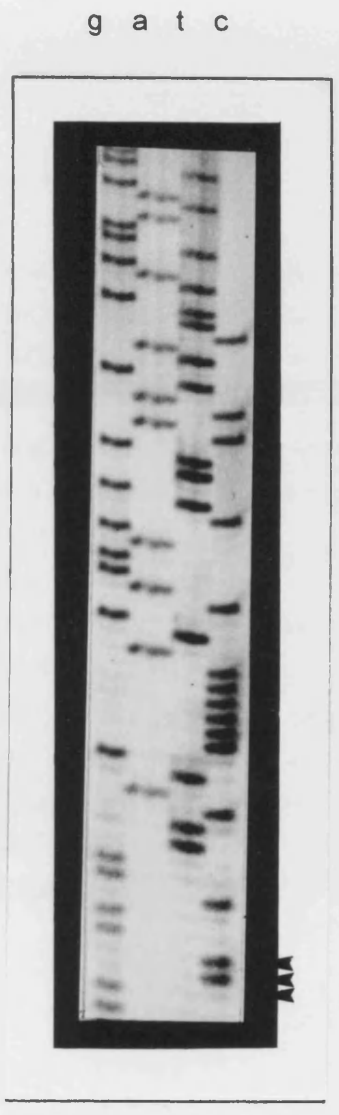


Figure 6.3 Rat *p53* exon 6 codon 243 sequence

Table 6.2

Histopathology, p53 and HBV status of human liver biopsy samples from the Gambia, an area with high exposure to aflatoxins.

Sample	[2]Histopathology	P53 status	HBV status
Control liver	Sections of this liver biopsy show no significant histological abnormality. Normal liver.	-	[3]-
HT1	The sections show infiltrating hepatocellular carcinoma.	-	nd
HT2	Sections show liver with extensive necrosis. A nodule of tumour is seen forming a pseudoglandular pattern. Bile is seen in the tumour indicating a hepatic carcinoma, most likely to be a hepatocellular carcinoma.	+	nd
HT3	The sections show liver which is not cirrhotic but shows marked cholestasis and portal tract inflammation. A large proportion of the specimen consists of a multifocal malignant tumour. The tumour cells resemble hepatocytes and form a sinusoidal pattern which blends into non malignant liver. There are many bizarre mitoses and bile is seen within the tumour. Features most likely represent a hepatocellular carcinoma.	-	nd
HT4	The sections of liver biopsy show a tumour composed of hepatocyte-like cells arranges in trabeculae and rosettes with focal acinus formation. There is marked decrease in reticulin. Many of the cells display a clear cell appearance with displacement of the nucleus to the periphery of the cell. This is due partly to fat accumulation and partly to glycogen accumulation. The appearances are of a well differentiated hepatoma.	-	nd
HT5	The liver is fragmented but it contains a tumour with surrounding fibrosis. The cytological features are most like a hepatocellular carcinoma and bile is seen within some of the tumour cells.	-	nd
HT6	The sections show an obvious malignant tumour with virtually no surviving normal liver. The cells are extremely pleomorphic, some	-	nd

[2] Histology taken from surgical pathology reports kindly provided by Dept. of Histopathology, Northwick Park Hospital.

[3] viral serology and auto antibodies negative

Sample	[2]Histopathology	P53 status	HBV status
	resemble hepatocytes but others are large giant cells with abundant large irregular nuclei. Many abnormal mitoses are seen. Almost certainly a HCC.		
HT7	The sections show cores of liver infiltrated by a HCC in which bile has been identified.	-	nd
HT8	The tissue is almost entirely necrotic with a few small foci of viable tissue with the appearance of HCC.	+	[4]-

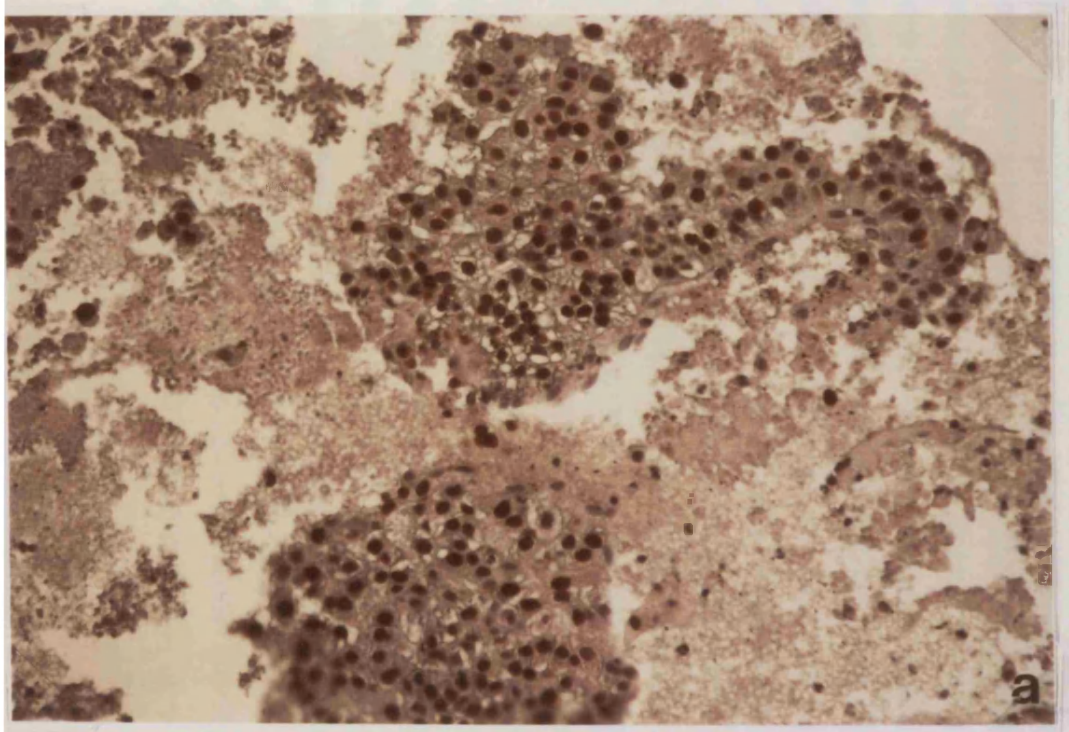
- = negative staining with P53 antibody
+ = positive staining with P53 antibody
nd = no data

[4] hepatitis B antigen negative

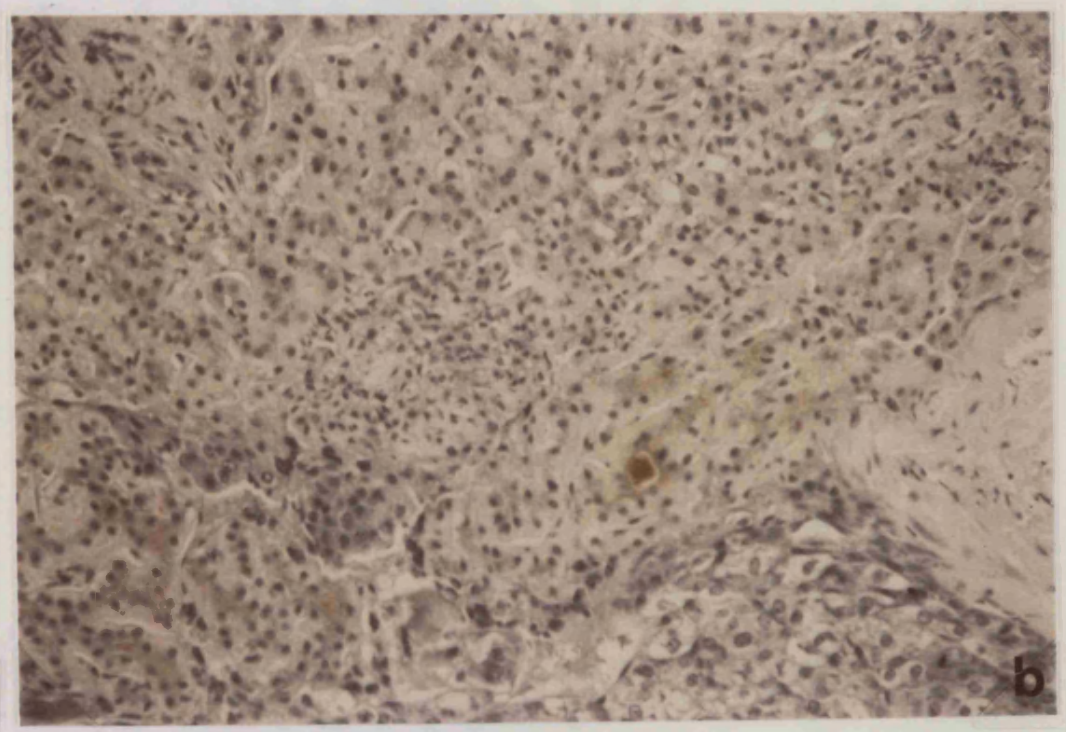
Figure 6.4 Immunohistochemical staining of human liver biopsy samples displaying HCC, from the Gambia, using an antibody recognising the amino terminal end of the human P53 protein.

a) Human HCC displaying strong nuclear staining in all cells present.

b) Human HCC displaying no staining in any of the cells present.



a



b

6.3 Summary and Discussion

Activated c-Ki-*ras* oncogene was detected in 83% of the AFB_1 induced rat liver tumours grown in nude mice by the injection of cells from each of two cell lines. This is in accordance with reports by McMahon *et al.,* 1986 and 1990; Sinha, 1988; Soman and Wogan, 1993. The incidence of c-Ki-*ras* mutations varies greatly among each of these reports with the lowest incidence of 25% reported by McMahon *et al.,* 1986 and the highest incidence of 100% reported by Soman and Wogan, 1993. The most obvious variation in each of these studies is the method of analysis and it would appear with the development of more sensitive techniques for oncogene activation analysis a greater number of mutations in the c-Ki-*ras* gene have been reported in tumours induced in rodents by exposure to AFB_1. The predominant mutation reported is a G:C to A:T base transition in codon 12 of c-Ki-*ras* which is in accordance with the mutation type reported in this thesis. Although mutations in N-*ras* and c-Ha-*ras* have also been reported, c-Ki-*ras* mutations are by far the most common in the AFB_1 induced rat hepatocarcinogenesis model (Buchmann *et al.,* 1991; Sinha *et al.,* 1988). No mutations were detected in the N-*ras* gene in either exon 1 or 2 in the rat liver tumours grown as transplants in nude mice which were analysed. Mutations in c-Ha-*ras* were not examined as no reports of activation were previously found *in vivo* using this model of hepatocarcinogenesis in the rat.

Despite a high frequency of c-Ki-*ras* mutations in the rat liver tumour cell lines grown in nude mice, no Ki- or N-*ras* mutations were found in any of the primary tumours induced in the AFB_1 dietary study presented in this thesis. This could perhaps be attributed to the late presentation of tumours, after 77 weeks, compared to 50+ weeks in other studies (Soman and Wogan, 1993; McMahon *et al.,* 1990). This was despite a lower exposure to AFB_1 over a shorter period of time in some of the previously reported studies, for example, Soman and Wogan (1993) exposed their animals to a total of 1.0 mg of AFB_1 via intraperitoneal injections over a period of eight weeks compared with the present AFB_1 dietary study where the rats were exposed to a total of 4.12 mg of AFB_1 in the diet over a period of 12 weeks. Previous studies have also indicated a decreased latency for tumour development with increasing exposure to AFB_1 (Wogan *et al.,* 1974). In addition analysis of an archival liver tumour sample (HTT 3) displaying a high level of aggression, which also produced metastases in the lung (HTT 4) did not display c-Ki-*ras* or N-*ras* activation. These two results would indicate a different mechanism of hepatocarcinogenesis in these animals resulting from exposure to AFB_1 that does not involve activation of c-Ki- or N-*ras* through mutation at codons 12/13 or 61. Analysis of foci of altered hepatocytes in this study at a number of stages of development as defined by their altered biochemical phenotype also did not indicate any c-Ki- or N-*ras* activation demonstrating that mutation in these genes is not an essential prerequisite for hepatocarcinogenesis induced by exposure to AFB_1. If the technique was to be questioned

on the grounds that the method may not be sensitive enough to detect a mutation in only a small subpopulation of the cells comprising the early foci examined, it would be predicted that if these mutations were essential in the carcinogenic process there would be a greater proportion of cells carrying the mutation present in the final tumours. The results are not consistent with this. Also the technique was clearly able to detect Ki-*ras* codon 12 mutations in the rat tumours grown in nude mice. It is possible, however, that the number of tumours analysed in the present study was not sufficient to detect a low frequency of c-Ki- or N-*ras* mutations. Tokusashi *et al.,* (1994) report only one c-Ki-ras codon 12 mutation in thirteen HCC and none in twenty-one hyperplastic nodules, induced by AFB_1 in rats examined. In fact of all the reports of *ras* mutations in AFB_1 induced rat HCC only Soman and Wogan (1993) report a high frequency of c-Ki-*ras* mutation. However a study by Donnelly *et al.,* (1996) has also described a 100% incidence of c-Ki-*ras* mutations resulting from AFB_1 carcinogenesis, in that case involving lung adenomas and carcinomas in mice. All of the reported point mutations occurred at G:C base pairs, 99% at codon 12 and 1% at codon 13. Of those at codon 12, 80% were G → T transversions and 18% were G → A transitions. This mutational pattern appears to be specific for AFB_1-induced lung tumours in this study as a comparison with spontaneous tumours indicated up to five different types of base substitutions. It appears both of the reported types of mutations i.e. G → T transversions and G → A transitions account for almost all of the mutations reported in animals and humans exposed to AFB_1, but there does seem to be some preference depending on gene, species and maybe even tissue involved. As discussed above the differences in reported incidences in *ras* mutations in AFB_1 carcinogenesis, may indicate differences in sensitivity of techniques used but may also indicate the lack of an essential role of *ras* mutations in the process of hepatocarcinogenesis induced by AFB_1 in the rat. It is possible that a lack of DNA stability in the tumour cells results in mutations in the *ras* genes resulting from and not preceding the oncogenic change. This is supported by the expectation that these mutations would be an early event but have not been reported in cases of AFB_1 induced hyperplastic nodules in the rat either in this study or that carried out by Tokusashi *et al.,* (1994), although in the study by Soman and Wogan (1993) both adenomas and HCC were found to contain the codon 12 c-Ki-*ras* mutation. In conclusion it would appear in some hepatocarcinogenic models there is a link between c-Ki-*ras* disruption and AFB_1 induced tumours in rodents. In others, for example the study presented in this thesis, either current methodology is not sensitive enough to detect heterozygous c-Ki-*ras* mutations in low populations of cells or another gene (or genes) plays a greater role in the control of processes involved in AFB_1 induced carcinogenesis.

Of the human liver biopsy samples analysed 2/8 samples displayed strong nuclear staining using an antibody recognising the N terminal end of the *p53* gene which indicates the presence of a mutated P53 protein or increased expression of wild-type P53. This

incidence of 25% correlates well with that predicted by Gerbes and Caselmann (1993) who suggested that an average *p53* mutation rate of 25% is assumed for areas with high AFB_1 exposure, double the rate seen in other areas. Although from the present results it is not possible to know if it is a mutant P53 protein which is being detected by the antibody or over-expression of the wild type it is probable that it is the former from the reported high level of mutation in the *p53* gene in human HCC from areas with high exposure to AFB_1.

Despite the frequency and apparent specificity of a point mutation in the *p53* gene at codon 249 in human HCC in areas of high exposure to AFB_1, no reports have established a similar phenomenon in HCC induced in rodents or non-human primates by AFB_1 (Hulla *et al.,* 1993; Fujimoto *et al.,* 1992). The absence of point mutations in codon 243 of the rat *p53* gene in the HCC induced in the present AFB_1 dietary study is in agreement with these reports. A study carried out by Lilliberg *et al.,* (1992) also examined changes in *p53* gene and gene expression in AFB_1 induced HCC in rats. It was reported that in these tumours alterations in the *p53* gene were common but not through point mutation in codon 243. These workers found gross genomic rearrangements or deletions, in one allele only, which resulted in dramatically reduced levels of *p53* mRNA and protein. It is possible that these aberrations were accompanied by subtle point mutations in the other allele resulting in inefficient transcription or mRNA processing or the expression of a mutated protein product, which interfered with the normal expression of *p53.* Examination for such mutations however was not carried out in that study. These results therefore indicate the possibility of the involvement of altered *p53* in AFB_1 induced HCC other than codon 243 mutations which could be due to species differences in metabolism of AFB_1, *p53* gene structure and/or the involvement of the hepatitis virus (Eaton *et al.,* 1994; Hulla *et al.,*1993 and Teramoto *et al.,* 1994). Recent work has cast doubt on the suggested link between mutation of codon 249 in *p53* in human carcinogenesis and infection with HBV or HCV. Kirby *et al.,* (1996) examined human non-neoplastic liver using a very sensitive technique to detect *p53* codon 249 mutations in HBsAg or HBcAg positive samples from Mozambique, an area of high exposure to AFB_1 and in samples from North Americans with HBV or HCV involved hepatitis and cirrhosis, with no known exposure to AFB_1. These workers discovered mutations in 5 of the 6 samples from Mozambican patients but none in the North American samples. Imazeki *et al.,* (1995) also report a lack of *p53* mutations in this codon in duck HCC induced by exposure to AFB_1 and positive for HBV infection.

It is most likely that species differences in metabolism and/or *p53* gene structure account for the lack of *p53* mutations at codon 243 in the rat HCC induced by AFB_1, in contrast to the codon 249 mutations observed in human AFB_1-associated HCC (Hulla and Schneider, 1993). All the evidence implies the presence of other mechanisms of hepatocarcinogenesis induced by AFB_1 in the rat, not involving the "hotspot" codon of *p53,* which may involve Ki-*ras* codon 12 mutations, but not as essential components. It has been

suggested that disruption of the signal transduction pathway through mutation in codons 12, 13 or 61 of one of the *ras* genes could be involved in the carcinogenic process. However in light of the results of the current study in which no mutations in c-Ki- and N-*ras* were detected in the preneoplastic or neoplastic lesions it would appear that disruption of P21-related signalling pathways by the specified point mutations is not an essential step for the production of tumours in rats following exposure to AFB_1.

To prove this further work could examine the possibility of increased expression of the c-*ras* genes in the tumours produced in the AFB_1 study in particular with the increased expression/ activation of other oncogenes such as c-*myc* throughout the process of hepatocarcinogenesis. Previous work has suggested the possibility of a co-operative effect of increased transcription of c-Ha-*ras* and c-*myc* in AFB_1 induced HCC in rats and c-Ki-*ras* and c-*myc* in the process of hepatocarcinogenesis in a choline-deficient diet model containing 0.1% ethionine (Tashiro *et al.,* 1986; Yaswen *et al.,* 1985), although mutational analysis of these genes or examination of the transforming ability of the transcripts was not carried out. With regard to the *p53* gene, further analysis of these tumour samples could perhaps concentrate on structural changes and examine a much greater area of the gene with regard to point mutational analysis and also follow changes in expression of this gene throughout the process of hepatocarcinogenesis. Lack of any abnormalities regarding the c-*ras* or *p53* genes would strongly support the hypothesis of different mechanisms of hepatocarcinogenesis which may involve other c-oncogenes/ tumour suppressor genes.

Chapter Seven

GST Yc_2 Monoclonal Antibody

(production and characterisation)

7.1 Introduction

Hayes *et al.,* (1991a) reported a novel alpha class glutathione *S*-transferase present in the liver of rats exposed to AFB_1, a potent hepatocarcinogen. The importance of this enzyme, named GST Yc_2, appeared to lie in its ability to be induced by AFB_1 and antioxidants in preneoplastic lesions displaying a resistant phenotype and in its high specific activity towards the AFB_1-epoxide. There are two inducible Yc_2 alpha class GST's both of which exist as heterodimers Ya_1Yc_2 and Yc_1Yc_2. To study this interesting and unique enzyme further Hayes *et al.,* (1994) produced a polyclonal antibody which recognised the GST Yc_2 subunit. However this antibody did not only recognise the GST Yc_2 enzyme but also two other constituitively expressed rat GST subunits, GST Yc_1 and GST Ya which lack the GST Yc_2 enzymes abilities to conjugate the AFB_1-epoxide effectively (Hayes *et al.,* 1991a). The two proteins Yc_2 and Yc_1 show very high homology toward each other differing in only a few amino acids (see Fig 2.12).

Because of the obvious importance of this GST Yc_2 enzyme in AFB_1 induced carcinogenesis it was decided to pursue the production of an antibody which would be specific for GST Yc_2 and show little or no cross-reactivity to the GST Yc_1 enzyme. After suitable characterisation of such an antibody to establish its specificity and versatility of use in different experimental procedures it could be used in this study to demonstrate the GST Yc_2 pattern of induction, redifferentiation, if any, after withdrawal of the carcinogen and the pattern apparent in tumours if possible in different tumour types.

Due to the high homology between the GST Yc_2 and Yc_1 proteins it might also be possible to use such a specific antibody to identify the regions of the Yc_2 protein which could be involved in the specific ability to conjugate the AFB_1-epoxide. If the areas of disparity between the two proteins were accounted for by a conformational change within the protein structure which preferentially allowed substrate (AFB_1-epoxide) binding or induced metabolism of the substrate they may be reflected in the epitope recognised by the Yc_2 specific antibody.

7.2 Results

7.2.1 Preparation of an immunogen for use in the monoclonal antibody production of GST Yc2 antibody

Two approaches were attempted to provide a suitable antigen for the production of this antibody. One was to use the already existing recombinant Yc_2 protein (prepared by Dr JD Hayes, BRC, University of Dundee), the second was to identify and synthesise a suitable polypeptide with maximum possible difference to the Yc_1 sequence (see section 2.11).

Sequence	accession number
GST Yc_1	X78848
GST Yc_2	X78847

Figure 7.1 Accession numbers for glutathione-*S*-transferases Yc_1 and Yc_2

Antipeptide antibodies crossreact with intact native proteins with high frequency and have the advantage of a well defined epitope (Hancock and Evan, 1992).

A. Identification of peptide for use as antigen in production of GST Yc_2 monoclonal antibody

The rat amino acid sequences of GST Yc_2 and GST Yc_1 were obtained using the *Entrez*[1] CD ROM (updated 1995) database system and aligned using MacVector™ 4.1[2] Sequence Analysis Software (International Biotechnologies Incorporated) on a Power Macintosh computer to determine the areas of disparity between them (Fig 7.1).

Hydrophilicity and potential antigenicity of each sequence was evaluated using Hopp-Woods hydropathy scale. The Hopp-Woods scale was designed to predict the location of antigenic determinants in a protein, assuming that the antigenic determinants would be exposed on the surface of the protein and would be located in hydrophilic regions. Its values are derived from the transfer free-energies for amino acid side groups between ethanol and water (Hopp and Woods, 1981). From this the most antigenic area with greatest hypervariability to Yc_1 was determined (Fig 7.2). A peptide of optimal length (13 amino acids) was commercially synthesised for use as the immunogen (see Fig 2.12).

7.2.2 Production of the GST YC_2 monoclonal antibody

Only one of the four exposed animals, one which had been exposed to the recombinant Yc_2 protein as an immunogen, displayed specific antibody production against GST Yc_2 above that seen in the preimmunised serum. Of the four animals used two were exposed to the recombinant Yc_2 protein and two were exposed to the KLH-coupled peptide, all were by i.p. injection (for further details see section 2.11.2), The serum of this animal exhibited a twelve-fold greater response against Yc_2 protein compared to the results obtained using BSA as the control antigen and a seven-fold greater response against Yc_2 protein compared to the Yc_1 protein (Fig 7.3e and f).

[1] Entrez is a registered trademark of the National Library of Medicine.

[2] MacVector is a trademark of Biotechnologies Incorporated.

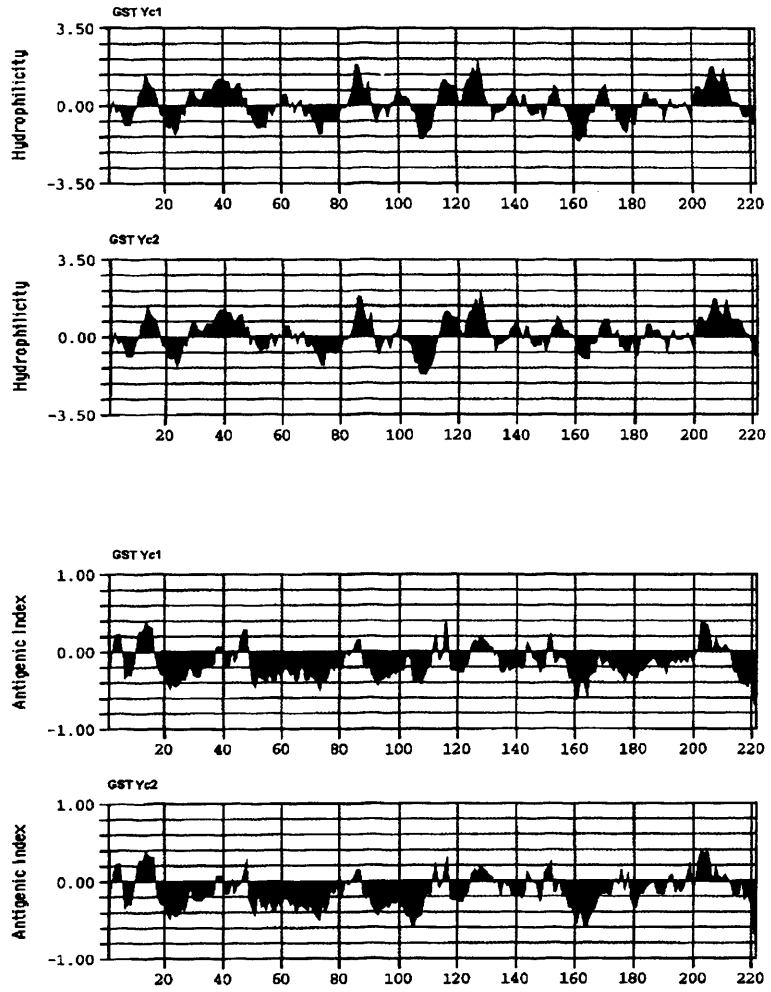


Figure 7.2 Hydrophilicity and antigenicity charts for GST Yc_1 and Yc_2 amino acid sequences. Amino acid position is represented on the x-axis, antigenic index and hydrophilicity according to Hopp-Woods scale is represented on the y-axis.

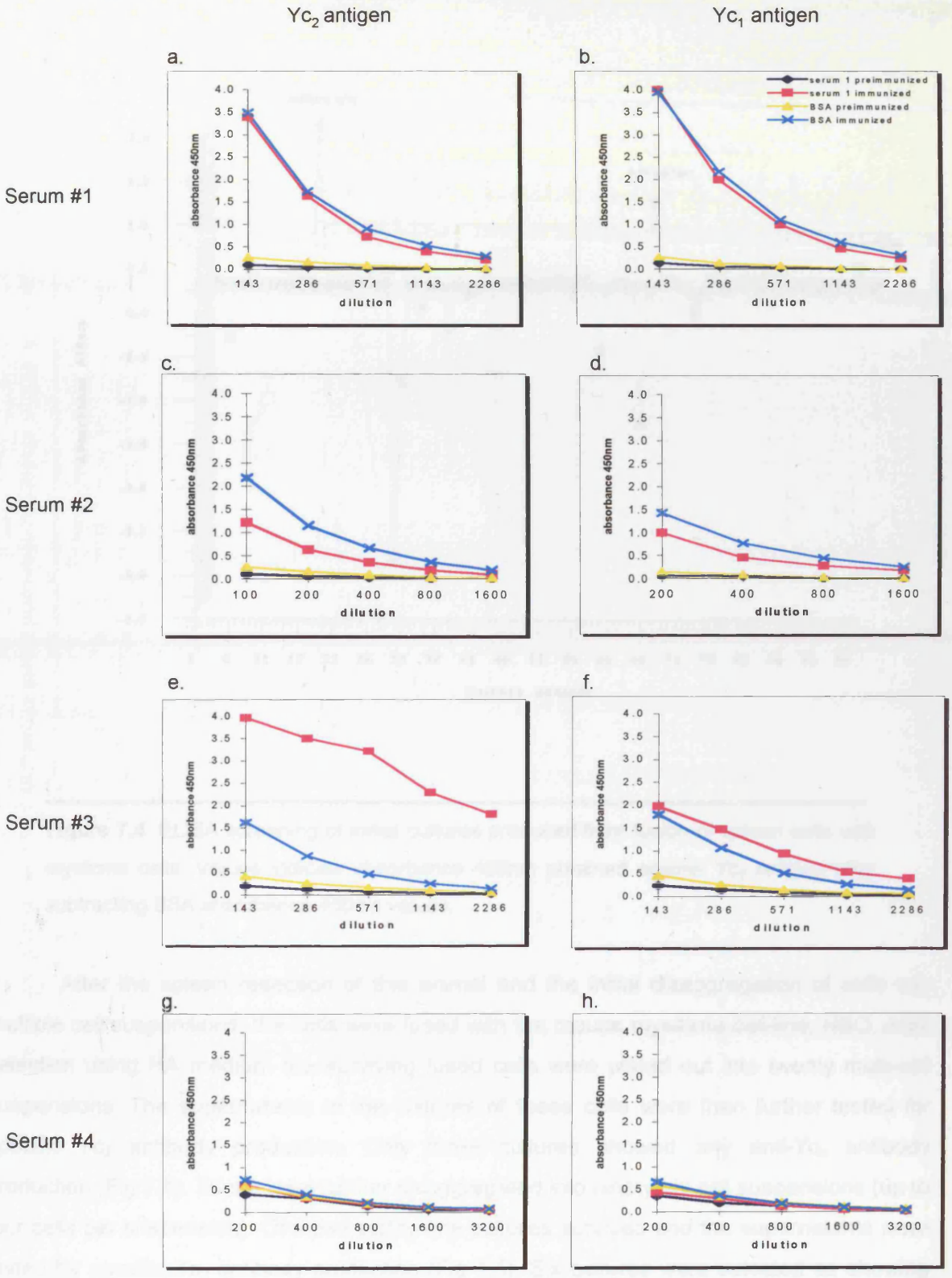


Figure 7.3 ELISA's of the four sera using Yc_1, Yc_2 or BSA as the immobilised antigen. Sera one and two were from animals exposed to the synthetic peptide. Sera three and four were from animals exposed to the recombinant protein. Each serum was analysed before and after exposure to the immunogen (preimmunized and immunized).

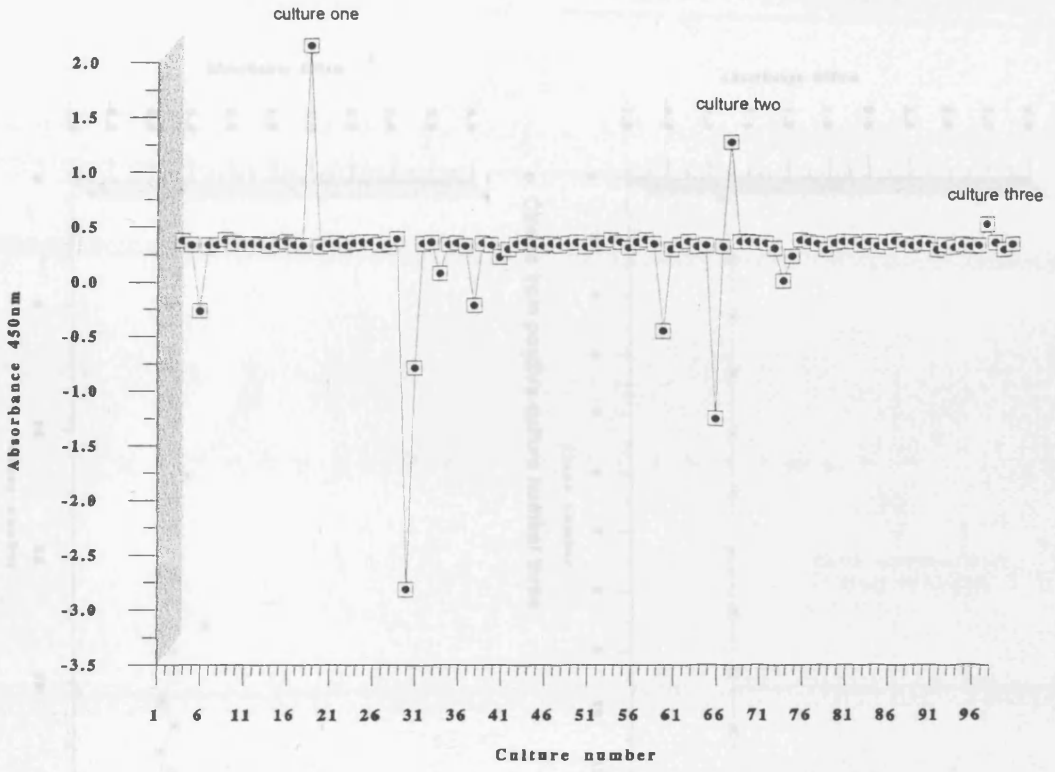


Figure 7.4 ELISA screening of initial cultures produced from fusion of spleen cells with myeloma cells. Values indicate absorbance 450nm obtained against Yc_2 antigen after subtracting BSA absorbance 450nm values.

After the spleen resection of this animal and the initial disaggregation of cells into multiple cell suspensions, the cells were fused with the mouse myeloma cell-line, NSO. After selection using HA medium the surviving fused cells were plated out into twenty multi-cell suspensions. The supernatants of the cultures of these cells were then further tested for specific Yc_2 antibody production. Only three cultures showed any anti-Yc_2 antibody production (Fig 7.4). These were further disaggregated into ninety-six cell suspensions (up to four cells per suspension). Of these eighty-one cultures survived and the supernatants were tested for specific Yc_2 antibody production (Fig 7.5). Six cultures were selected as showing the highest specific Yc_2 antibody level (Fig 7.5b). These were further cloned by splitting into twenty-two single cell suspensions and tested as before. Two of these clones were chosen as having the highest level of specific antibody to Yc_2 (Fig 7.6) for further culture to produce unlimited quantities of supernatant containing the specific GST Yc_2 antibody. These clones were used in further experiments to characterise the antibody.

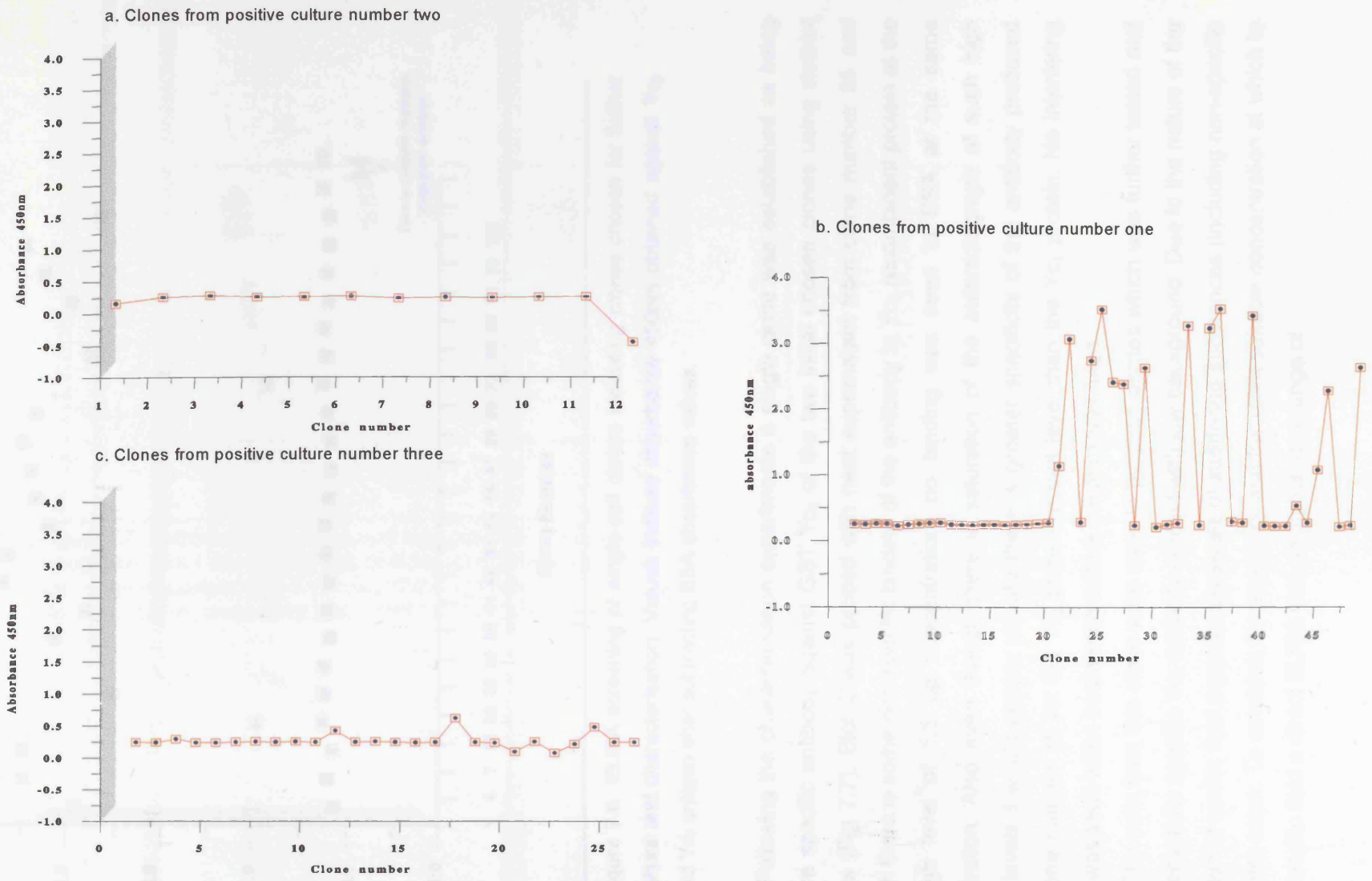


Figure 7.5 ELISA screening of second round of cultures prepared from three clones selected in first round screening. Values indicate absorbance 450 nm obtained against Yc_2 antigen after subtracting BSA absorbance 450 nm values.

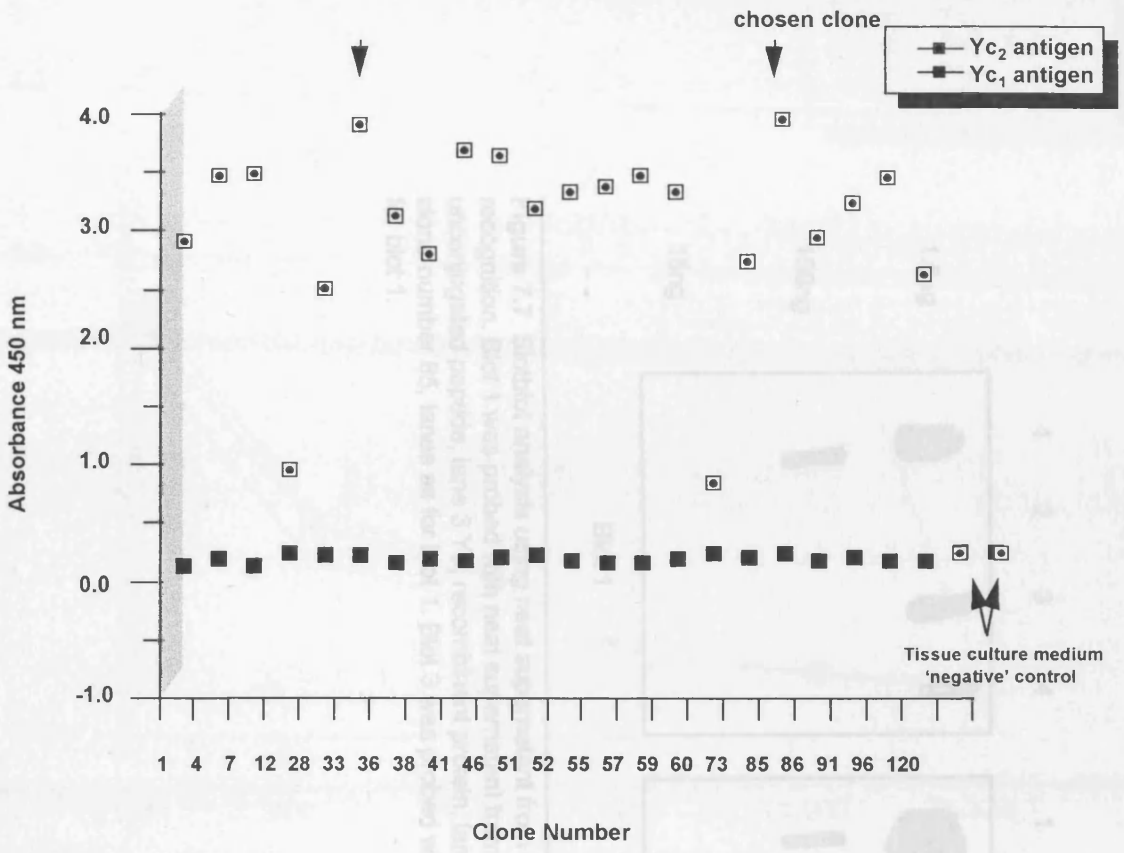


Figure 7.6 ELISA screening of single-cell clones indicating clones chosen for further culture and characterisation. Values indicate absorbance 450nm obtained against Yc_2 and Yc_1 antigen after subtracting BSA absorbance values.

Following the characterisation experiments a single clone was established as being the more specific antibody against GST Yc_2 of the two initial chosen clones using slotblot analyses (Fig 7.7). Blot 2 was probed with neat supernatant from clone number 85 and although there is some non-specific binding of the antibody to Yc_1 recombinant protein at the very high level of 1.5 μg concentration, no binding was seen to BSA at the same concentration. Also even though there is saturation of the autoradiograph at such high protein levels it would appear visually there is greater specificity of the antibody produced from clone number 85 for the Yc_2 protein at that level than the Yc_1 protein. No interfering binding was seen when neat tissue culture medium was used.

The cells from this clone were used to produce ascites which was further tested and characterised for specific Yc_2 antibody content with low background. Due to the nature of the production of ascitic fluid occasionally levels of interfering substances (including non-specific antibodies) occur. To establish the specificity and the most suitable concentration at which to use the ascitic fluid a direct ELISA was set up using a range of

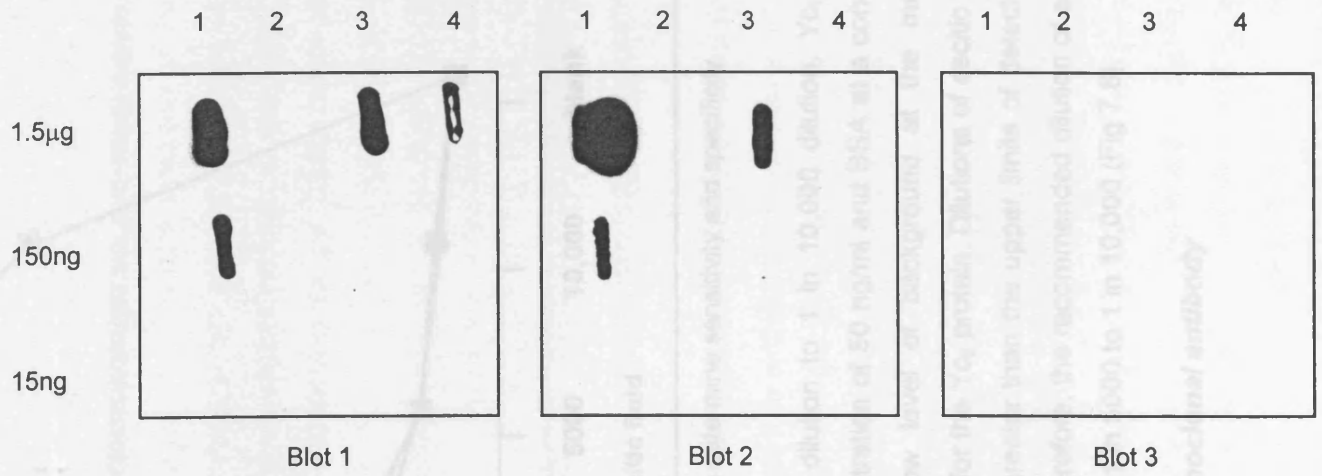


Figure 7.7 Slotblot analysis using neat supernatant from clones 33 and 85 to characterise specificity of antigen recognition. Blot 1 was probed with neat supernatant from clone number 33, lane 1 Yc_2 recombinant protein, lane 2 unconjugated peptide, lane 3 Yc_1 recombinant protein, lane 4 BSA. Blot 2 was probed with neat supernatant from clone number 85, lanes as for blot 1. Blot 3 was probed with tissue culture supernatant as a negative control, lanes as for blot 1.

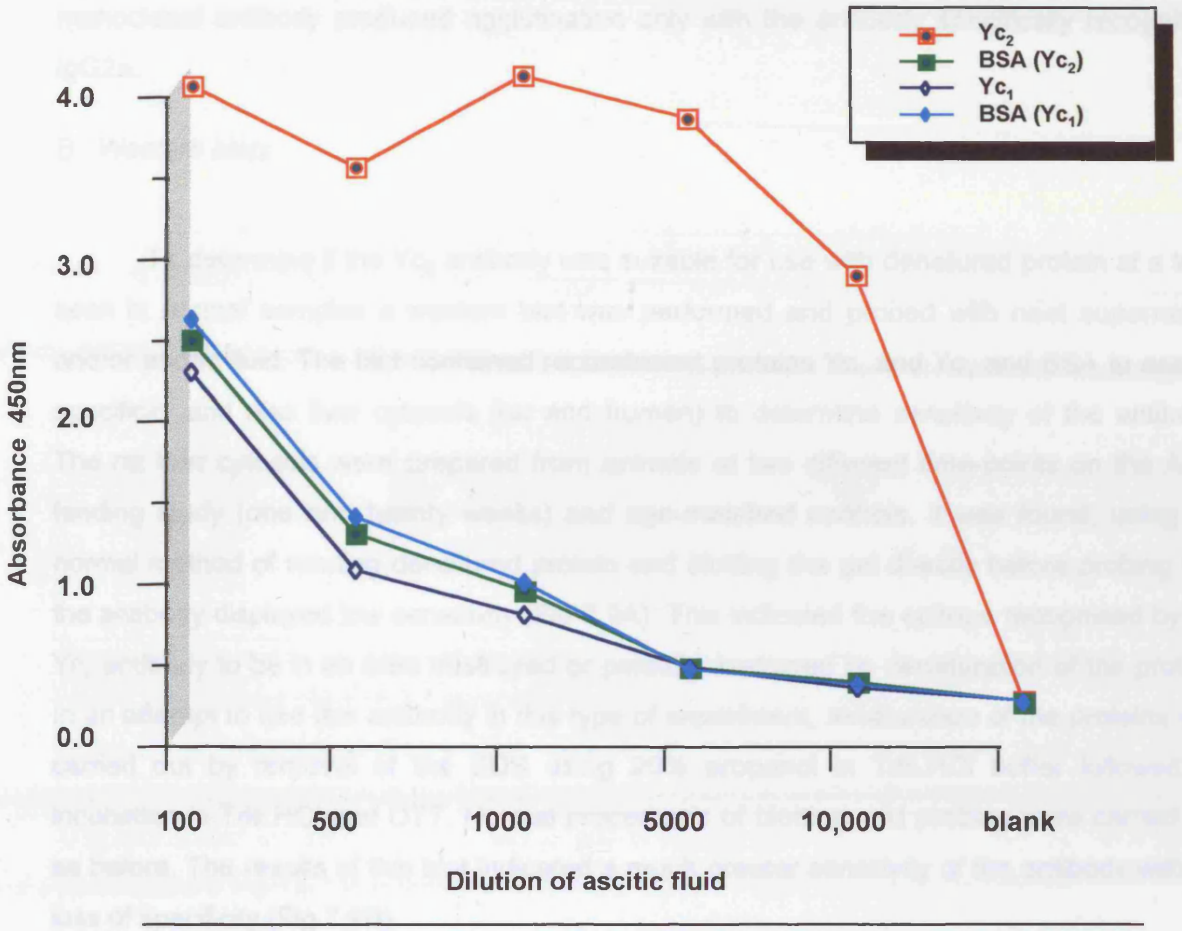


Figure 7.8 Direct ELISA of ascitic fluid to determine sensitivity and specificity.

dilutions of the ascitic fluid from 1 in 100 dilution to 1 in 10,000 dilution. Yc_1 and Yc_2 recombinant proteins were used at a concentration of 50 ng/ml and BSA at a concentration of 100 ng/ml. The results indicated a low level of background at the more dilute concentrations and a high level of specificity for the Yc_2 protein. Dilutions of ascitic fluid used in the range of 1 in 100 to 1 in 1000 were greater than the upper limits of detection of the plate reader and hence too concentrated, therefore, the recommended dilution of ascitic fluid for use in direct ELISA fell within the range of 1 in 5000 to 1 in 10,000 (Fig 7.8).

7.2.3 Characterisation of the GST YC_2 monoclonal antibody

A. Isotyping

Isotyping of the antibody was carried out using an anti-mouse monoclonal isotyping kit (Serotec) specifically designed to identify the class and subclass of monoclonal antibodies. The principle of this system is based on red cell agglutination. A positive, agglutinated result occurs when a highly specific antibody, coupled onto sheep red blood cells, recognises and binds to a particular isotype. The antibodies included in the kit recognised the following isotypes: IgG1, IgG2a, IgG2b, IgG3, IgA and IgM. GST Yc_2

monoclonal antibody produced agglutination only with the antibody specifically recognising IgG2a.

B. Western blots

To determine if the Yc_2 antibody was suitable for use with denatured protein at a level seen in normal samples a western blot was performed and probed with neat supernatant and/or ascitic fluid. The blot contained recombinant proteins Yc_1 and Yc_2 and BSA to assess specificity and also liver cytosols (rat and human) to determine sensitivity of the antibody. The rat liver cytosols were prepared from animals at two different time-points on the AFB_1 feeding study (one and twenty weeks) and age-matched controls. It was found, using the normal method of running denatured protein and blotting the gel directly before probing that the antibody displayed low sensitivity (Fig 7.9A). This indicated the epitope recognised by the Yc_2 antibody to be in an area destroyed or partially destroyed by denaturation of the protein. In an attempt to use this antibody in this type of experiment, renaturation of the proteins was carried out by removal of the SDS using 20% propanol in Tris.HCl buffer followed by incubation in Tris.HCl and DTT. Normal procedures of blotting and probing were carried out as before. The results of this blot indicated a much greater sensitivity of the antibody with no loss of specificity (Fig 7.9B).

To determine if these antibodies could recognise a human GST alpha protein with high homology to the rat GST Yc_2, human foetal liver cytosols were prepared and probed. No staining was detected on these samples probed with the Yc_2 monoclonal antibody but a band similar in size to that seen with the rat Ya protein (M_r 25,500) was detected when probed with the Yc_1/Yc_2 antibody (Fig 7.10, lanes 9 and 10).

When the liver cytosols from animals on the AFB_1 feeding study were examined using both these antibodies as probes the following results were obtained. Using the Yc_2 antibody as a probe the strongest staining was seen using the cytosols from the animals after one week on the study (that is, six weeks old), either exposed only to the control diet or to the AFB_1 treated diet for one week (Fig 7.9, lane 4). Comparing the staining intensity obtained using the cytosols from animals after twenty weeks on the study (that is, twenty-five weeks old), there appears to be a significant induction or retention of the Yc_2 protein in the animal exposed to the AFB_1 diet compared to the age-matched control exposed only to the control diet (Fig 7.9B, lanes 2 and 3). The level of Yc_2 present in this normal adult rat was at a much lower level at that seen in the control animal at six weeks old. A similar

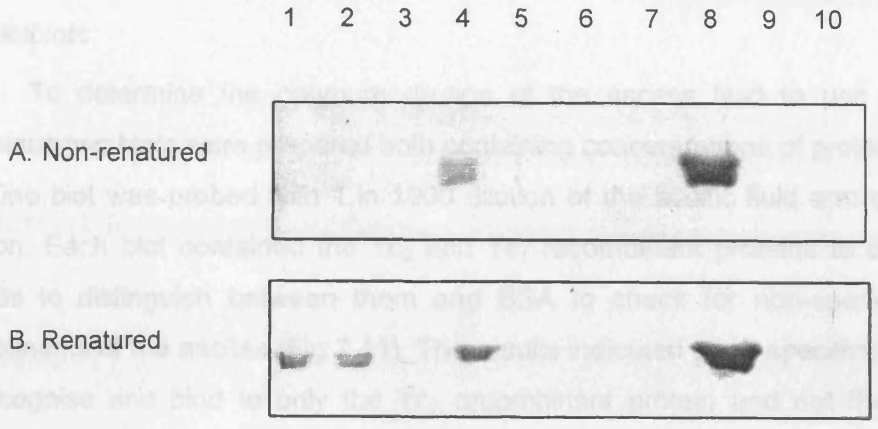


Figure 7.9 Western blot of non-renatured and renatured recombinant Yc_1 and Yc_2 proteins and liver cytosol preparations using neat supernatant containing Yc_2 specific antibody.

Lanes 1-4 rat liver cytosols 20μg protein – lane 1 AFB_1-treated animal 1 week on study, lane 2 AFB_1-treated animal 20 weeks on study, lane 3 age-matched control animal 20 weeks on study, lane 4 age-matched control animal 1 week on study, lane 6 20μg protein BSA, lane 7 1μg Yc_1 recombinant protein, lane 8 1μg Yc_2 recombinant protein, lanes 9 and 10 20μg human foetal cytosols.

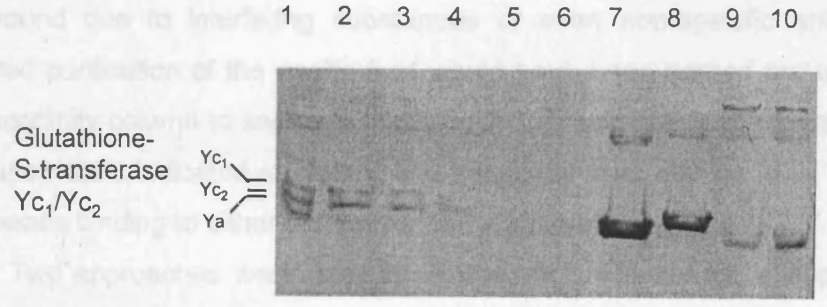


Figure 7.10 Western blot of recombinant Yc_1 and Yc_2 proteins and liver cytosol preparations using Yc_1/ Yc_2 antibody.

Lanes 1-4 rat liver cytosols 20 μg – lane 1 AFB_1-treated animal 1 week on study, lane 2 age-matched control animal 1 week on study, lane 3 AFB_1–treated animal 20 weeks on study, lane 4 age-matched control animal 20 weeks on study, lane 6 20μg BSA, lane 7 1μg Yc_2 recombinant protein, lane 8 1μg Yc_1 recombinant protein, lanes 9 and 10 20μg human foetal cytosols.

pattern of staining intensity was seen when the polyclonal Yc_1/Yc_2 antibody was used as the probe (Fig 7.10, lanes 3 and 4).

C. Slotblots

To determine the optimum dilution of the ascites fluid to use in the slotblotting technique two blots were prepared both containing concentrations of protein from 100 ng to 5 ng. One blot was probed with 1 in 1000 dilution of the ascitic fluid and one with 1 in 5000 dilution. Each blot contained the Yc_2 and Yc_1 recombinant proteins to check ability of the ascites to distinguish between them and BSA to check for non-specific binding of any components of the ascites (Fig 7.11). The results indicated good specificity of the ascitic fluid to recognise and bind to only the Yc_2 recombinant protein and not the Yc_1 recombinant protein at a dilution of 1 in 1000 with excellent sensitivity down to 5 ng of protein. However non-specific binding to BSA was seen at this dilution. At the greater dilution of 1 in 5000 specific binding to Yc_2 and not Yc_1 was also indicated but the sensitivity was reduced to 50 ng of protein. Again however some non-specific binding to BSA was also seen though at a reduced level due to the higher dilution factor. It was decided on the basis of these results not to use the ascitic fluid for the slotblot experiments as there was a more suitable alternative available, that is, the supernatant. The advantage of using the ascites lies in its ability to be diluted up to 5000 fold therefore requiring much less material without the continued requirement of cell culture, however it can have the disadvantage of high levels of background due to interfering substances or even non-specific antibodies. If time had permitted purification of the ascitic fluid would have been carried out using for example, an immunoaffinity column to separate the specific Yc_2 antibody from the interfering substances. The supernatant indicated specificity and sensitivity from 400 ng to 25 ng of protein with no non-specific binding to either the Yc_1 recombinant protein or BSA (Fig 7.12).

Two approaches were used in an attempt to identify the epitope recognised by the antibody. The first was to determine if binding would occur to the synthetic peptide which would indicate this area as containing the epitope recognised by the antibody. Binding to the peptide alone was unsuccessful and this was thought to be due to the possible configuration of the peptide in this form lacking the secondary structure portrayed *in vivo*. Therefore a blot was prepared containing the peptide conjugated to keyhole limpet haemocyanin (KLH) which might mimic better the structure *in vivo* and also KLH alone was included to determine any possible cross-reactivity of the antibody (Fig 7.12). No cross-reactivity of the antibody was seen with KLH alone and no binding was seen with the peptide conjugated to KLH. Although these results may indicate this area of the protein sequence was not the epitope recognised

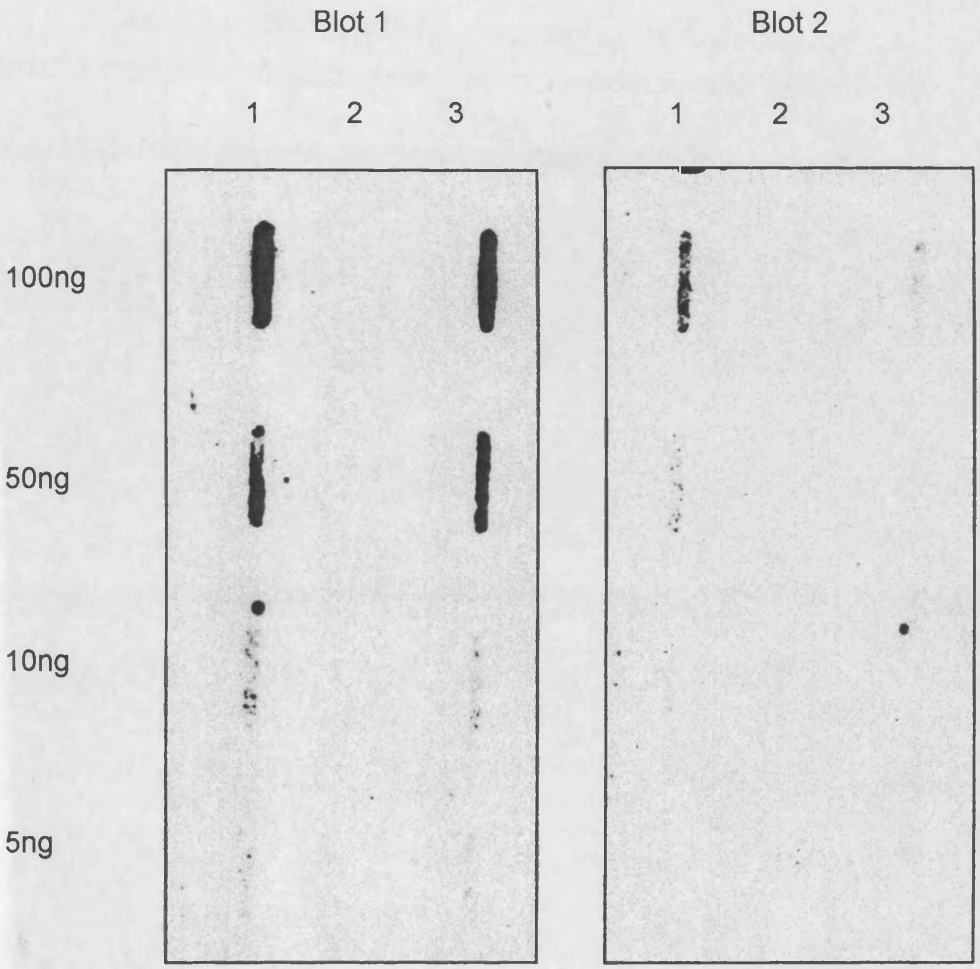


Figure 7.11 Slot blot of Yc_1 and Yc_2 recombinant proteins and BSA using varying dilutions of ascitic fluid containing Yc_2 specific antibody.
Blot 1 was probed with 1 in 1000 dilution of Yc_2 ascites fluid. Lane 1 Yc_2 recombinant protein, lane 2 Yc_1 recombinant protein lane 3 BSA. Blot 2 was probed with 1 in 5000 dilution of Yc_2 ascites fluid. Lanes as for blot 1.

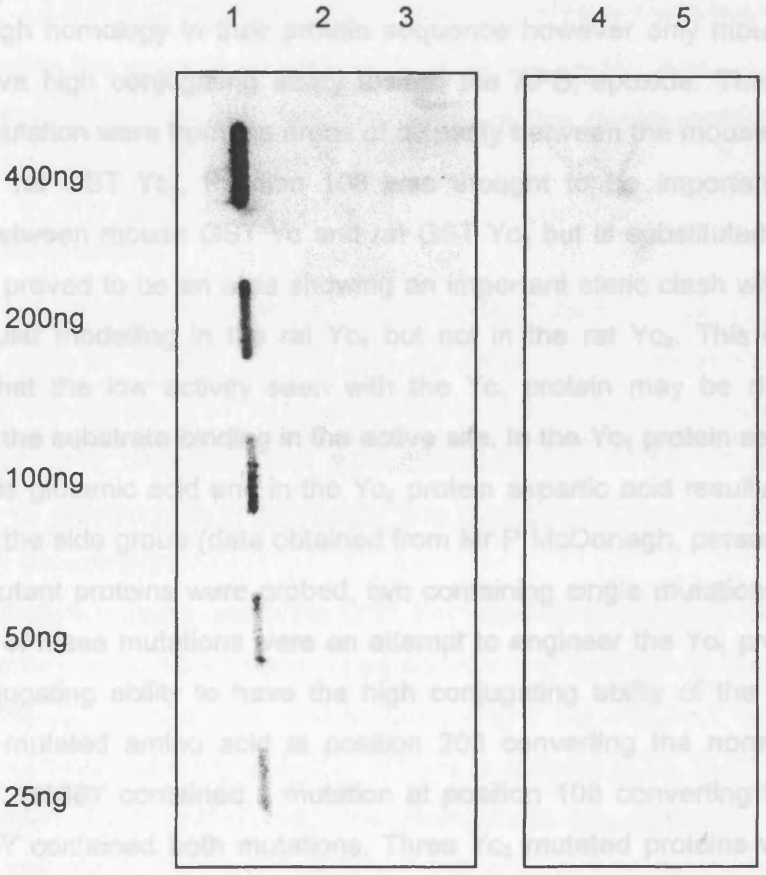


Figure 7.12 Slotblot probed with neat supernatant.
Lane 1 Yc_2 recombinant protein, lane 2 Yc_1 recombinant protein, Lane 3 BSA, Lane 4 keyhole limpet haemocyanin (KLH), Lane 5 peptide conjugated to KLH.

by the Yc_2 antibody, it cannot be determined that the peptide conjugated to KLH has formed the identical secondary structure as it would in the native Yc_2 protein. Therefore further experiments are required to exclude this area as containing the epitope.

The second approach was to establish whether the antibody would recognise any of the mutant Yc_1 and Yc_2 recombinant proteins, which had been prepared, by Mr P McDonagh, Dept. Of Nuclear Magnetic Resonance, University of Leicester. Two amino acid positions were chosen to form the basis of these mutations. All three GST's (mouse Yc, rat Yc_1 and Yc_2) show high homology in their protein sequence however only mouse GST Yc and rat GST Yc_2 have high conjugating ability toward the AFB_1-epoxide. Therefore the positions chosen for mutation were from the areas of disparity between the mouse GST Yc or rat GST Yc_2 and the rat GST Yc_1. Position 108 was thought to be important because Y108 is conserved between mouse GST Yc and rat GST Yc_2 but is substituted to H108 in rat Yc_1. Position 208 proved to be an area showing an important steric clash with the AFB_1-epoxide using molecular modelling in the rat Yc_1 but not in the rat Yc_2. This data has led to the hypothesis that the low activity seen with the Yc_1 protein may be due in part to steric hindrance of the substrate binding in the active site. In the Yc_1 protein sequence the position 208 residue is glutamic acid and in the Yc_2 protein aspartic acid resulting in a difference in the length of the side group (data obtained from Mr P McDonagh, personal communication). Three Yc_1 mutant proteins were probed, two containing single mutations and one a double mutation. All of these mutations were an attempt to engineer the Yc_1 protein with low AFB_1-epoxide conjugating ability to have the high conjugating ability of the Yc_2 protein. E208D contained a mutated amino acid at position 208 converting the normal glutamic acid to aspartic acid. H108Y contained a mutation at position 108 converting histidine to tyrosine. E208D/H108Y contained both mutations. Three Yc_2 mutated proteins were also used with mutations at the same amino acid positions. These mutations were devised to knock-out the AFB_1-epoxide conjugating ability from Yc_2. D208M contained a mutated amino acid at position 208 converting aspartic acid to methionine. Y108L contained a mutation at position 108 converting tyrosine at this position to leucine. Y108L/D208M contained both mutations. As negative controls- wild-type Yc_1 protein, BSA and the protein elution buffer (neat) were also applied to the slotblot.

Blot 1 indicates the results obtained by probing the Yc_1 mutated proteins (Fig 7.13, blot 1, lanes 4-6) with the Yc_2 antibody, only the positive control wild type Yc_2 recombinant protein showed any binding of the antibody (Fig 7.13, blot 1). Blot 2 indicates the results obtained by probing the Yc_2 mutated proteins (Fig 7.13, blot 2, lanes 5-7) and as a positive control a wild type Yc_2 recombinant protein prepared in a similar way to the mutant Yc_2 proteins (Fig 7.13, blot 2, lane 4) with the Yc_2 antibody. The Yc_2 antibody recognised and bound to both wild-type Yc_2 proteins and the three mutated Yc_2 proteins, no binding occurred

with either wild-type Yc_1 protein, BSA or the protein elution buffer (Fig 7.13, blot 2). There were some differences in the intensity of signal obtained for each of the different mutated proteins, for example, Yc_2 mutant D208M and Yc_2 double mutant D208M/Y108L showed a less intense signal, losing sensitivity at 50 ng of protein, compared to both the wild-type and Yc_2 mutant Y108L which retained a strong signal at 50 ng of protein, in particular, the Yc_2 mutant Y108L appeared to show a very strong signal. It is thought this was an artefact due to more protein being loaded. Compared to the other mutant proteins the recovery of the Yc_2 mutant Y108L protein was eight-fold greater than that obtained with the Yc_2 wild-type protein and it is possible the amount determined and hence loaded was underestimated due to incorrect dilution of this protein during analysis of the concentration (Mr P McDonagh, personal communication).

D. Immunohistochemistry

Initial experiments were carried out using the tissue culture supernatant containing the GST Yc_2 antibody diluted 1 in 50, 100, 500 and 1000 to determine the most suitable dilution at which to use the GST Yc_2 monoclonal antibody in immunohistochemical staining of tissue sections. Little difference was seen between each of the dilutions, the 1 in 1000 dilution however appeared to show slightly less background and therefore this dilution was used in further experiments.

Comparative staining, using the GST Yc_2 monoclonal antibody and the GST Yc_1/ Yc_2 polyclonal antibody, of serial sections prepared from liver and kidney tissue demonstrate the specificity of the GST Yc_2 monoclonal antibody (Fig 7.14). Figure 7.14a and b indicate at magnification x4 areas strongly staining with the polyclonal GST Yc_1/ Yc_2 antibody are the only areas which are identified with the monoclonal Yc_2 antibody. When amplified to magnification x20 this is more obvious (Fig 7.14c and d). The area to the left of the portal tract has stained much more strongly with the monoclonal GST Yc_2 antibody. In addition some cells located to the right show strong cytoplasmic staining. Similarly in the cortex area of the kidney only some of the cells lining the proximal tubules show strong positive staining when probed with the Yc_2 monoclonal antibody compared to the staining indicated when probed with the Yc_2 polyclonal antibody (Fig 7.14e-h).

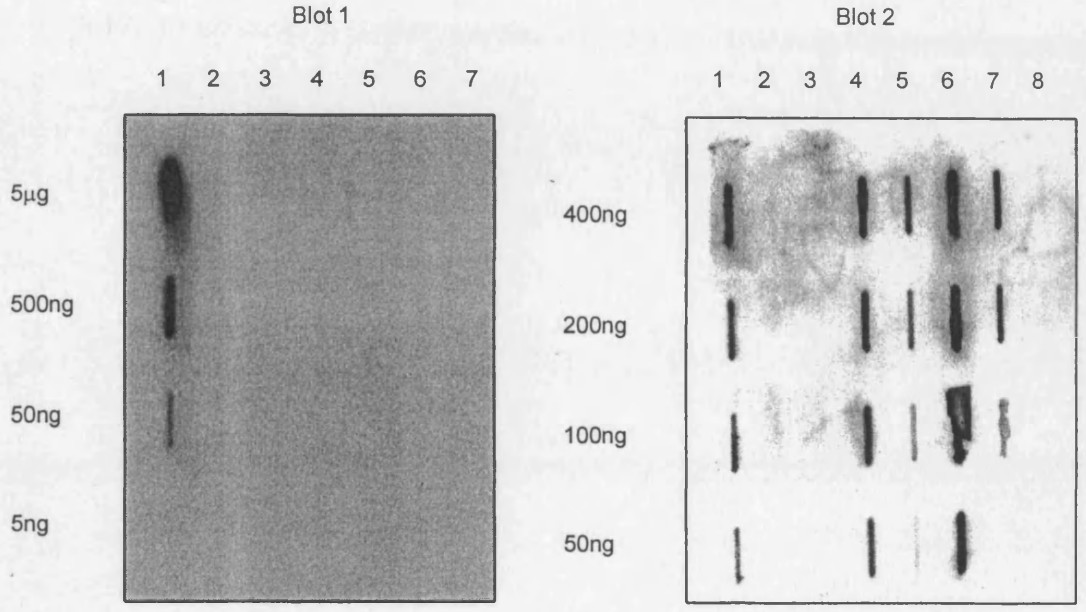


Figure 7.13 Slotblot of mutant Yc_1 and Yc_2 recombinant proteins using neat supernatant containing Yc_2 specific antibody.

Blot 1: lane 1 Yc_2 recombinant protein, lane 2 Yc_1 recombinant protein, lane 3 BSA, lane 4 Yc_1 double mutant E208D/H108Y, lane 5 Yc_1 mutant H108Y, lane 6 Yc_1 mutant E208D, lane 7 protein elution buffer. Blot 2: lane 1 Yc_2 recombinant protein, lane 2 Yc_1 recombinant protein, lane 3 BSA, lane 4 wildtype Yc_2 (prepared in the same manner as the Yc_2 mutant proteins), lane 5 Yc_2 mutant D208M, lane 6 Yc_2 mutant Y108L, lane 7 double Yc_2 mutant D208M/Y108L, lane 8 protein elution buffer (neat).

Figure 7.14 Immunohistochemical staining for GST Yc_2 compared to GST Yc_1/ Yc_2/ Ya in liver and kidney serial sections from rats fed AFB_1. The symbol >> or > indicates the same point on each of the sections.

a) GST Yc_2 (monoclonal) staining of rat liver seventy-seven weeks following treatment with AFB_1 (4 ppm), demonstrating much more specific staining to the left of the main portal tract located in the centre of the section. Magnification x4.

b) GST Yc_1 / Yc_2 (polyclonal) staining of rat liver seventy-seven weeks following treatment with AFB_1 (4 ppm). Magnification x4.

c) GST Yc_2 (monoclonal) staining of rat liver seventy-seven weeks following treatment with AFB_1 (4 ppm), demonstrating much more specific staining to the left of the main portal tract located in the centre of the section. Magnification x20.

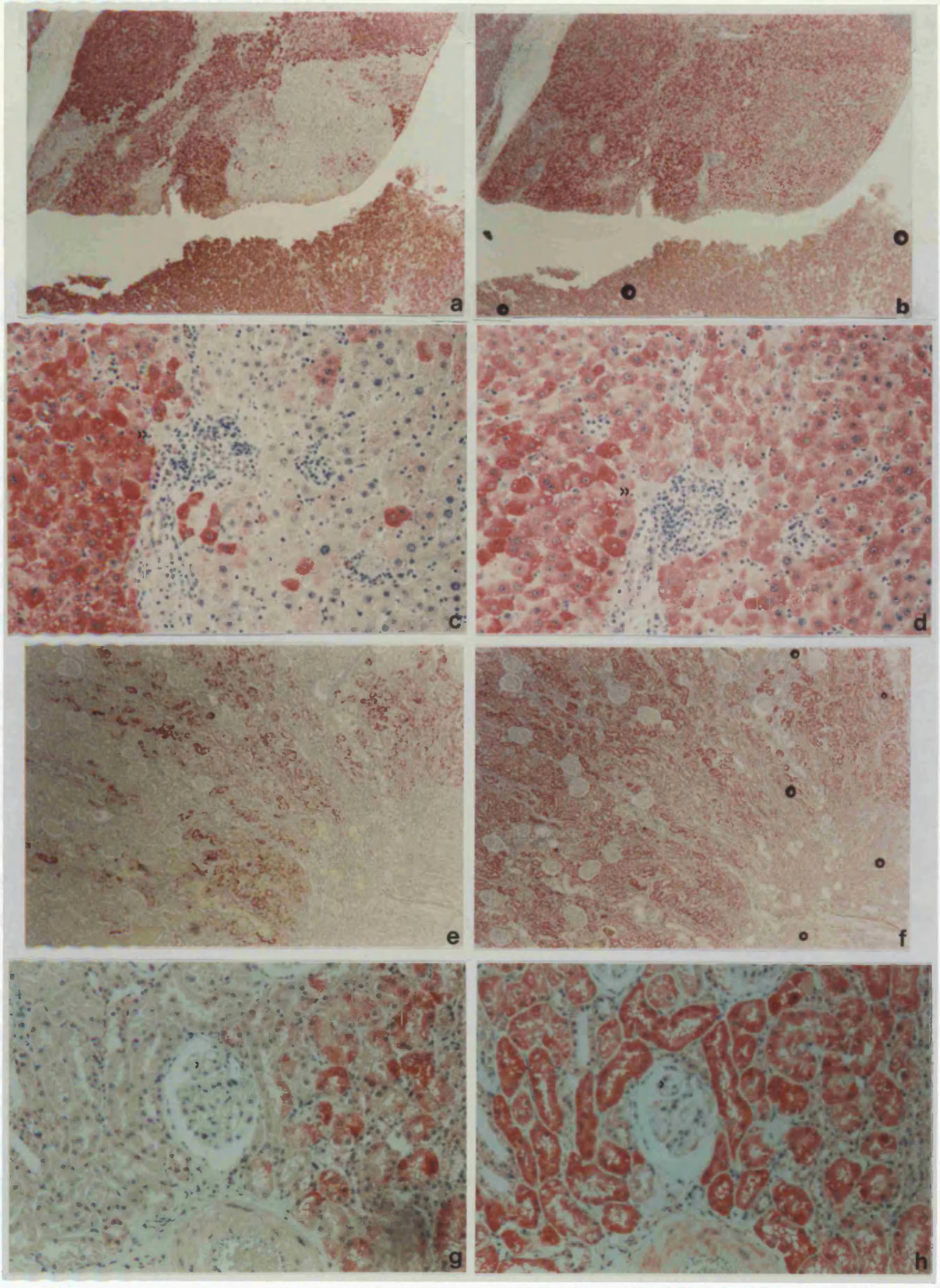
d) GST Yc_1 / Yc_2 (polyclonal) staining of rat liver seventy-seven weeks following treatment with AFB_1 (4 ppm). Magnification x20.

e) GST Yc_2 (monoclonal) staining of rat kidney seventy-seven weeks following treatment with AFB_1 (4 ppm). Magnification x4.

f) GST Yc_1/ Yc_2 (polyclonal) staining of rat kidney seventy-seven weeks following treatment with AFB_1 (4 ppm). Magnification x4.

g) GST Yc_2 (monoclonal) staining of rat kidney seventy-seven weeks following treatment with AFB_1 (4 ppm), demonstrating much more specific staining to the right of the section. Magnification x20.

h) GST Yc_1/ Yc_2 (polyclonal) staining of rat kidney seventy-seven weeks following treatment with AFB_1 (4 ppm). Magnification x20.


a
b
c
d
e
f
g
h

7.4 Summary and Discussion

The production of a rat GST Yc_2 antibody recognising with great specificity only the GST Yc_2 protein and not GST Yc_1 protein was very successful. Characterisation of this antibody has revealed an antibody of great versatility which can successfully and with ease be used to specifically identify the rat GST Yc_2 protein by using ELISA or slotblotting experimental methods. It can also accurately identify regions of acetone fixed sections which contain the rat GST Yc_2 protein using immunohistochemistry. Although the antibody is suitable for use in western blotting procedures optimisation of the technique may be necessary to obtain sensitive reproducible results. The isotype of the antibody proved to be an IgG class of immunoglobulin which is found in significantly higher concentrations than IgM, in plasma.

The ascitic fluid proved to have very high avidity for the GST Yc_2 protein but the higher level of background non-specific binding seen even at quite dilute concentrations suggested the supernatant to be more suitable for the method of slotblotting in which higher levels of protein were used. However the ascitic fluid was found to be sufficient for the immunohistochemistry experiments in very dilute concentrations. Purification of the ascites is required to remove the interfering components to eliminate the non-specific background.

In the Western blotting experiments the band obtained using the Yc_2 antibody representing the Yc_2 protein (M_r 25,800) appeared almost as a doublet with a decreasing tail, this remained consistent throughout the experiments. This is an artefact which occurs commonly in protein electrophoresis and the reasons for this are unsure but there are a few possible explanations. If there was degradation of the protein samples this would appear as a smear or multiple bands, perhaps of less intensity, below the major band. Alternatively if the level of reducing agent, that is, 2-mercaptoethanol, was insufficient in the sample buffer the reformation of some of the disulphide bridges may have occurred resulting in the presence of bands of different size after electrophoresis. Some proteins would be more susceptible to this effect than others depending on their amino acid content. The other less likely possible explanation is that it may be an artefact of the electrophoresis, if the protein sample runs obliquely in the gel it would give the appearance of a doublet with this tailing effect. Three bands were present in the rat liver cytosols when the polyclonal Yc_1/Yc_2 antibody was used as a probe. This antibody recognised the Yc_1 (M_r 27,500) and Yc_2 (M_r 25,800) recombinant proteins and also the Ya protein (M_r 25,500) (Fig 7.10, lanes 7 and 8). These results are in accordance with those reported by Hayes *et al.*, in 1991(a).

The Western blots demonstrating the level of Yc_2 present in untreated rat cytosols (Figs 7.9 and 7.10) were compared to those seen by Hayes *et al.*, in 1994. They demonstrated a level of Yc_2 protein present in foetal and immature untreated male rat

hepatic cytosol which decreased gradually until it became completely undetectable, by ten weeks of age. The results obtained in this study correlate well with this decrease in level of Yc_2 protein, being detected only at extremely low levels in the untreated animal at twenty-five weeks of age. The relative level of intensity of staining for Yc_2 in this study in the untreated animal at six weeks of age however appears to be greater than that seen in the study by Hayes *et al.,* as by seven weeks of age in their study it is barely detectable. This may indicate the later maturation of the animals in this study and hence prolonged retention of enzymes such as GST Yc_2.

From the results shown it would appear the Yc_2 monoclonal antibody does not recognise the peptide (which was synthesised and used as an immunogen see section 2.11.1) and therefore in all probability does not have its epitope in this region. This is based on the assumption however that the peptide conjugated to KLH has been presented to the antibody in the secondary structural form in which it is present in the native Yc_2 protein. Further experiments need to be carried out perhaps using cleaved fractions of Yc_2 to determine the region of antigenicity. Recently several chimeric proteins have become available, containing both the mouse Yc and rat Yc_1 proteins in varying proportions, from the laboratory of Dr D Eaton, Seattle, Washington, USA . These proteins would more accurately indicate the area containing the epitope recognised by the Yc_2 antibody as the oligopeptides are present in their native form.

The specific activities of the mutant Yc_1 and Yc_2 proteins for the conjugation of AFB_1-epoxide were analysed and preliminary experiments showed that the mutations in Yc_2 were successful in knocking-out the AFB_1 conjugating activity that was present but the mutations in Yc_1 failed to induce any activity (data obtained from Mr P McDonagh, personal communication). In the experiments carried out using the mutant proteins the Yc_2 antibody showed affinity for the wild type and all of the mutant Yc_2 proteins but no affinity for the Yc_1 wild type or mutant Yc_1 proteins. These results indicated that although in the case of the Yc_2 mutant proteins the specific conjugating activity was reduced, this was not reflected in a decreased affinity of the Yc_2 antibody for the protein. One conclusion would be that the epitope recognised by the anti Yc_2 antibody was not in an area affected by these mutations and therefore not in an area necessary for either AFB_1-epoxide binding or conjugation. However mutations in this area in Yc_1 did not induce conjugating activity and so the significance of these results in terms of enzymatic activity remain unclear. As there was affinity of the Yc_2 antibody for the Yc_2 proteins mutated at positions 108 and 208 indicating the epitope to be outside this area it is perhaps not surprising the antibody did not bind to the Yc_1 proteins mutated at the same positions. Determination of binding to the mouse Yc could also be determined as it too has high conjugating activity toward the AFB_1-epoxide.

Immunohistochemical staining using both the GST Yc_1/ Yc_2 polyclonal antibody and the GST Yc_2 monoclonal antibody indicates the monoclonal antibody to be a suitable specific antibody for use in determining the expression of GST Yc_2 both in liver and kidney. The difference in specific staining patterns indicating expression was most obvious in liver and kidney sections undergoing the carcinogenic process seventy-seven weeks following exposure to AFB_1. The expression GST Yc_2 during other stages of the study are presented in Chapter five.

Conclusion

"Read not to contradict and confute, nor to believe and take for granted, nor to find talk and discourse, but to weigh and consider."

Francis Bacon (1561-1626)

Conclusion

This part of the thesis is divided into two sections; section one summarises the objectives and results of the study with emphasis on the original aspects of the work followed by section two, which discusses the present findings in the wider context of the mechanism of aflatoxin-induced hepatocarcinogenesis.

8.1 Section I - Review

This study achieved its aims of examining changes in cellular and genetic expression during the stages of hepatocarcinogenesis using AFB_1 exposure as a model, in male Fischer rats. The model employing a stop exposure protocol was designed such that any regression of lesions were also examined. The approach usually adopted is an examination of rat livers continuously exposed to AFB_1, from very early stages of hepatocarcinogenesis through to final tumours. The regression model used in the present study has received limited previous attention. To achieve the objectives of this study new enzymatic markers, GST Yc_2 and aldehyde reductase, which have been suggested as having an important role in the metabolism of AFB_1 were used. The lack of a specific antibody to detect one of these enzymes, GST Yc_2, prompted efforts to produce a specific monoclonal antibody to detect this enzyme which proved to be successful. The results of the analyses provided novel data on the expression of these enzymes in livers exposed to AFB_1. In parallel to the studies on expression of Yc_2 and aldehyde reductase which is probably involved in the mechanism of resistance in the livers of the AFB_1-exposed animals, the levels of AFB_1 adducts resulting from dietary exposure or to injection of [^{3}H]AFB_1 were measured. These experiments provided data correlating the biochemical, genetic and mechanistic aspects of the induction of resistance to cytotoxicity present in the livers of the animals exposed to AFB_1. A unique aspect of the present study was the attempt to correlate the biochemical and genotypic profiles of the livers of animals from early exposure through to tumour production.

The progression of the successive stages in hepatocarcinogenesis were demonstrated using this model in several ways. Also the amount of AFB_1 consumed was more than sufficient to produce a toxic effect as reported in previous studies (Table 3.4) (Wogan and Newberne, 1967; Butler and Barnes, 1968; Godoy *et al.*, 1976). There was strong evidence of bile duct proliferation, an acute toxic effect of exposure to AFB_1. This was readily demonstrated immunohistochemically by the expression of GGT and CK-19 in the proliferated bile duct epithelial cells. In addition, the presence of an early toxic response was indicated by the reduction in body weight gain of the test animals compared to the control animals almost immediately following exposure to AFB_1. Although the difference in body weight gain was not statistically different over the period of the twelve week exposure when compared to the control animals it was accompanied by indications of toxicity; macroscopic lesions were evident from week three which progressed to large nodules by week eleven and

week eleven and tumours by week seventy-seven. The immunohistochemical staining experiments indicated the presence of single hepatocytes as well as foci of altered hepatocytes expressing phase II enzymes not expected to be present in parenchymal cells as early as one week following exposure to AFB_1. The progression of these lesions subsequently involving the expression of additional enzymes with increasing time of exposure to AFB_1. As each additional enzyme was expressed the phenotypic similarity of these lesions to that seen in the final tumours was increased (Table 5.1). However the expression of some of the proteins examined that is, GGT and CK-19 in the foci of altered hepatocytes was delayed or altered in this study when compared to previous studies. That binding of AFB_1 to nucleic acids in the liver had taken place was demonstrated by measuring the level of AFB_1 adducts present in nucleic acids extracted from animals exposed to AFB_1, although this level of binding was somewhat lower than expected in the initial stages of the study (Figure 4.6). While all the evidence suggests superficially the expected toxic response as a result of exposure to AFB_1, the lower initial level of AFB_1 binding to nucleic acids in the liver, the lack of a significant difference in body weight gain between the control and test animals and the anomalies in expression of certain enzymes suggests this toxic response to be in fact less marked than expected given the level of AFB_1 administered.

8.2 Section II – Final Conclusion

One of the most interesting outcomes of this project was the contrast between some of the results in this AFB_1 feeding study compared with those previously reported. This is especially true of one of the major objectives of the study which was to examine the relationship between the expression of certain enzymes as early markers of carcinogenesis and the presence of genetic abnormalities in the c-*ras* oncogene family and the *p53* tumour suppressor gene, previously indicated to be of importance in the process of AFB_1 induced hepatocarcinogenesis. An observation which has been discussed earlier in the relevant sections of this thesis is the late appearance of tumours in this study despite an exposure to AFB_1 greater than that previously reported as inducing tumours in a much shorter period of time. Previous dose-response studies have suggested that increasing the dose of AFB_1 administered decreased the latency for tumour development (Wogan *et al.*, 1974). What are the possible hypotheses for the differences in the results of the present and previous studies? One hypothesis is strongly supported throughout the thesis by certain findings. The phenotypic profile of the livers of the animals used in this study, at the time of initial exposure to AFB_1, showed a higher level of glutathione S-transferase than the animals used previously in the same laboratory before 1994 as indicated by the increased level of GSH-AFB_1-epoxide conjugate (Figure 5.12). Immunohistochemical analysis of GST Yc_2 also supported this finding by demonstrating the expression of this enzyme in both parenchyma and bile duct epithelia of animals up to seven weeks of age which was in contrast to a study by Hayes *et*

al., (1994) where the disappearance of GST Yc_2 expression from the parenchyma as early as five weeks of age was reported. Aldehyde reductase was also shown to be expressed at a higher level for a greater length of time in the animals used in this study compared to previous studies (Hayes *et al.,* 1993). In view of the involvement of these enzymes in the detoxification of AFB_1, their presence at the early stages of the study would be expected to have a profound effect on the progression of AFB_1 induced carcinogenesis.

Measurement of the level of AFB_1 adducts formed during the initial weeks of exposure to AFB_1 demonstrated a delay in accumulation of adducts until the third week of exposure when a sharp increase in AFB_1-adduct levels took place (Figure 4.6). Since the level of adducts would have been expected to be highest at the initial stages of the study these data support the presence of a resistance mechanism, due to the high prevailing levels of GST 7-7, GST Yc_2 and AR in the liver parenchyma, preceding insult by AFB_1.

Analysis of genetic mutations in the cellular oncogenes c-Ki and N-*ras* failed to find mutations in codons 12/13 and 61 at any stage from the earliest foci to the hepatocellular carcinomas in this study. These mutations have previously been reported as occurring frequently in the livers of rats exposed to AFB_1 (McMahon *et al.,* 1986; Sinha, 1988; Soman and Wogan, 1993)(Table A3.1). However the considerable variation in reported experimental results raises doubts as to whether these mutations are prevalent events in the process of all AFB_1-induced hepatocarcinogenesis, certainly they cannot be an essential component of AFB_1–induced rodent hepatocarcinogenesis. Since it is evident that mutations in c-Ki- or N-*ras* genes are not essential in AFB_1-induced hepatocarcinogenesis in the rat it can be concluded that different mechanisms of malignant transformation must be possible in the same model of carcinogenesis. In the case of the study presented in this thesis while AFB_1 remained a complete carcinogen, the mechanism by which carcinogenesis occurred may have been altered by the resistant status of the animals during the initial period of exposure to AFB_1. The major differences between the study presented here and some reported studies where c-*ras* mutations have been identified are the method of administration, dietary exposure compared to intraperitoneal injection and the level of AFB_1 exposure 4.12 mg compared to 1.0 mg, respectively (Soman and Wogan, 1993; McMahon *et al.,* 1986).

It is intriguing to compare the similarity of the lack of c-*ras* mutations demonstrated in this rodent study to the low incidence of these mutations reported in human hepatocellular carcinoma (Challen *et al.,* 1992; Tada *et al.,* 1990, Nikolaidou *et al.,* 1993 and Leon *et al.,* 1995). In particular the study by Leon *et al.,* 1995 who reported no mutations in codons 12,13 and 61 of all three *ras* genes in South African people despite the fact that dietary exposure to AFB_1 is a risk factor in this population. The carcinogenic protocol used in this study may have greater similarity to the chronic low level exposure to AFB_1 seen in humans and may indicate the involvement of mechanisms of carcinogenesis not involving mutations in c-*ras* genes in both cases. The fact that they do not exactly parallel each other however is indicated by the

failure to find *p53* codon 243 mutations in the liver tumours produced in this rodent study.

Consequently while an acute toxic response to AFB_1 exposure occurred the carcinogenic effect of AFB_1 appears to have been delayed and even possibly changed by the presence of an enhanced AFB_1-conjugating ability of the animals, due to the high levels of phase II enzymes present in the liver during the initial exposure to AFB_1. It would appear the level and period of AFB_1 administration was sufficient to induce initiation but the promotional effects were delayed and/or reduced. The animal model of carcinogenesis presented in this thesis in provoking a limited delayed carcinogenic response may therefore provide a more realistic model to use if the results are to be extrapolated to carcinogenic processes occurring in humans than protocols involving extensive liver damage due the use of high levels of carcinogen.

A novel area of work examined the expression of a new aldehyde reductase which metabolises the AFB_1-dihydrodiol, in the livers of AFB_1 exposed animals. The results indicated a strong correlation in the expression of AR with the expression of GST 7-7, an early marker of carcinogenesis. Both proteins were induced as an early response in the form of single positively stained cells as determined by immunohistochemistry with the relevant antibodies (Figures 5.2 and 5.4). The presence of these single positive cells were soon replaced, in the case of both proteins, with minifoci and eventually foci of altered hepatocytes. A calculation of the numbers of foci per area of liver, stained using the appropriate antibodies for GST 7-7 and AR, also demonstrated a very close correlation for each of the respective proteins (Figure 5.10). These results strongly indicate a similar mechanism of control for both of these proteins. The AR enzyme, dissimilar from any previously described reductase, is known to be inducible by antioxidants including ethoxyquin (Hayes *et al.,* 1993). What is not clear in the absence of further work is whether the expression of this novel AR is a specific response to the exposure of AFB_1 or whether it is a more general response to toxic and carcinogenic insult as is the case in the expression of GST 7-7. Further studies identifying the structure of the AR genes and examining the regulation of expression of AR in alternative models of hepatocarcinogenesis will reveal whether transcriptional control similarities existing between GST 7-7 and AR.

Whilst the importance of the inducible enzyme GST Yc_2 in the metabolism of AFB_1 in rat liver has been reported by Hayes *et al.,* (1991), no previous study has followed its pattern of induction using immunohistochemistry to accurately locate the enzyme in the pathology of hepatocellular carcinoma. This has been partly due to the lack of a specific antibody capable of distinguishing between the inducible GST Yc_2 and the constituitively expressed GST Yc_1. As part of this study a very specific antibody recognising only the GST Yc_2 protein was produced. This antibody was found to be suitable for use in the experimental methods of ELISA, slotblotting and immunohistochemistry. Using this antibody the present study revealed GST Yc_2 positive foci of altered hepatocytes in AFB_1 treated liver sections following

eight weeks of treatment. This pattern of expression was in contrast to that of GST 7-7 and AR which showed expression in single cells as early as the second week following treatment with AFB_1. It is likely both of these proteins, GST 7-7 and AR, are induced as an early general protective response following insult to a toxic compound and it would appear the induction of GST Yc_2 is also as a protective response. However the data also suggests that these enzymes are not co-ordinately regulated. A publication by Hayes *et al.,* (1996) examining the regulation of GST's and AR by antioxidants supports this observation as they describe a variation in the relative increase in hepatic GST Yc_2 and AR following treatment of rats with certain chemoprotectors. Manson *et al.,* (in press) describes a co-ordinate expression of phase II drug metabolising enzymes following exposure to certain chemoprotectors with a concurrent decrease following removal of the inducing agent. These findings imply different regulatory mechanisms controlling the expression of these genes depending on the inducing agent. This is likely to be through the antioxidant response element in the case of chemoprotectors but in the case of AFB_1 different control mechanisms are being activated which are not switched off following removal of the carcinogen. In light of the low constituitive ability of humans to conjugate the AFB_1–epoxide (Raney *et al.,* 1992), as in the rat, it raises the question as to whether there is an inducible GST in humans with high specificity for AFB_1. Such an inducible GST would presumably have high homology to the mouse Yc or rat Yc_2 GST's and it may be possible to utilise the specific antigenic determinant site present on the rat GST Yc_2 antibody to identify it. This is a subject requiring further investigation.

Appendix One

Statistical Formulae

NOTE: The Minitab®[1] system was used to carry out the analysis of variance and correlation statistics.

A1.1 Analysis of Variance

Analysis of variance was used to compare the means of two populations, assuming a random sample from each population. The AOVONEWAY minitab command was used. The first part of the output is an analysis of variance table: DF = degrees of freedom, SS = sum of squares, MS = mean of squares, F = F-ratio (equivalent to the students t-test) and P = calculated from the tables - if $p < 0.05$ the null hypothesis is rejected. The second part of the output indicates: N = number of values, MEAN = sample mean, STDEV = standard deviation. A plot of the 95 percent confidence intervals is also drawn for the means based on pooled standard deviation.

A1.2 Correlation

The correlation values were calculated to measure the association between two sets of variables. The CORRELATION minitab command was used. The measure of correlation used was the Pearson product moment correlation coefficient, designated *r*. The correlation coefficient is positive if *y* tends to increase as *x* increases. Conversely, the correlation is negative if *y* tends to decrease as *x* increases. If all points fall exactly on a straight line, then if $r = +1$ the slope points upwards and if $r = -1$ the slope points downwards. If there is no association between *x* and *y* then *r* will be near 0.

A1.3 Number of Adducts per mmole DNA

A1.3.1 Quenching factor

The quenching factor of [^{3}H]AFB$_1$ was determined using an external tritium standard:

$$\frac{170{,}642 \text{ cpm}}{277{,}700 \text{ dpm}} \times \frac{100}{1} = 61\%$$

A1.3.2 Converting counts to dpm

$$\frac{100}{\text{Quenching factor}} \times \frac{\text{cpm}}{1} = \text{dpm}$$

[1] Minitab is a registered trademark of Minitab, Inc.

A1.3.3 Number of molecules of AFB_1 to DNA

A. Factors

- Specific activity of $[^3H]AFB_1$ = 18 Ci/mmole (Moravek, data product sheet)
- 1 μCi = 2.2×10^6 dpm
- 50 μg/ml DNA = 7.87×10^{-11} moles/ml (Sambrook, 1989)
- AFB_1 = M_w 312.3 g

B. Formula

i) Convert sample counts to dpm

ii) $\frac{1}{2.2 \times 10^6 \text{ dpm}} \times \frac{\text{sample dpm}}{1}$ = number of μCi in sample

iii) Convert μCi to Ci (*i.e.* 10^6)

iv) $\frac{1}{18 \text{ Ci / mmole}} \times \frac{\text{Ci in sample}}{1}$ = number of AFB_1 molecules (mmole) in x μg of nucleic acid

v) Calculate number of pmole (*i.e.* 10^{-12}) of AFB_1 adducts in x μg of nucleic acid

vi) Convert AFB_1 adducts pmole to pg (multiply by 312.2)

vii) Calculate number of AFB_1 adducts pg per mg nucleic acid

$$\frac{AFB_1 \text{ molecules (pg)}}{\text{nucleic acid molecules (μg)}} \times \frac{1000}{1} = AFB_1 \text{ adducts (pg) per mg nucleic acid}$$

Appendix Two

Raw data from AFB_1 adduct experiments

Table A2.1

Raw data values for competitive ELISA experiments to quantitate the total number of AFB_1 nucleic acid adducts present in animals chronically fed AFB_1 (4 ppm). Standard deviation is indicated in brackets.

Sample	AFB_1 adducts pmoles/mg nucleic acid	AFB_1 adducts /mmole nucleic acid	AFB_1 adducts pg/mg nucleic acid
Negative and Positive Controls			
16	0.695 (0.27)	4.4×10^{-4} (1.7×10^{-4})	218.5 (83.8)
20	0.51 (0.34)	3.2×10^{-4} (2.2×10^{-4})	159 (105.7)
HS	0.67 (0.39)	3.2×10^{-4} (2.5×10^{-4})	209 (121.2)
RK	0.455 (0.21)	2.9×10^{-4} (1.3×10^{-4})	142.5 (64.5)
EQ	0.455 (0.34)	2.9×10^{-4} (2.2×10^{-4})	142.5 (108)
Aden	0.39 (0.23)	2.5×10^{-4} (1.5×10^{-4})	123 (72)
Guan	0.465 (0.29)	2.9×10^{-4} (1.8×10^{-4})	146 (91)
PC	6.61 (0)	4.2×10^{-3} (0)	2065 (0)
Test Animals Number of Weeks on AFB_1 Diet			
1	1.55 (0.45)	9.9×10^{-4} (2.9×10^{-4})	485 (140)
2	1.64 (0.36)	1.0×10^{-3} (2.3×10^{-4})	512.5 (113)
3	3.34 (0.94)	2.1×10^{-3} (5.9×10^{-4})	1043 (293)
4	3.19 (0.70)	2.0×10^{-3} (4.5×10^{-4})	995 (220)
5	2.67 (0.27)	1.7×10^{-3} (1.7×10^{-4})	835 (85)
6	1.46 (0.18)	9.3×10^{-4} (1.1×10^{-4})	455 (55)
7	1.39 (0.48)	8.9×10^{-4} (3.1×10^{-4})	435 (150)
8	2.56 (1.00)	1.6×10^{-3} (6.4×10^{-4})	800 (330)
9	0.9 9 (0.30)	5.7×10^{-4} (1.9×10^{-4})	280 (93)
10	0.79 (0.40)	5.0×10^{-4} (2.5×10^{-4})	248 (125)
11	0.64 (0.40)	4.1×10^{-4} (2.5×10^{-4})	200 (130)
12	0.98 (0.77)	6.2×10^{-4} (4.9×10^{-4})	305 (240)

Sample	AFB_1 adducts pmoles/mg nucleic acid	AFB_1 adducts /mmole nucleic acid	AFB_1 adducts pg/mg nucleic acid
Control Animals			
Number of Weeks on AFB_1 Study			
1	0.33 (0.11)	2.1×10^{-4} (7×10^{-5})	102 (33.8)
2	0.30 (0.13)	1.9×10^{-4} (8.3×10^{-5})	95 (40)
3	0.34 (0.09)	2.2×10^{-4} (5.7×10^{-5})	108 (27.5)
4	1.14 (0.86)	7.3×10^{-4} (5.5×10^{-4})	355 (270)
5	0.93 (0.43)	5.9×10^{-4} (2.7×10^{-4})	290 (135)
6	0.78 (0.01)	4.9×10^{-4} (6.4×10^{-6})	245 (3.75)
7	0.99 (0.19)	6.3×10^{-4} (1.2×10^{-4})	310 (60)
8	0.56 (0.21)	3.6×10^{-4} (1.3×10^{-4})	175 (65)
9	0.98 (0.43)	6.2×10^{-4} (2.7×10^{-4})	305 (135)
10	0.32 (0.06)	2.0×10^{-4} (3.8×10^{-5})	100 (19.5)
11	0.34 (0.07)	2.2×10^{-4} (4.5×10^{-5})	105 (22)
12	0.56 (0.10)	3.6×10^{-4} (6.4×10^{-5})	175 (33)

Table A2.2

Amount of [^{3}H]AFB_1 (μg/kg) injected per animal on the AFB_1 study, two hours before cull. Amount of radioactivity (μCi) injected per animal is indicated in brackets.

Number of weeks on AFB_1 study	Test animals	Control animals
3	17.4 (100)	not injected
4	30.6 (176)	36.7 (211)
5	36.7 (211)	not injected
6	34.6 (199)	47 (270)
7	43.3 (249)	not injected
8	43.3 (249)	not injected
9	43 (246)	42.6 (245)
10	44.5 (256)	not injected
11	48.4 (278)	not injected
12	50.3 (289)	49.2 (283)

Appendix Three

Protein expression and *ras* and p53 analysis results

Table A3.1 Biochemical pattern of foci and tumours using immunohistochemical staining and *ras* and *p53* gene mutation analysis

Focus/Tumour (all tissues are liver except where indicated)		GST 7.7	AR	Yc_1/Yc_2 (polyclonal)	Yc_2 (monoclonal)	CK-19	P450	GGT	N-ras codon 12/13 (ggt/ggc)	N-ras codon 61 (caa)	Ki-ras codon 12 (ggt)	Ki-ras codon 61 (caa)	p53 codon 243 (agg)
ARCHIVAL TUMOUR AND CONTROL TISSUE													
Nude mice tumours													
JB1	1	[1]+	+	+	[2]nd	weakly+	+	weakly+/-	ggt/ggc	caa	g(g/a)t	caa	nd
JB1	2	+	+	+	nd	weakly+	+	weakly+/-	ggt/ggc	caa	g(g/a)t	caa	nd
JB1	3	+	+	+	nd	weakly+	+	weakly+/-	ggt/ggc	caa	g(g/a)t	caa	nd
JB1	4	+	+	+	nd	weakly+	+	weakly+/-	ggt/ggc	caa	g(g/a)t	caa	nd
BL10	1	+	+/-	+	nd	+	-	+	ggt/ggc	caa	ggt	caa	nd
BL10	2	+	+/-	+	nd	+	-	+	ggt/ggc	caa	g(g/a)t	caa	nd
Archival tumour tissue (HTT)													
HTT	1	+	+	+	nd	weakly+	+	weakly+	ggt/ggc	caa	ggt	caa	nd
HTT	2	+	+	+	nd	-	+	weakly +/-	ggt/ggc	caa	ggt	caa	nd
HTT	3	+	+	+	+	-	+	+	ggt/ggc	caa	ggt	caa	agg
HTT	3 Lung	+	+	+	+	-	+	+	ggt/ggc	caa	ggt	caa	agg

[1] + or - indicates positive or negative expression, respectively.
[2] nd = no data

Focus/Tumour (all tissues are liver except where indicated)		GST 7.7	AR	Yc_1/Yc_2 (polyclonal)	Yc_2 (monoclonal)	CK-19	P450	GGT	N-ras codon 12/13 (ggt/ggc)	N-ras codon 61 (caa)	Ki-ras codon 12 (ggt)	Ki-ras codon 61 (caa)	p53 codon 243 (agg)
Archival control tissue (HCT)													
HCT	1	[3]nad	nad	nad	nd	nad	nad	nad	ggt/ggc	caa	ggt	caa	agg
HCT	2	nad	nad	nad	nad	nad	nad	nad	ggt/ggc	caa	ggt	caa	nd
FOCI OF ALTERED HEPATOCYTES AND TUMOURS INDUCED BY AFB_1													
Foci of altered hepatocytes (FAH)													
FAH	1	+	+	+	nd	+	+	+	ggt/ggc	caa	ggt	caa	nd
FAH	2	+	+	+	nd	+/-	+/-	+	ggt/ggc	caa	ggt	caa	nd
FAH	3	+	+	+	nd	-	+/-	-	ggt/ggc	caa	ggt	caa	nd
FAH	4	+	+	+	nd	-	+	+	ggt/ggc	caa	ggt	caa	nd
FAH	5	+	+	+	nd	-	+	+	ggt/ggc	caa	ggt	caa	nd
FAH	6	+	+	+	nd	+	-	+	ggt/ggc	caa	ggt	caa	nd
FAH	7	+	+	+	nd	-	-	-	ggt/ggc	caa	ggt	caa	nd
FAH	8	+	+	+	nd	-	-	nd	ggt/ggc	caa	ggt	caa	nd
FAH	9	+	+	+	nd	+	+/-	+	ggt/ggc	caa	ggt	caa	nd
FAH	10	+	+	+	nd	+	+/-	+	ggt/ggc	caa	ggt	caa	nd
FAH	11	+	+	+	nd	+	-	+	ggt/ggc	caa	ggt	caa	nd
FAH	12	+	+	+	nd	-	-	+	ggt/ggc	caa	ggt	caa	nd

[3] nad = no abnormalities detected

Focus/Tumour (all tissues are liver except where indicated)		GST 7.7	AR	Yc_1/Yc_2 (polyclonal)	Yc_2 (monoclonal)	CK-19	P450	GGT	N-ras codon 12/13 (ggt/ggc)	N-ras codon 61 (caa)	Ki-ras codon 12 (ggt)	Ki-ras codon 61 (caa)	p53 codon 243 (agg)
FAH	13	+	+	+	nd	-	-	+	ggt/ggc	caa	ggt	caa	nd
FAH	14	+	+	+	nd	-	-	+	ggt/ggc	caa	ggt	caa	nd
FAH	15	+	+	+	nd	+	-	+	ggt/ggc	caa	ggt	caa	nd
FAH	16	+	+	+	nd	+	-	+	ggt/ggc	caa	ggt	caa	nd
FAH	17	+	+	+	nd	+	nd	+	ggt/ggc	caa	ggt	caa	nd
FAH	18	+	+	+	nd	-	-	+	ggt/ggc	caa	ggt	caa	nd
FAH	19	+	+	+	nd	-	-	+	ggt/ggc	caa	ggt	caa	nd
FAH	20	+	+	+	nd	-	-	+	ggt/ggc	caa	ggt	caa	nd
FAH	21	+	+	+	nd	+	-	+	ggt/ggc	caa	ggt	caa	nd
FAH	22	+	+	+	nd	+	-	+	ggt/ggc	caa	ggt	caa	nd
FAH	23	+	+	+	nd	+	-	+	ggt/ggc	caa	ggt	caa	nd
		Tumour tissue (TT)											
TT	1	+	+	+	+	-	+	+/-	ggt/ggc	caa	ggt	caa	agg
TT	1 Lung	+	+	+	+	-	+	+	ggt/ggc	caa	ggt	caa	agg
TT	2	+	+	+	+	+	+	+	ggt/ggc	caa	ggt	caa	agg
TT	3	+	+	+	+	+	+	+	ggt/ggc	caa	ggt	caa	agg
TT	4	+	+	+	+	+	+	+	ggt/ggc	caa	ggt	caa	agg
TT	5	+	+	+	+	-	-	+	ggt/ggc	caa	ggt	caa	agg

Focus/Tumour (all tissues are liver except where indicated)	GST 7.7	AR	Yc_1/Yc_2 (polyclonal)	Yc_2 (monoclonal)	CK-19	P450	GGT	N-ras codon 12/13 (ggt/ggc)	N-ras codon 61 (caa)	Ki-ras codon 12 (ggt)	Ki-ras codon 61 (caa)	p53 codon 243 (agg)
TT 6	+	+	+	+	+	+	+	ggt/ggc	caa	ggt	caa	agg
Control Tissue (CT)												
CT 1 ([4]45 wks)	nad	nad	nad	nad	nad	nad	nad	ggt/ggc	caa	ggt	caa	agg
CT 2 (49 wks)	nad	nad	nad	nad	nad	nad	nad	ggt/ggc	caa	ggt	caa	agg
[5]CT 3 (82 wks)	+foci	+foci	nad	+[6]bd +foci	nad	nad	nad	ggt/ggc	caa	ggt	caa	agg

[4] Indicates age of control animals in weeks.
[5] Two small (<10 cells) foci were noted in control animal 82 weeks of age.
[6] bd = bile duct area

Bibliography

1. Aguilar, F., Harris, C.C., Sun, T., Hollstein, M., and Cerutti, P. Geographic variation of p53 mutational profile in nonmalignant human liver. Science, *264*: 1317-1319, 1994.
2. Aoyama, T., Yamano, S., Guzelian, P.S., and Gelboin, H.V. Five of 12 forms of vaccinia virus-expressed human hepatic cytochrome P450 metabolically active aflatoxin B_1. Proc. Natl. Acad. Sci. USA, *87*: 4790-4793, 1990.
3. Asao, T., Buchi, G., Abdel-Kader, M.M., Chang, S.B., Wick, E.L., and Wogan, G.N. The structures of aflatoxins B and G_1. Journal of The American Chemical Society, *87*: 882-886, 1965.
4. Ashby, J., Lefevre, P.A., Burlinson, B., and Penman, M.G. An assessment of the in vivo rat hepatocyte DNA-repair assay. Mutation Research, *156*: 1-18, 1985.
5. Autrup, H., Seremet, T., and Wakhisi, J. Evidence for human antibodies that recognize an aflatoxin epitope in groups with high and low exposure to aflatoxins. Archives of Environmental Health, *45*: 31-34, 1990.
6. Avarameas, S. Coupling of enzymes to proteins with glutaraldehyde. Immunochemistry, *6*: 43-52, 1969.
7. Bailey, G.S. Role of aflatoxin DNA adducts in the cancer process. In: D.L. Eaton and J.D. Groopman (eds.), The Toxicology of Aflatoxins: human health, veterinary and agricultural significance, pp. 137-148, San Diego: Academic Press, INC. 1994.
8. Bailey, G.S., Williams, D.E., Wilcox, J.S., Loveland, P.M., Coulombe, R.A., and Hendricks, J.D. Aflatoxin B_1 carcinogenesis and its relation to DNA adduct formation and adduct persistence in sensitive and resistant salmonid fish. Carcinogenesis, *9*: 1919-1926, 1988.
9. Balmain, A. and Brown, K. Oncogene activation in chemical carcinogenesis. Advances in Cancer Research, *57*: 147-182, 1988.
10. Bannasch, P. and Zerban, H. Predictive value of hepatic preneoplastic lesions as indicators of carcinogenic response. In: H. Vaino, P.N. Magee, D.B. McGregor and A.J. McMichael (eds.), Mechanisms of Carcinogenesis in Risk Identification, pp. 389-427, International Agency for Research on Cancer: Lyon. 1992.
11. Bannasch, P. Preneoplastic lesions as end points in carcinogenicity testing. I. Hepatic preneoplasia. Carcinogenesis, *7*: 689-695, 1986.
12. Barbacid, M. *ras* GENES. Ann. Rev. Biochem. *56*: 779-827, 1987.
13. Barnes, J.M. and Butler, W.H. Carcinogenic activity of aflatoxin to rats. Nature, *202*: 10-16 1964.
14. Barrett, J.C. and Fletcher, W.F. Cellular and molecular mechanisms of multistep carcinogenesis in cell culture models. In: Mechanisms of environmental carcinogenesis, volume II, pp. 73-111, 1986.
15. Bauer-Hofmann, R., Buchmann, A., Wright, A.S., and Schwarz, M. Mutations in the Ha-*ras* proto-oncogene in spontaneous and chemically induced liver tumours of the CF1 mouse. Carcinogenesis, *11*: 1875-1877, 1990.
16. Blount, W.P. Turkey 'X' disease. Turkeys, 52-77, 1961.
17. Bos, J.L. *ras* oncogenes in human cancer: a review. Cancer Research, *49*: 4682-4689, 1988.

18. Bourne, J.A. Handbook of Immunoperoxidase staining methods. Dako Corporation, 1993.
19. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. analytical biochemistry, *72*: 248-254, 1976.
20. Bratton, A.C. and Marshall, E.K. A new coupling component for sulfanil-amide determination. J. Biol. Chem. *128*: 537-550, 1939.
21. Bressac, B., Kew, M., Wands, J., and Ozturk, M. Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. Nature, *350*: 429-431, 1991.
22. Buchmann, A., Bauer-Hofmann, R., Mahr, J., Drinkwater, N.R., Luz, A., and Schwarz, M. Mutational activation of the *c*-Ha-*ras* gene in liver tumors of different rodent strains: Correlation with susceptibility to hepatocarcinogesis. P. N. A. S. *88*: 911-915, 1991.
23. Buetow, K.H. Genetic studies of human primary hepatocellular carcinoma. Comparative Molecular Carcinogenesis, 155-172, 1992.
24. Burlison, B. An *in vivo* unscheduled DNA synthesis (UDS) assay in the rat gastric mucosa: preliminary development. Carcinogenesis, *10*: 1425-1428, 1989.
25. Busby, W.F. and Wogan, G.N. Aflatoxins. In: C.E. Searle (ed.), Chemical Carcinogens, pp. 945-1136, American Chemical Society. 1984.
26. Butler, W.H. and Barnes, J.M. Carcinogenic action of groundnut meal containing aflatoxin in rats. Food Cosmet Toxicol, *6*: 135-141, 1967.
27. Cabral, J.R.P. and Neal, G.E. The inhibitory effects of ethoxyquin on the carcinogenic action of aflatoxin B_1 in rats. Cancer Letters, *19*: 125-132, 1983.
28. Carthew, P., Edwards, R.E., Hill, R.J., and Evans, J.G. Cytokeratin expression in cells of the rodent bile duct cells developing under normal and pathological conditions. Br. J. Exp. Path. *70*: 717-725, 1989.
29. Cerutti, P., Hussain, P., Pourzzand, C., and Aguilar, F. Mutagenesis of the H-*ras* proto-oncogene and *p53* tumor suppressor gene. Cancer Research, *54*: 1934s-1938s, 1994.
30. Challen, C., Guo, K., Collier, J.D., Cavanagh, D., and Bassendine, M.F. Infrequent point mutations in codons 12 and 61 of ras oncogenes in human hepatocellular carcinomas. J. Hepatol, *14*: 342-346, 1992.
31. Chetsanga, C.J. and Frenette, G.P. Excision of aflatoxin B_1-imidazole ring opened guanine adducts from DNA by formamidopyrimidine-DNA glycosylase. Carcinogenesis, *4*: 997-1000, 1983.
32. Chu, N., Janckila, A.J., Wallace, J.H., and Yam, L.T. Assessment of a method for immunochemical detection of antigen on nitrocellulose membranes. The Journal of Histochemistry and Cytochemistry, *37*: 257-263, 1989.
33. Cotran, R.S., Kumar, V., and Robbins, S.L. The Liver and Biliary Tract. In: W.B.S. Staff (ed.), Robbins Pathologic Basis of Disease, pp. 911-980, Philadelphia: W.B. Saunders Company. 1989.
34. Coursaget, P., Depril, N., Chabaud, M., Nandi, R., Mayelo, V., LeCann, P., and Yvonnet, B. High prevalence of mutations at codon 249 of the p53 gene in hepatocellular carcinomas from Senegal. Br. J. Cancer, *67*: 1395-1397, 1993.
35. Croy, R.G. and Wogan, G. Temporal patterns of covalent DNA adducts in rat liver after single and multiple doses of aflatoxin B_1. Cancer Research, *41*: 197-203, 1981.

36. Croy, R.G., Essigmann, J.M., Reinhold, V.N., and Wogan, G.N. Identification of the principle aflatoxin B_1-DNA adduct formed *in vivo* in rat liver. P. N. A. S. *75*: 1745-1749, 1978.

37. Cullen, J.M. and Newberne, P.M. Experimental toxicology of aflatoxins. In: D.L. Eaton and J.D. Groopman (eds.), The Toxicology of Aflatoxins: human health, veterinary and agricultural significance, pp. 3-21, San Diego: Academic Press, INC. 1994.

38. D'Andrea, A.D. and Haseltine, W.A. Modification of DNA by aflatoxin B_1 creates alkali-labile lesions in DNA positions of guanine and adenine. P. N. A. S. *75*: 4120-4124, 1978.

39. Dashwood, R.H., Arbogast, D.N., Fong, A.T., Pereira, C., Handricks, J.D., and Bailey, G.S. Quantitative inter-relationships between aflatoxin B_1 carcinogen dose, indole-3-carbinol anti-carcinogen dose, target organ DNA adduction and final tumour response. Carcinogenesis, *10*: 175-181, 1989.

40. Davis, L.G. Preparation of DNA from eukaryotic cells: general method. Methods in Molecular Biology, *86*: 44-46, 1986.

41. Degen, G.H. and Neumann, H. The major metabolite of aflatoxin B_1 in the rat is a glutathione conjugate. Chem. Biol. Interactions, *22*: 239-255, 1978.

42. Dix, K.M. The development of hepatocellular tumours followed by aflatoxin B_1 exposure of the partially hepatectomised mouse. Carcinogenesis, 385-390, 1984.

43. Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K., and Mattick, J.S. 'Touchdown' PCR to circumvent spurious priming during gene amplification. N. A. R. *19*: 40081991.

44. Donehower, L.A. and Bradley, A. The tumor suppressor p53. Biochemica et biophysica, *1155*: 181-205, 1993.

45. Donnelly, P.J., Devereux, T.R., Foley, J.F., Maronpot, R.R., Anderson, M.W., and Massey, T.E. Activation of K-*ras* in aflatoxin B_1-induced lung tumours from AC3F1 (A/JXC3H/HeJ) mice. Carcinogenesis, *17*: 1735-1740, 1996.

46. Dumenco, L., Oguey, D., Wu, J., Messier, N., and Fausto, N. Introduction of a murine p53 mutation corresponding to human codon 249 into a murine hepatocyte cell line results in growth advantage, but not in transformation. Hepatology, *22*: 1279-1288, 1995.

47. Dwivedi, Y., Rastogi, R., Mehrotra, R., Garg, N.K., and Dhawan, B.N. Picroliv protects against aflatoxin B_1 acute hepatotoxicity in rats. Pharmocological Research, *27*: 189-199, 1993.

48. Eaton, D.L. and Gallagher, E.P. Mechanisms of aflatoxin carcinogenesis. Annu. Rev. Pharmacol. Toxicol. *34*: 135-172, 1994a.

49. Eaton, D.L., Ramsdell, H.S., and Neal, G.E. Biotransformation of aflatoxins. In: D.L. Eaton and J.D. Groopman (eds.), The Toxicology of Aflatoxins: human health, veterinary and agricultural significance, pp. 45-73, San Diego: Academic Press, INC. 1994b.

50. Egan, S.E. and Weinberg, R.A. The pathway to signal achievement. Nature, *365*: 781-783, 1993.

51. Ellman, G.L. Tissue sulfhydryl groups. Archives of Biochemistry and Biophysics, *82*: 70-77, 1959.

52. Essigmann, J.M., Croy, R.G., Nadzan, A.M., Busby, W.F., Reinhold, V.N., Buchi, G., and Wogan, G.N. Structural identification of the major DNA adduct formed by aflatoxin B_1 *in vitro*. Proc. Natl. Acad. Sci. *74*: 1870-1874, 1977.

53. Essigmann, J.M., Green, C.L., Croy, R.G., Fowler, K.W., Buchi, G.H., and Wogan, G.N.

Interactions of aflatoxin B_1 and alkylating agents with DNA: Structural and functional studies. Quant. Biol. *47*: 327-337, 1982.

54. Farber, E. Cellular biochemistry of the stepwise development of cancer with chemicals: G.H.A. Clowes memorial lecture. Cancer Research, *44*: 5463-5474, 1984.
55. Farber, E. The biochemistry of preneoplastic liver: a common metabolic pattern in hepatocyte nodules. Can. J. Biochem. Cell Biol. *62*: 486-494, 1984.
56. Feinberg, A.P. and Vogelstein, B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. analytical biochemistry, *132*: 6-13, 1983.
57. Feitelson, M.A., Zhu, M., Duan, L., and London, W.T. Hepatitis B X antigen and p53 are associated *in vitro* and in liver tissues from patients with primary hepatocellular carcinoma. Oncogene, *8*: 1109-1117, 1993.
58. Festing, M.F.W. A case for using inbred strains of laboratory animals in evaluating the safety of drugs. Fd. Cosmet. Toxicol. *13*: 369-375, 1975.
59. Forrester, L.M., Neal, G.E., Judah, D.J., Glancey, M.J., and Wolf, C.R. Evidence for involvement of multiple forms of cytochrome P-450 in aflatoxin B_1 metabolism in human liver. Proc. Natl. Acad. Sci. USA, *87*: 8306-8310, 1990.
60. Foster, P.L., Eisenstadt, E., and Miller, J.H. Base substitution mutations induced by metabolically activated aflatoxin B1. P. N. A. S. *80*: 2695-2698, 1983.
61. Franke, W.W., Mayer, D., Schmid, E., Denk, H., and Borenfreund, E. Differences of expression of cytoskeletal proteins in cultured rat hepatocytes and hepatoma cells. Experimental Cell Research, *134*: 345-365, 1981.
62. Fujimoto, K., Yamada, Y., Okajima, E., Kakizoe, T., Sasaki, H., Sugimara, T., and Terada, M. Frequent association of p53 gene mutation in invasive bladder cancer. Cancer Research, *52*: 1393-1398, 1992.
63. Fujimoto, Y., Hampton, L.L., Luo, L., Wirth, P., and Thorgeirsson, S.S. Low frequency of *p53* gene mutation in tumours induced by aflatoxin B_1 in nonhuman primates. Cancer Research, *52*: 1044-1046, 1992.
64. Fujimoto, Y., Hampton, L.L., Wirth, P.J., Wang, N.J., Xie, J.P., and Thorgeirsson, S.S. Alterations of tumour suppressor genes and allelic losses in human hepatocellular carcinomas in China. Cancer Research, *54*: 281-285, 1994.
65. Gallagher, E.P., Wienkers, L.C., Stapleton, P.L., Kunze, K.L., and Eaton, D.L. Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the bioactivation of aflatoxin B_1. Cancer Research, *54*: 101-108, 1994.
66. Gerbes, A.L. and Caselmann, W.H. Point mutations of the p53 gene, human hepatocellular carcinoma and aflatoxins. Journal of Hepatology, *19*: 312-315, 1993.
67. Godoy, H.M., Judah, D.J., Arora, H.L., Neal, G.E., and Jones, G. The effects of prolonged feeding with aflatoxin B_1 in adult rat liver. Cancer Research, *36*: 2399-2497, 1976.
68. Goplan-Kriczky, P., Hiruma, S., and Lotlikar, P.D. Effect of glutathione levels on aflatoxin B_1-DNA binding in livers and kidneys of male rats and hamsters pretreated with buthionine sulfoximine and diethylmaleate. Cancer Letters, *76*: 25-30, 1994.
69. Green, J.A., Carthew, P., Heuillet, E., Simpson, J.L., and Manson, M.M. Cytokeratin expression during AFB_1-induced carcinogenesis. Carcinogenesis, *11*: 1175-1182, 1990.

70. Haffner, R. and Oren, M.X. Biochemical properties and biological effects of p53. Current Opinion in Genetics and Development, *5*: 84-90, 1995.

71. Halver, J.E. Aflatoxicosis and trout hepatoma. In: L.A. Goldblatt (ed.), Aflatoxin: Scientific Background, Control and Implications, pp. 265-306, New York: Academic Press. 1969.

72. Halvorson, M.R. Aflatoxin B_1 hydroxylation by the pregnenolone-16□-carbonitrile-inducible form fo rat liver microsomal cytochrome P-450. Carcinogenesis, *9*: 2103-2108, 1988.

73. Hancock, D.C. and Evan, G.I. Production and Characterisation of Antibodies Against Synthetic Peptides. In: M.M. Manson (ed.), Immunochemical Protocols, pp. 33-42, Totowa: Humana Press. 1992.

74. Hanigan, M.H. and Pitot, H.C. Gamma-glutamyl transpeptidase- its role in hepatocarcinogenesis. Carcinogenesis, *6*: 165-172, 1985.

75. Harrison, D.J., May, L., Hayes, J.D., and Neal, G.E. Glutathione *S*-transferase localization in aflatoxin B_1-treated rat livers. Carcinogenesis, *11*: 927-931, 1990.

76. Hatch, M.C., Chen, C., Levin, B., Ji, B., Yang, G., Hsu, S., Wang, L., Hsieh, L., and Santella, R.M. Urinary aflatoxin levels, hepatitis-B virus infection and hepatocellular carcinoma in Taiwan. Int. J. Cancer, *54*: 931-934, 1993.

77. Hayashi, H., Sugio, K., Matsumata, T., Adachi, E., Urata, K., Tanaka, S., and Sugimachi, K. The mutation of codon 249 in the *p53* gene is not specific in Japanese hepatocellular carcinoma. Liver, *13*: 279-281, 1993.

78. Hayes, J.D., Judah, D.J., and Neal, G.E. Resistance to Aflatoxin B_1 is associated with the expression of a novel aldo-keto reductase which has catalytic activity towards a cytotoxic aldehyde-containing metabolite of the toxin. Cancer Research, *53*: 3887-3894, 1993.

79. Hayes, J.D., Judah, D.J., McLellan, L.I., and Neal, G.E. Contribution of the glutathione-*S*-transferases to the mechanisms of resistance to aflatoxin B_1. Pharmac. Ther. *50*: 443-472, 1991a.

80. Hayes, J.D., Judah, D.J., McLellan, L.I., Kerr, L.A., and Peacock, S.D. Ethoxyquin-induced resistance to aflatoxin B_1 in the rat is associated with the expression of a novel alpha-class glutathione *S*-transferase subunit, Yc_2, which possesses a high catalytic activity for aflatoxin B_1-8,9-epoxide. Biochem. J. *279*: 385-398, 1991b.

81. Hayes, J.D., Judah, D.J., Neal, G.E., and Nguyen, T. Molecular cloning and heterologous expression of a cDNA encoding a mouse glutathione *S*-transferase Yc subunit possessing high catalytic activity for aflatoxin B_1-8,9-epoxide. Biochem. J. *285*: 173-180, 1992.

82. Hayes, J.D., McLeod, R., Ellis, E.M., Pulford, D.J., Ireland, L.S., McLellan, L.I., Judah, D.J., Manson, M.M., and Neal, G.E. Regulation of glutathione *S*-transferases and aldehyde reductase by chemoprotectors: studies of mechanisms responsible for inducible resistance to aflatoxin B_1. IARC Scientific Publication, *139*: 175-187, 1996.

83. Hayes, J.D., Nguygen, T., Judah, D.J., Petersson, D.G., and Neal, G.E. Cloning of cDNAs from fetal rat liver encoding glutathione *S*-transferase Yc polypeptide. The Journal of Biological Chemistry, *269*: 20707-20717, 1994a.

84. Hertzog, P.J., Smith, J.R., and Garner, R.C. Characterisation of the imidazole ring-opened forms of *trans*-8, 9-dihydro-8-(7-guanyl)9-hydroxy aflatoxin B_1. Carcinogenesis, *3*: 723-725, 1982.

85. Hesketh, R. (ed.), *RAS*. In: The Oncogene Handbook, 1994.

86. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. p53 mutations in human cancers. Science, *253*: 49-53, 1991.

87. Hollstein, M.C., Wild, C.P., Bleicher, F., Chutimataewin, S., Harris, C.C., Sricatanaku, P., and Montesano, R. p53 mutations and aflatoxin B_1 exposure in hepatocellular carcinoma patients from Thailand. Int. J. Cancer, *53*: 51-55, 1993.

88. Hopp, T.P. and Woods, K.R. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA, *78*: 3824-3828, 1981.

89. Hornes, E. and Korsnes, L. Magnetic DNA hybridization properties of oligonucleotide probes attached to superparamagnetic beads and their use in the isolation of poly(A) mRNA from eukaryotic cells. GATA, *7*: 145-150, 1990.

90. Hsu, I.C., Metcalf, R.A., Sun, T., Welsh, J.A., Wang, N.J., and Harris, C.C. Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature, *350*: 427-428, 1991.

91. Hulla, J.E. and Schneider, R.P. Structure of the rat p53 tumor suppressor gene. N. A. R. *21*: 713-717, 1993.

92. Hulla, J.E., Chen, Z.Y., and Eaton, D.L. Aflatoxin B_1- induced rat hepatic hyperplastic nodules do not exhibit a site-specific mutation within the *p53* gene. Cancer Research, *53*: 9-11, 1993.

93. IARC Monographs. Evaluation of carcinogenic risk to humans. In: pp. 83-87, IARC Press. 1987.

94. Imazeki, F., Yokosuka, O., Ohto, M., and Omato, M. Aflatoxin and p53 abnormality in duck hepatocellular carcinoma. J. Gastroenterol. Hepatol. *10*: 646-649, 1995.

95. Iyer, R.S., Coles, B.F., Raney, K.D., Tier, R., Guengerich, F.P., and Harris, T.M. DNA Adduction by the Potent Carcinogen Aflatoxin B1: Mechanistic Studies. Journal of The American Chemical Society, *116*: 1603-1609, 1994.

96. Jakobsen, K.S., Breivold, E., and Hornes, E. Purification of mRNA directly from crude plant tissues in 15 minutes using magnetic oligo dT microspheres. Nucleic Acids Research, *18*: 36691990.

97. Judah, D.J., Hayes, J.D., Yang, J., Lian, L., Roberts, G.C., Farmer, P.B., Lamb, J.H., and Neal, G.E. A novel aldehyde reductase with activity towards a metabolite of aflatoxin B_1 is expressed in rat liver during carcinogenesis and following the administration of an anti-oxidant. Biochem. J. *292*: 13-18, 1993.

98. Kensler, T.W., Davis, E.F., and Bolton, M.G. Strategies for chemoprotection against aflatoxin-induced liver cancer. In: D.L. Eaton and J.D. Groopman (eds.), The Toxicology of Aflatoxins: human health, veterinary and agricultural significance, pp. 281-308, San Diego: Academic Press, INC. 1994.

99. Kensler, T.W., Egner, P.A., Davidson, N.E., Roebuck, B.D., Pikul, A., and Groopman, J.D. Modulation of aflatoxin metabolism, aflatoxin-N^7-guanine formation, and hepatic tumorigenesis in rats fed ethoxyquin: role of induction of glutathione *S*-transferases. Cancer Research, *46*: 3924-3931, 1986.

100. Kirby, G.M., Batist, G., Fotouhi-Ardakani, N., Nakazawa, H., Yamasaki, H., Kew, M., Cameron, R.G., and Alaoui-Jamali, M.A. Allele-specific PCR analysis of p53 codon 249 AGT transversion in liver tissues from patients with viral hepatitis. Int. J. Cancer, *68*: 21-25, 1996.

101. Kirby, G.M., Wolf, C.R., Neal, G.E., Judah, D.J., Henderson, C.J., Srivatanakul, P., and Wild,

C.P. *In vitro* metabolism of aflatoxin B_1 by normal and tumorous liver tissue from Thailand. Carcinogenesis, *14*: 2613-2620, 1993.

102. Kohler, G. and Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature, *256*: 495-497, 1975.
103. Krishnamachari, K.A.V.R., Bhat, R.V., Nagarajan, V., and Tilak, T.B.G. Hepatitis due to aflatoxicosis - An outbreak in Western India. The Lancet, 1061-1063, 1975.
104. Leadon, S.A., Tyrrell, R.M., and Cerutti, P.A. Excision repair of aflatoxin B_1-DNA adducts in human fibroblasts. Cancer Research, *41*: 5125-5129, 1981.
105. Leon, M. and Kew, M.C. Analysis of ras gene mutations in hepatocellular carcinoma in southern African blacks. Anticancer Research, *15*: 859-861, 1995.
106. Levine, A.J., Momand, J., and Finlay, C.A. The p53 tumour suppressor gene. Nature, *351*: 453-456, 1991.
107. Lieberman, M.W., Barrios, R., Carter, B.Z., Habib, G.M., Lebovitz, R.M., Rajagopalan, S., Sepulveda, A.R., Shi, Z., and Wan, D. g-Glutamyl Transpeptidase. American Journal of Pathology, *147*: 1175-1185, 1995.
108. Lilleberg, S.L., Cabonce, M.A., Raju, N.R., Wagner, L.M., and Kier, L.D. Alterations in the structural gene and the expression of *p53* in rat liver tumors induced by aflatoxin B_1. Molecular Carcinogenesis, *6*: 159-172, 1992.
109. Lord, P.G., Hardaker, K.J., Loughlin, J.M., Marsden, A.M., and Orten, T.C. Point mutation analysis of *ras* genes in spontaneous and chemically induced C57Bl/10J mouse liver tumours. Carcinogenesis, *13*: 1383-1387, 1992.
110. Macdonald, F. and Ford, C.H.J. Oncogenes and Tumor suppressor genes. BIOS Scientific Publishers Limited: Oxford, 1991.
111. Makarananda, K. Immunological detection of human exposure to aflatoxins. 1989.(Abstract)
112. Mandel, H.G., Judah, D.J., and Neal, G.E. Effect of dietary protein level on aflatoxin B_1 actions in the liver of weanling rats. Carcinogenesis, *13*: 1853-1857, 1992.
113. Mandel, H.G., Manson, M., Judah, D.J., Simpson, J.L., Green, J.A., Forrester, L.M., Wolf, C.R., and Neal, G.E. Metabolic basis for the protective effect of the antioxidant ethoxyquin on aflatoxin B_1 hepatocarcinogenesis in the rat. Cancer Research, *47*: 5218-5223, 1987.
114. Manson, M.M. Biphasic early changes in rat liver gamma glutamyl transpeptidase in response to aflatoxin B_1. Carcinogenesis, *4*: 467-472, 1983.
115. Marshall, M. The effector interactions of p21 ras. T. I. B. S. *18*: 250-254, 1993.
116. Mason, P.J., Enver, T., Wilkinson, D., and Williams, J.G. Assay of transcription *in vitro.* In: B.D. Hames and S.J. Higgins (eds.), Gene Transcription, pp. 5-64, Oxford: IRL Press. 1993.
117. Maxuitenko, Y.Y., MacMillan, D.L., Kensler, T.W., and Roebuck, B.D. Evaluation of the post-initiation effects of oltipraz on aflatoxin B1-induced preneoplastic foci in a rat model of hepatic tumorigenesis. Carcinogenesis, *14*: 2423-2425, 1993.
118. McCormick, F. How receptors turn Ras on. Nature, *363*: 15-16, 1993.
119. McGlynn, K.A., Rosvold, E.A., Lustbader, E.D., Hu, Y., Clapper, M.L., Zhou, T., Wild, C.P., Xia, X., Baffoe-Bonnie, A., Ofori-Adjei, D., Chen, G., London, W.T., Shen, F., and Buetow, K.H. Susceptibility to hepatocellular carcinoma is associated with genetic variation in the enzymatic detoxification of aflatoxin B1. Proc. Natl. Acad. Sci. USA, *92*: 2384-2387, 1995.

120. McLean, M. and Dutton, M.F. Cellular interactions and metabolism of aflatoxin: an update. Pharmac. Ther. *65*: 163-192, 1995.

121. McLellan, L.I., Judah, D.J., Neal, G.E., and Hayes, J.D. Regulation of aflatoxin B_1-metabolising aldehyde reductase and glutathione S-transferase by chemoprotectors. Biochemical Journal, *300*: 117-124, 1994.

122. McMahon, G., Davis, E.F., Huber, L.J., Kim, Y., and Wogan, G.N. Characterization of c-Ki-*ras* and N-*ras* oncogenes in aflatoxin B_1-induced rat liver tumors. Proc. Natl. Acad. Sci. USA, *87*: 1104-1108, 1990.

123. McMahon, G., Hanson, L., Lee, J., and Wogan, G.N. Identification of an activated c-Ki-*ras* oncogene in rat liver tumors induced by aflatoxin B_1. Proc. Natl. Acad. Sci. USA, *83*: 9418-9422, 1986.

124. Moll, R., Franke, W.W., and Schiller, D.L. The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell, *31*: 11-24, 1982.

125. Montesano, R., Bresil, H., Planche-Martel, G., Margison, G.P., and Pegg, A.E. Effect of chronic treatment of rats with dimethylnitrosamine on the removal of O6-methylguanine from DNA. Cancer Research, *40*: 452-458, 1980.

126. Moore, M., Nakagawa, K., Satoh, K., Ishikawa, T., and Sato, K. Single GST-P positive liver cells - putative initiated hepatocytes. Carcinogenesis, *8*: 483-486, 1987.

127. Moss, E.J., Manson, M.M., and Neal, G.E. Effect of manipulation of g-glutamyl transpeptidase levels on biliary excretion of aflatoxin B_1 conjugates. Carcinogenesis, *5*: 869-874, 1984.

128. Muench, K.F., Misra, R.P., and Humayun, M.Z. Sequence specificity in aflatoxin B_1-DNA interactions. P. N. A. S. *80*: 6-10, 1983.

129. Naftalin, L., Sexton, M., Whitaker, J.F., and Tracey, D. A routine procedure for estimating serum gamma-glutamyl-transpeptidase activity. Clinica Chimica Acta, *26*: 293-296, 1969.

130. Neal, G.E. and Judah, D.J. Effect of hypophysectomy and aflatoxin B_1 on rat liver. Cancer Research, *38*: 3460-3467, 1978.

131. Neal, G.E. Inhibition of rat liver RNA synthesis by Aflatoxin B_1. Nature, *244*: 432-435, 1973.

132. Neal, G.E., Manson, M.M., and Legg, R.F. The production of carcinomas *in vivo* by the intravenous or subcutaneous injection of a hepatocellular tumour cell line. Cancer Letters, *19*: 325-332, 1983.

133. Neal, G.E., Metcalfe, S.A., Legg, R.F., Judah, D.J., and Green, J.A. Mechanism of the resistance to cytotoxicity which precedes aflatoxin B_1 hepatocarcinogenesis. Carcinogenesis, *2*: 457-461, 1981.

134. Neal, G.E., Moss, E.J., and Manson, M.M. Glutathione conjugation in oncogenesis. In: Glutathione conjugation, pp. 279-314, London: Academic Press Limited. 1988.

135. Neal, G.E., Nielsch, U., Judah, D.J., and Hulbert, P.B. Conjugation of model substrates or microsomally-activated aflatoxin B_1 with reduced glutathione, catalysed by cytosolic glutathione-*S* transferases in livers of rats, mice and guinea pigs. Biochemical Pharmacology, *36*: 4269-4276, 1987.

136. Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Ganzalez, F.J., Coon, M.J., Gunslaus, I.C., Gotoh, O., Okuda, K., and Nebert, D.W. The P450 superfamily: Update on new sequences, gene mapping, accession numbers, early trivial names

of enzymes, and nomenclature. DNA and Cell Biology, *12*: 1-51, 1993.

137. Neveu, M.J., Hully, J.R., Babcock, K.L., Hertzberg, E.L., Nicholson, B.J., Paul, D.L., and Pitot, H.C. Multiple mechanisms are responsible for altered expression of gap junction genes during oncogenesis in rat liver. Journal of Cell Science, *107*: 83-95, 1994.
138. Newberne, P.M. and Butler, W.H. Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: A review. Cancer Research, *29*: 236-250, 1969.
139. Newberne, P.M. and Wogan, G.N. Sequential morphological changes in aflatoxin B_1 carcinogenesis in the rat. Cancer Research, *28*: 770-781, 1968.
140. Ng, I.O.L., Chung, L.P., Tsang, S.W.Y., Lam, C.L., Lai, E.C.S., Fan, S.T., and Ng, M. p53 gene mutation spectrum in hepatocellular carcinomas in Hong Kong Chinese. Oncogene, *9*: 985-990, 1994.
141. Ng, I.O.L., Srivastava, G., Chung, L.P., Tsang, S.W.Y., and Ng, M.M.T. Overexpression and point mutations of p53 tumor suppressor gene in hepatocellular carcinomas in Hong Kong Chinese people. Cancer, *74*: 30-37, 1994.
142. Nigam, S.K. and Ghosh, S.K. Aflatoxin, its metabolism and carcinogenesis- a historical review. J. Toxicol. - Toxin Reviews, *13*: 179-203, 1994.
143. Nikolaidou, A., Liloglou, T., Malliri, A., Ergazaki, M., Tiniakos, G., Tiniakos, D., and Spandidos, D.A. Detection of hepatitis B virus DNA and mutations in K-*ras* and p53 genes in human hepatocellular carcinomas. international journal of oncology, *3*: 593-596, 1993.
144. Nose, H., Imazeki, F., Ohto, M., and Omato, M. p53 gene mutations and 17p allelic deletions in hepatocellular carcinoma from Japan. Cancer, *72*: 355-360, 1993.
145. Ormerod, M.G. and Imrie, S.F. Enzyme-antienzyme method for immunohistochemistry. In: M.M. Manson (ed.), Immunochemical protocols, pp. 117-124, Humana Press: Towota,New Jersey. 1992.
146. Osborn, M. and Weber, K. Intermediate filament proteins: a multigene family distinguishing major cell lineages. TIBS, *11*: 469-472, 1986.
147. Ozturk, M. *et. al.,* p53 mutation in hepatocellular carcinoma after aflatoxin exposure. The Lancet, *30*: 1356-1359, 1991.
148. Pitot, H.C. and Siria, A.E. The stages of initiation and promotion in hepatocarcinogenesis. Biochemica et Biophysica Acta, *605*: 191-215, 1980.
149. Pitot, H.C., Goldsworthy, T., Moran, S., Sirica, A.E., and Weeks, J. Properties of incomplete carcinogens and promoters in hepatocarcinogenesis. Carcinogenesis, *7*: 85-98, 1982.
150. Piva, G., Galvano, F., Pietri, A., and Piva, A. Detoxification methods of aflatoxins. A review. Nutrition Research, *15*: 767-776, 1995.
151. Raney, K.D., Meyer, D.J., Ketterer, B., Harris, T.M., and Guengerich, F.P. Glutathione conjugation of aflatoxin B_1 *exo*- and *endo*-epoxides by rat and human glutathione *S*-transferase. Chem. Res. Toxicol. *5*: 470-478, 1992.
152. Righter, H.F., Shalkop, W.T., Mercer, H.D., and Leffel, E.C. Influence of age and sexual status on the development of toxic effects in the male rat fed aflatoxins. Toxicology and Applied Pharmacology, *21*: 435-439, 1972.
153. Robertson, I.G.C., Zeiger, E., and Goldstein, J.A. Specificity of rat liver cytochrome P-450 isozymes in the mutagenic activation of benzo[a]pyrene, aromatic amines and aflatoxin B_1.

Carcinogenesis, *4*: 93-96, 1983.

154. Roomi, M.W., Ho, R.K., Sarma, D.S.R., and Farber, E. A common biochemical pattern in preneoplastic hepatocyte nodules generated in four different models in the rat. Cancer Research, *45*: 564-571, 1985.
155. Ross, R.K., Yuan, J., Yu, M.C., Wogan, G.N., Qian, G., Tu, J., Groopman, J.D., Gao, Y., and Henderson, B.E. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. The Lancet, *339*: 943-946, 1992.
156. Rutenburg, A.M., Kim, H., Fischbein, J.W., Hanker, J.S., Wasserkrug, H.L., and Seligman, A. Histochemical and ultrastructural demonstration of gamma-glutamyl transpeptidase activity. The Journal of Histochemistry and Cytochemistry, *17*: 517-526, 1969.
157. Sabbioni, G., Skipper, P.L., Buichi, G., and Tannenbaum, S.R. Isolation and characterization of the major serum albumin adduct formed by aflatoxin B_1 *in vivo* in rats. Carcinogenesis, *8*: 819-824, 1987.
158. Sagher, D. and Strauss, B. Insertion of nucleotides opposite apurinic/apyrimidinic sites in deoxyribonucleic acid during *in vitro* synthesis: uniqueness of adenine nucleotides. Biochemistry, *22*: 4518-4526, 1983.
159. Salmon, W.D. and Newberne, P.M. Occurrence of hepatomas in rats fed diets containing peanut meal as a major source of protein. Cancer Research, *23*: 571-575, 1962.
160. Sambrook, J., Fritsch, E.F., and Maniatis, T. Molecular Cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory Press, 1989.
161. Sanger, F., Nicklen, S., and Coulson, R. DNA sequencing with chain-terminating inhibitors. P. N. A. S. *74*: 5463-5467, 1977.
162. Sato, K. Glutathione S-transferases and hepatocarcinogenesis. Jpn. J. Cancer Res. *79*: 556-572, 1988.
163. Sato, K., Kitahara, A., Satoh, K., Ishikawa, T., Tatematsu, K., and Ito, N. The placental form of glutathione *S*-transferase as a new marker protein for preneoplasia in rat chemical hepatocarcinogenesis. Gann, *75*: 199-202, 1995.
164. Satoh, T., Nakafuku, M., and Kaziro, Y. Function of Ras as a molecular switch in signal transduction. The Journal of Biological Chemistry, *267*: 24149-24152, 1992.
165. Selivanova, G. and Wiman, K.G. *p53*: A cell cycle regulator activated by DNA damage. Advances in Cancer Research, *66*: 143-179, 1995.
166. Sell, S. Cellular origin of cancer: Dedifferentiation or stem cell maturation arrest? Environmental health Perspectives, *101*: 15-26, 1993.
167. Senstag, C. and Wurgler, F.E. DNA recombination induced by aflatoxin B_1 activated by cytochrome P450 1A enzymes. Molecular Carcinogenesis, *11*: 227-235, 1994.
168. Serck-Hanssen, A. Aflatoxin-induced fatal hepatitis? Arch. Environ. Health, *20*: 729-731, 1970.
169. Shank, R.C. Epidemiology of aflatoxin carcinogenesis. In: Environmental Cancer, pp. 291-318, Washington London: Hemisphere Publishing Corporation. 1977.
170. Shank, R.C., Bourgeois, C.H., Keschamras, N., and Chandavimol, P. Aflatoxins in autopsy specimens form Thai children with acute disease of unknown aetiology. Food Cosmet. Toxicol. *9*: 501-507, 1971.
171. Sheabar, F.Z., Groopman, J.D., Qian, G., and Wogan, G.N. Quantitative analysis of aflatoxin-

albumin adducts. Carcinogenesis, *14*: 1203-1208, 1993.

172. Shimada, T. and Nakamura, S. Cytochrome P-450-mediated activation of procarcinogens and promutagens to DNA-damaging products by measuring expression of *umu* gene in *salmonella typhimurium* TA1535/pSK1002. Biochemical Pharmacology, *36*: 1979-1987, 1987.
173. Shimada, T., Nakamura, S., Imaoka, S., and Funae, Y. Genotoxic and mutagenic activation of Aflatoxin B_1 by constitutive forms of cytochrome *P*-450 in rat liver microsomes. Toxicology and Applied Pharmacology, *91*: 13-21, 1987.
174. Sinha, S., Webber, C., Marshall, C.J., Knowles, M.A., Proctor, A., Barrass, N.C., and Neal, G.E. Activation of *ras* oncogene in aflatoxin-induced rat liver carcinogenesis. P. N. A. S. *85*: 3673-3677, 1988.
175. Sizaret, P., Malaveille, C., Monteasno, R., and Frayssinet, C. Detection of aflatoxins and related metabolites by radiommunoassay. J. N. C. I. *69*: 1375-1381, 1982.
176. Slagle, B.L., Zhou, Y., and Butel, J.S. Hepatitis B virus integration event in human chromosome 17p near the p53 gene identifies the region of the chromosome commonly deleted in virus-positive hepatocellular carcinomas. Cancer Research, *51*: 49-54, 1991.
177. Solt, D. and Farber, E. New principle for the analysis of chemical carcinogens. Nature, *263*: 701-703, 1976.
178. Soman, N. and Wogan, G. Activation of the c-Ki-*ras* oncogene in aflatoxin B_1-induced hepatocellular carcinoma and adenoma in the rat: Detection by denaturing gradient gel electrophoresis. P. N. A. S. *90*: 2045-2049, 1993.
179. Steinberg, P., Schramm, H., Schladt, L., Roberston, L.W., Thomas, H., and Oesch, F. The distribution, induction and isoenzyme profile of glutathione S-transferase and glutathione peroxidase in isolated rat liver parenchymal, Kupffer and endothelial cells. Biochem. J. *264*: 734-744, 1989.
180. Stryer, L. Hormone Action. In: Biochemistry, pp. 975-1004, New York: WH Freeman and company. 1988.
181. Swenson, D.H., Miller, E.C., and Miller, J.A. Aflatoxin B_1 -2,3-oxide: Evidence for its formation in rat liver in vivo and by human liver microsomes in vitro. Biochemical and Biophysical Research Communications, *60*: 1036-1043, 1974.
182. Symonds, H., Krall, L., Remington, L., Saenz-Robles, M., Lowe, S., Jacks, T., and Van Dyke, T. p53-Dependent apoptosis suppresses tumor growth and progression *in vivo*. Cell, *78*: 703-711, 1994.
183. Tada, M., Omato, M., and Ohto, M. Analysis of *ras* gene mutations in human hepatic malignant tumours by polymerase chain reaction and direct sequencing. Cancer Research, *50*: 1121-1124, 1990.
184. Tashiro, F., Morimura, S., Hayashi, K., Makino, R., Kawamura, H., Horikoshi, N., Nemoto, K., Ohtsuba, K., Sugimura, T., and Ueno, Y. Expression of the c-Ha-ras and c-myc genes in aflatoxin B1-induced hepatocellular carcinomas. Biochemical and Biophysical Research Communications, *138*: 858-864, 1986.
185. Teich, N.M. Oncogenes and Cancer. In: L.M. Franks and N.M. Teich (eds.), Introduction to the cellular and molecular biology of cancer, pp. 230-268, Oxford: Oxford University Press. 1993.
186. Teramato, T., Satonaka, K., Kitazawa, S., Fujimori, T., Hayashi, K., and Maeda, S. *p53* gene

abnormalities are closely related to hepatoviral infections and occur at a late stage of hepatocarcinogenesis. Cancer Research, *54*: 231-235, 1994.

187. Tokusashi, Y., Fukuda, I., and Ogawa, K. Absence of *p53* mutations and various frequencies of Ki-*ras* exon 1 mutations in rat hepatic tumors induced by different carcinogens. Molecular Carcinogenesis, *10*: 45-51, 1994.
188. Towbin, H., Staehelin, T., and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA, *76*: 4350-4354, 1979.
189. Truckess, M.W. and Wood, G.E. Recent methods of analysis for aflatoxins in food and feeds. In: D.L. Eaton and J.D. Groopman (eds.), The Toxicology of Aflatoxins: human health, veterinary and agricultural significance, pp. 409-432, San Diego: Academic Press, INC. 1994.
190. Tsuda, H., Moore, M.A., Asamoto, M., Inoue, T., Ito, N., Satoh, K., Ichihara, A., Nakamura, T., Amelizad, Z., and Oesch, F. Effect of modifying agents on the phenotypic expression of cytochrome P-450, glutathione *S*-transferase molecular forms, microsomal epoxide hydrolase, glucose-6-phosphate dehydrogenase and gamma-glutamyltranspeptidase in rat liver preneoplastic lesions. Carcinogenesis, *9*: 547-554, 1988.
191. Unsal, H., Yakicier, C., Marcais, C., Kew, M., Volkmann, M., Zentgraf, H., Isselbacher, K.J., and Ozturk, M. Genetic heterogeneity of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA, *91*: 822-826, 1994.
192. Vesselinovitch, S.D., Mihailovich, N., Wogan, G.N., Lombard, L.S., and Rao, K.V.N. Aflatoxin B_1, a hepatocarcinogen in the infant mouse. Cancer Research, *32*: 2289-2291, 1972.
193. Vojtesek, B., Bartek, J., Midgley, C.A., and Lane, D.P. An immunochemical analysis of the human nuclear phosphoprotein p53. Journal of Immunological Methods, *151*: 237-244, 1992.
194. Volkmann, M., Hofmann, W.J., Muller, M., Rath, U., Otto, G., Zentgraf, H., and Galle, P.R. p53 overexpression is frequent in european hepatocellular carcinoma and largely independent of the codon 249 hot spot mutation. Oncogene, *9*: 195-204, 1994.
195. Wang, X.W., Forrester, K., Yeh, H., Feitelson, M.A., Gu, J., and Harris, C.C. Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. Proc. Natl. Acad. Sci. USA, *91*: 2230-2234, 1994.
196. Weber, K. and Osborn, M. The reliability of molecular weight determinations by dodecyl-sulfate-polyacrylamide gel electrophoresis. The Journal of Biological Chemistry, *244*: 4406-4412, 1969.
197. Whetsell, L., Maw, G., Nadon, N., Ringer, D.P., and Schaefer, F.V. Polymerase chain reaction microanalysis of tumors from stained histological slides. Oncogene, *7*: 2355-2361, 1992.
198. Wild, C.P., Garner, R.C., Montesano, R., and Tursi, F. Aflatoxin B_1 binding to plasma albumin and liver DNA upon chronic administration to rats. Carcinogenesis, *7*: 853-858, 1986.
199. Wild, C.P., Shrestha, S.M., Anwar, W.A., and Montesano, R. Field studies of aflatoxin exposure, metabolism and induction of genetic alterations in relation to HBV infection and hepatocellular carcinoma in the Gambia and Thailand. Toxicology Letters, *64/65*: 455-461, 1992.
200. Williams, J.G. and Mason, P.J. Hybridisation and the analysis of RNA. In: B.D. Hames and S.J. Higginsm (eds.), Nucleic acid hybridisation, pp. 139-160, Oxford: IRL Press. 1991.

201. Wilson, D.M. and Payne, G.A. Factors affecting *Aspergillus flavus* group infection and aflatoxin contamination of crops. In: D.L. Eaton and J.D. Groopman (eds.), The Toxicology of Aflatoxins: human health, veterinary and agricultural significance, pp. 309-326, San Diego: Academic Press, INC. 1994.
202. Wogan, G. Aflatoxins and their relationship to hepatocellular carcinoma. In: pp. 25-41, John Wiley and sons: 1976.
203. Wogan, G.N. and Newberne, P.M. Dose-response characteristics of Aflatoxin B_1 carcinogenesis in the rat. Cancer Research, *27*: 2370-2376, 1967.
204. Wogan, G.N. Mycotoxins and other naturally occurring carcinogens. In: Environmental Cancer, pp. 263-290, Washington London: Hemisphere Publishing Corporation. 1977.
205. Wogan, G.N., Paglialunga, S., and Newberne, P.M. Carcinogenic effects of low dietary levels of aflatoxin B_1 in rats. Food Cosmet Toxicol, *12*: 681-685, 1974.
206. Wong, J.J. and Hsieh, D.P.H. Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential. Proc. Natl. Acad. Sci. USA, *73*: 2241-2244, 1976.
207. Yaswen, P., Goyette, M., Shank, P., and Fausto, N. Expression of c-Ki-*ras*, c-Ha-*ras*, and c-*myc* in specific cell types during hepatocarcinogenesis. Mol. Cell. Biol. *5*: 780-786, 1985.
208. Zuckerman, A.J., Tsiquaye, K.N., and Fulton, F. Tissue culture of human embryo liver cells and the cytotoxicity of aflatoxin B1. British Journal of Experimental Pathology, *48*: 20-27, 1966.