

**MYCOTOXICOLOGICAL PROPERTIES OF *FUSARIUM VERTICILLIOIDES* AND  
THE FUMONISINS – MECHANISMS AND IMPLICATIONS FOR SETTING RISK  
ASSESSMENT PARAMETERS IN HUMANS**

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PhD (University of Stellenbosch)

Submitted in the fulfillment of the degree

**DOCTOR OF SCIENCE  
(Biochemistry)**



In the Faculty of Natural Sciences  
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**March 2009**

## **DECLARATION**

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Date: March 2009

## ABSTRACT

The fumonisin mycotoxins are known to be the causative principle for several animal diseases and are associated with the development of liver and oesophagus cancer and neural tube defects in humans. The thesis focuses mainly on the characterisation of the compounds from maize cultures of the fungus *Fusarium verticillioides*, isolated from maize, the toxicological effects in animals, mechanism involved in hepato- and nephrocarcinogenicity and discussing the major differences and contradictions in the literature together with their impact on setting relevant risk assessment parameters to safeguard human health. Controversies include the importance of non-genotoxicity vs genotoxicity in the development of cancer, the role of threshold effects in carcinogenesis and the establishment of realistic risk assessment parameters that will also be applicable in developing countries. Recent approaches suggest that thresholds should also apply for genotoxic carcinogens as interaction with the DNA is only one event in the multi-step process of cancer development and therefore could not be taken as the basis for applying a no-effect threshold for genotoxins. It would appear that a carcinogen such as fumonisin, whether it is labeled genotoxic or non-genotoxic *per se*, exhibits some degree of risk at any level due to additive or synergistic interactions with other xenobiotics and/or dietary constituents. The underlying mechanisms of fumonisin-induced carcinogenicity includes the disruption of sphingolipid, phospholipids and fatty acid metabolism, which plays a major role in the modulation of apoptotic and cell proliferative pathways related to cancer development. Interactive responses between arachidonic acid and ceramide affect downstream cell signal transduction pathways and depending on the cell type the disruption of these pathways could either stimulate or inhibit cell proliferation which eventually will determine the induction of apoptosis and hence affect cell survival. The modulating roles of dietary constituents such as vitamins, protein and the South African herbal teas are also highlighted as they affected the outcome of toxicological assays, thus determining thresholds of the adverse effects in specific target organs that will impact risk assessment parameters. Regulation of the fumonisins in food and the associated risk are debated from many perspectives. In developing countries there is a lack of quality control implying that maize highly contaminated with mycotoxins may directly enter the food chain of adults and children as control of mycotoxins is difficult or in some cases totally absent. The interaction of politics, economy and technology will eventually determine the impact on health as the regulation of fumonisin in food differs between countries. Knowledge about the biological effects of the fumonisins is currently playing an important role in the development of simple and inexpensive methods to reduce the levels of the fumonisin in maize by targeting specific populations at risk.

## OPSOMMING

Die fumonisien mikotoksiene veroorsaak verskeie siektes by diere en is geassosieer met die ontwikkeling van lewer en slukderm kanker en geboorte defekte by mense. Die tesis handel oor die karakterisering van die fumonisiene in mielie kulture van die swam *Fusarium verticillioides* oorspronklik geïsoleer uit mielies, die toksikologiese effekte in diere, die onderliggende meganismes betrokke in die kankerontwikkeling in die lewer en niere en 'n bespreking van die verskille en teenstrydighede in die literatuur rakende die toksikologiese effekte asook hoe dit die risiko bepaling in mense en regulering in voedsel kan beïnvloed. Sekere van die kontroversiële aspekte sluit in die rol wat verbindinge wat met genetiese materiaal (DNA) bind en beskadig (genotoksies) teenoor die wat kanker induseer volgens ander meganismes (nie-genotoksies) en die rol in die bepaling van drumpelwaardes wat belangrik is in die vaststelling van risikoparameters in ontwikkelde en ontwikkelende lande. Onlangse benaderings dui daarop dat vanweë die kompleksiteit van kankerontwikkeling, karsinogene wat nie DNA beskadig nie ook 'n drumpelwaarde besit soos in die geval met genotoksiese karsinogene en behoort dus tot dieselfde mate gereguleer word. Dit blyk dat 'n karsinogeen soos fumonisien, of dit nou genotoksies of nie-genotoksies beskou word, 'n mate van risiko by enige vlak inhou as gevolg van additiewe en sinergistiese interaksies met verskeie ander toksiene en voedselkomponente wat die drumpelwaarde en dus regulering in voedsel kan beïnvloed. Studies rakende die meganismes van kanker-induksie handel ondermeer oor die effek van die fumonisiene op fosfolipied-, sfingolipied- en vetsuurmetabolisme. Hierdie veranderings speel 'n belangrike rol in selgroei en geprogrammeerde seldood, twee belangrike aspekte wat die oorlewing van selle bepaal en onderliggend is aan die ontwikkeling van kanker. 'n Interaksie tussen arachidoonsuur en seramied, twee belangrike molekules in die vetsuur en sfingolipied metabolisme paaie, word selektief deur die fumonisiene geïmmuniseer en speel dus 'n belangrike rol in sein transduksie paaie geassosieer met sel groei en geprogrammeerde seldood. Die modulering van die karsinogene effekte van die fumonisiene deur verskeie voedselkomponente soos vitamienes, proteïen en Suid Afrikaanse kruiekruid word ook bespreek in die lig van moontlike intervensies van die toksikologiese effekte in mense. Die finale gedeeltes van die tesis handel oor die regulering van die fumonisiene in voedsel en die verskille wat bestaan tussen ontwikkelende en ontwikkelde lande. Hierdie verskille kan nadelige effekte hê in ontwikkelende lande waar regulering nie streng toegepas word nie en die toksien in die voedselketting van volwassenes en kinders kan beland. Die interaksie tussen die politiek, ekonomie en tegnologie sal uiteindelik die impak op gesondheid bepaal deur die vaststelling van spesifieke vlakke in voedsel. Kennis oor die biologiese effekte van die fumonisiene speel huidiglik 'n belangrike rol in die ontwikkeling van eenvoudige en goedkoop metodes om die vlakke in stapelvoedsel van hoë-risiko populasies te verlaag.

## PREFACE AND ACKNOWLEDGEMENTS

The dissertation summarises research undertaken following my PhD degree that was obtained during 1986 in the Department of Biochemistry, University of Stellenbosch and includes selected publications spanning the period of research for more than 20 years (1986 until 2008). The main thrust of the studies focused on the carcinogenicity of the fungus *Fusarium verticillioides*, the discovery, toxicological studies, possible biochemical mechanisms involved and the establishment of risk assessment parameters of the fumonisins. The outcomes reflect a multidisciplinary approach and collaboration between colleagues at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) Unit, MSc and PhD students from different local and international Universities, collaborators both locally and abroad and regulatory bodies including the World Health Organisation and the International Agency for Research on Cancer. During this period I have encountered prominent scientists in the field of mycotoxicology and carcinogenesis who, with their enthusiasm, have stimulated many of the ideas that culminated in the outputs that form part of the dissertation. My interaction with Prof WFO Marasas, retired Director of the PROMEC Unit, was central to the discovery of the fumonisins and it was an exiting journey in unraveling the mysteries of these mycotoxins in animal and human disease. My postdoctoral study visit under the guidance of Prof E Faber at the University of Toronto in 1988 has been extremely fruitful to conceptualize mechanisms involved in the cancer initiating and promoting properties of the fumonisins. Some aspects of this research were continued at IARC in Lyon France during 1996 whilst visiting the laboratories of Prof CP Wild. Several visits to the USA and the attendance of conferences on the toxicology and carcinogenicity of the fumonisins have been fruitful in defining certain hypotheses and I am indebted to Drs R Riley and K Vos of the United States Department of Agriculture (USDA) in Athens, Georgia for fruitful discussions and debates regarding fumonisin mycotoxicology.

The dissertation consists of commentaries of selected papers covering various topics of the fungus with the main focus on the fumonisins and perspectives of how the findings impact on existing knowledge in the literature. However, the dissertation should not be considered as a detailed review but the publications and comments provide certain perspectives, which are likely to stimulate some debate regarding the mechanisms of carcinogenesis involved and the extrapolation for assessing risk to

humans. Several aspects regarding the mechanisms of fumonisin-induced toxicological effects are unknown that could assist in determining more accurate risk assessment parameters in humans, especially in rural communities of developing countries.

I am indebted to Prof PS Swart of the Department of Biochemistry, Stellenbosch University for his guidance and the opportunity to graduate from the Department. To all my colleagues and friends, a special word of appreciation for their interest and support. To my wife Dalene, daughter Christa and son Wentzel for their love and understanding during the more than twenty years I conducted the research.

**Dedicated to Dalene, Christa and Wentzel (jr)**

**ABBREVIATIONS**

AFB<sub>1</sub> – aflatoxin B<sub>1</sub>  
AKt – protein kinase B  
AP/HFB<sub>1</sub> – aminopolyol derivatives of the fumonisins  
ATH – atypical tubule hyperplasia  
CCl<sub>4</sub> – carbontetrachloride  
CdK – cyclin dependent kinase  
Chol – cholesterol  
CPN - chronic progressive nephropathy  
DEN – diethylnitrosamine  
EGF – epidermal growth factor  
ELEM - equine leukoencephalomalacia  
FA – fatty acids  
FA<sub>1</sub> – N-acetyl derivative of FB<sub>1</sub>  
FB – fumonisin B mycotoxins  
GGT – gamma glutamyltranspeptidase  
GPx – glutathione reductase  
GR – glutathione reductase  
GSH – glutathione (reduced form)  
GSK-3 – glucocorticoid synthase kinase-3 $\beta$   
HGF – hepatocyte growth factor  
JECFA – joint expert committee on food additives  
LC PUFA – long chain PUFA  
LNT – linear non-threshold  
MAPK – mitogen activated protein kinase  
MONO-UNSATs – monounsaturated fatty acids  
MTLs – maximum tolerable levels  
NOEL – no-observed-effect-level  
NTD – neural tube defects  
NTP – national toxicology program  
OC – oesophageal cancer  
PB – phenobarbital  
PC –phosphatidylcholine  
PC/PE – phosphatidylethanolamine/phosphatidylcholine ratio

PDI – probable daily intake  
PE – phosphatidylethanolamine  
PGST<sup>+</sup> - placental form of glutathione-S-transferase positive  
PGE<sub>2</sub> – prostaglandin E<sub>2</sub>  
PI – phosphatidylinositol  
PI-3K – phosphoinositol-3 kinase  
PMTI – provisional tolerable daily intake  
P/S – polyunsaturated/saturated ratio  
PUFA – polyunsaturated fatty acids  
Rb – retinoblastoma protein  
Sa – sphinganine  
So – sphingosine  
Sa/So - sphinganine/sphingosine ratio  
SATS – saturated fatty acids  
SM – sphingomyelin  
TA/TB – *Alternaria* toxins  
TBARS – thiobarbituric acid reactive substances  
TDI – tolerable daily intake  
TGF $\alpha$  – tumor growth factor alpha  
TGF $\beta$  – tumor growth factor beta  
TAG – triacylglycerol  
8-OH-dG – 8 hydroxy guanine  
2-AAF/PH – 2-acetylaminofluorene/partial hepatectomy  
C20:4n-6 – arachidonic acid  
C18:2n-6 – linoleic acid  
C18:3n-3 - linolenic acid  
C18:1n-9 - oleic acid  
C16:0 – palmitic acid  
C18:0 – stearic acid  
C20:5n-3 – eicosapentanoic acid  
C20:3n-6 – gamma linoleic acid



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*....Where were you when I laid the earth's foundation?  
... have you ever give orders to the morning,  
or show the dawn its place?  
... Do you give the horse his strength  
or clothe his neck with a flowing mane?  
... Does the hawk take flight by your wisdom?  
... Does the eagle soar at your command?*

**Job 38 to 39**

## INTRODUCTION

Mycotoxins are mainly produced by species of the fungal genera *Aspergillus*, *Penicillium* and *Fusarium* on a wide range of food commodities worldwide (Marasas *et al.*, 2008). It is estimated that mycotoxins affect a quarter of the world's food crops resulting in economical losses amounting to approximately \$1.4 billion in the United States annually (Wu, 2004). Crop losses associated with aflatoxin contamination of maize in the United States was estimated \$75 to \$100 million in 1985 (Henry *et al.*, 1999). Stringent mycotoxin regulations resulted in the trade of the best quality foods whilst contaminated foods are marketed and consumed locally with increased the risk of mycotoxin exposure. The high regulatory standards adversely affect the economy of developing countries due to the rejection of certain food products, resulting in losses of over \$600 million when considering the regulation of aflatoxin. Apart from severe crop losses due to mycotoxin contamination it impacts on a large array of chronic health risks including cancer, digestive, blood and nerve disorders in humans and/or animals (Shephard *et al.*, 2006). The health impact of mycotoxins in developing countries is far more severe due to the chronic exposure to high levels of malnourished populations. The reduction of mycotoxin intake is therefore of major importance, especially in developing countries where exposure is extremely high due to malpractices regarding farming, storing and the utilisation of grains as the sole dietary staple.

Since the discovery of *Fusarium verticillioides* (previously known as *F. moniliforme* or *Oospora verticillioides*) isolated from moldy maize, it has been associated with disease development in animals and humans as it is the most prevalent fungus associated with maize intended for human and animal consumption (Marasas, 1985; 2001). The first remarkable disease induced by the fungus in animals was leukoencephalomalacia (LEM) in equines when either using naturally contaminated maize or maize fungal cultures (Wilson and Maronpot, 1971; Marasas *et al.*, 1976). Numerous studies in experimental animals showed that the fungus caused diverse toxic lesions with the main target organ that differs depending on the animal species. One of the most prominent findings was the induction of liver cancer in rats by chronic exposure to fungal culture material suggesting that carcinogenic principles are produced by the fungus (Marasas *et al.*, 1984). The first indication that the fungus is associated with a human disease was derived from studies conducted in the

Transkei region of the Eastern Cape Province, South Africa during the late 1970 and early 1980, showing that the fungus was correlated with the incidence of human oesophageal cancer (OC) (Marasas *et al.*, 1979, 1981, 1982). Similar findings were also noted in China in five counties with high OC incidence rates (Zhang, *et al.*, 1997). None of the known *Fusarium* mycotoxins, such as the trichothecenes, zearalenone and moniliformin is produced by *F. verticillioides*, although some conflicting reports have been published. Therefore for two decades the toxic and carcinogenic principles of the fungus remained unknown despite continued attempts to characterise the active metabolites.

The present dissertation commenced with studies related to the carcinogenicity of the potent mutagen, fusarin C, produced by the fungus *F. verticillioides* (Chapter II). There were strong indications that fusarin C could be responsible for the carcinogenic properties of the fungus but the heat instability and prominent deactivation by drug metabolizing enzymes in the liver were contra indicators. The ground-breaking paper regarding the characterisation of the newly discovered fumonisin B (FB) mycotoxins was the finding that culture material of the fungus promoted the formation of preneoplastic lesions in the liver of rats utilising a cancer initiation/promotion model with diethylnitrosamine (DEN) as the cancer initiator (Gelderblom *et al.*, 1988). Details regarding this finding and the subsequent isolation, purification, characterisation and radiolabelling are described in Chapter II. Studies in experimental animals, presented in Chapter III, showed that FB<sub>1</sub> is the causative principle of both LEM in horses and the hepatotoxic and hepatocarcinogenic effects in both male BD IX and Fischer 344 rats. Different dietary constituents, including iron, high protein diet and tea antioxidants modulate the carcinogenic properties of FB<sub>1</sub>. A synergistic interaction with aflatoxin B<sub>1</sub> was also demonstrated in a liver cancer model. These dietary constituents are likely to alter the biological response and site specific sensitivity of FB<sub>1</sub>-induced effects in different animal species.

The mechanism of cancer induction of the fumonisins in the liver is presented in Chapter IV. Two controversial aspects of FB<sub>1</sub>-induced hepatocarcinogenesis are addressed which include (i) the cancer initiating properties of this apparent non-genotoxin which should impact on risk assessment in humans and (ii) a unique model for cancer promotion involving fatty acids (FA), specifically arachidonic acid

(C20:4n-6). This is the first time that such a model is proposed for cancer promotion by a chemical carcinogen in the liver. The proposed mechanism for cancer promotion is critically debated with respect to known models that exist for cancer promotion in the liver. Cancer promotion by FB<sub>1</sub> is also discussed in relation to the biological effects obtained *in vitro* utilising primary hepatocyte cultures and *in vivo* in rat liver. The interactive mechanism that seems to exist between the FA, phospholipids and sphingolipid metabolism is presented in detail with discussions extending into Chapter V.

Aspects regarding parameters determining the risk assessment of the fumonisins and some controversies that exists are addressed in Chapter V. These include the role of thresholds in fumonisin-induced toxic and carcinogenic effects and the adoption of non-linear dose response extrapolations with respect to genotoxic and nongenotoxic carcinogens. A critical assessment of the current risk paradigm for the fumonisins and the interaction of health, economy, technology and politics are debated with specific reference to the potential impact on human populations in developing countries. These aspects are further developed in the short Summary and Discussion of the thesis, presented in Chapter VI.

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## CHAPTER I

### MUTAGENIC AND CARCINOGENIC PROPERTIES OF *FUSARIUM VERTICILLIOIDES*

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## **TOXICITY, MUTAGENICITY AND CARCINOGENICITY OF *FUSARIUM VERTICILLIOIDES***

### *1.1 Studies using maize culture material.*

#### *1.1.1 Studies in rats*

Initial studies on the toxicological effects of *F verticillioides* in male BD IX rats fed a commercial rat mash were performed by Kriek *et al.* (1981a). The culture material of 21 isolates of the fungus from Transkeian maize was incorporated at dietary levels of 8, 16 and 32 %. The two high dosage levels (32% and 16%) of the different isolates, including strain MRC 826, were toxic and all the rats died after 49 and 78 days, respectively, while surviving rats receiving the lower dosage level were killed after 78 days. Lesions were mainly restricted to the heart and liver. These include cirrhosis and development of hyperplastic nodules in the liver and prominent heart lesions in the left ventricular endocardium including acute and proliferative endocardial lesions and concurrent intraventricular thrombosis. Changes in the other organs were less common but endothelial proliferation of pulmonary arteries and low-grade toxic nephrosis were of interest. It was suggested that with respect to the cardiotoxicity the target cells appear to be the endothelial cells. The cardiotoxicity induced by cultures of *F verticillioides* also differed from that induced by moniliformin, a mycotoxin produced by *F. subglutinans*, which mainly affects the myocardium (Kriek *et al.*, 1977).

Two separate studies were performed regarding the long-term toxicological effects of maize cultures of *verticillioides* MRC 826 in male BD IX rats (Marasas *et al.*, 1984; Jaskiewicz *et al.*, 1987a). The fungal strain used in both studies was isolated from maize cultivated in a high incidence area of OC in the Transkei region on the Eastern Cape Province, South Africa. The first experiment was carried out over a period of 763 days using commercial rat feed containing mouldy maize meal at a level of 8% and 4 % for 286 days followed by 2 % for the remainder of the experiment (Marasas *et al.*, 1984). Both freeze-dried and oven-dried (45-50 °C) materials were used to obtain some information about the heat stability of the active compound(s). The maintenance diet was screened on a regular basis for the presence aflatoxin during the course of the experiment and found to be negative. None of the rats survived the 8 % dietary treatment and were terminated between 50 and 75 days following the onset of the experiment with pathological liver lesions including cirrhosis, nodular hyperplasia, bile duct hyperplasia and adenofibrosis. Other pathological changes include endothelial hyperplasia of the ventricular endocardium and/or intraventricular

thrombosis with oesophageal basal cell hyperplasia in about 50% of the rats that received the freeze-dried culture material. The most remarkable pathological features developed in the livers of the rats that received the lower dosage levels of both the freeze- and oven-dried culture material. In 80% of the rats the culture material caused hepatocellular carcinoma while 63% of the rats developed ductular carcinoma. These cancers invariably developed in severely cirrhotic livers showing nodular hyperplasia as well as a range of altered cell populations including clear cell, acidophilic and basophilic cell foci and areas. Pulmonary metastases developed in three rats of each treatment. Adenofibrosis also developed consistently in all the animals and they progressed into large expansile neoplastic lesions referred to as cholangiocarcinoma that were observed macroscopically to extend above the surface of the liver. Hyperplasia of the oesophageal basal cells was also detected commonly in the treated rats, with a higher incidence in those receiving the freeze-dried culture material. It was concluded that some of the causative principles, especially those responsible for the basal cell hyperplasia, are heat sensitive. The major finding, however, was that culture material of the fungus induced two types of liver cancer suggesting that the fungus, commonly occurring in maize which is a major human dietary staple, has the ability to produce a carcinogenic metabolite(s), some of which may be heat labile.

In a follow-up long-term study the same freeze dried culture material of *F. verticillioides* MRC 826 was fed to male BD IX rats at a low (0.5%) dietary level using a semi-synthetic diet marginally deficient in certain vitamins and minerals (Jaskiewicz *et al.*, 1987a). Culture material of another non-toxic fungal strain designated *F. verticillioides* MRC 1069 that lacks any detectable toxic effects in rats, although it produces the mutagen fusarin C, was included at a dietary level of 5% for comparative purposes. Following a feeding period of 23 to 27 months, 2 out of 21 rats fed culture material of strain MRC 826 developed hepatocellular carcinoma with lung metastases and 8 out of 21 animals developed cholangiocarcinoma. The livers of the rats also contained numerous hepatocyte nodules while adenofibrosis, ductular cell ("oval cell") hyperplasia and gamma glutamyltranspeptidase (GGT) positive foci were common features. Basal cell hyperplasia was prominent in the oesophageal epithelium of the rats, myocardial disseminated fibrosis occurred frequently while endocardial and subcardial fibrosis also occurred in 3 rats. Rats fed the non-toxic strain MRC 1069, containing high levels of fusarin C, developed very few lesions in the liver, including mild ductular hyperplasia and a hepatocyte nodule in one rat. The low outcome of liver tumours in this study was ascribed to the low to marginal hepatotoxic effects. As the non-toxic strain MRC 1069 failed to induce any

carcinogenic changes in the liver of the rats implies that the hepatotoxicity was closely associated with the carcinogenic effects of strain MRC 826.

### 1.1.2 A study in vervet monkeys

Studies with *F. verticillioides* strain MRC 826 amongst others, in nonhuman primates commenced during the 1980's. Initial studies were conducted in baboons using *F. verticillioides* strain MRC 602 with acute congestive heart failure and liver cirrhosis as the major pathological lesions (Kriek *et al.*, 1981b). Subsequent studies were conducted with the more toxic strain of the fungus *F. verticillioides* MRC 826, shown to effect equine leukoencephalomalacia, lung edema in pigs, acute nephrosis and hepatitis in sheep and cirrhosis, nephrosis and congested heart failure in rats. Based on this information long-term studies with strain MRC 826 were performed in rats, described above, and vervet monkeys that extended over a period of 13.5 years. Jaskiewicz *et al.* (1987b) and Fincham *et al.*, (1991) published findings regarding the hepatotoxic and arthrogenic effects of the fungal culture material, respectively. The time course of the study spans the pre- and post-fumonisin period, resulting in a retrospective evaluation of exposure after fumonisin analyses of the different cultured batches used (Gelderblom *et al.*, 2001). Dose selection, consisting of an initial low (0.1% culture material) and a high dose (0.25% culture material) was based on blood clinical chemical analyses associated with liver and kidney function. Three different maize culture batches of the fungus were used and feed and FB intake profiles were determined. Changes in the serum liver parameters were confirmed histopathologically by liver biopsies and were characteristic of a chronic toxic hepatitis. Threshold levels for liver and kidney damage were similar and ranged between 0.1 to 0.18 mg FB/kg bw/day and 0.18 to 0.2 mg FB/kg bw/day, respectively. Based on these data a no-observed-effect-level (NOEL) was estimated between 0.11 to 0.18 mg FB/kg bw/day representing maize contamination levels of 8.21 to 13.25 mg FB/kg. Changes in the sphinganine and sphinganine/-sphingosine ratio, possible biomarkers of exposure were detected at an intake of 0.29 to 0.64 mg FB/kg bw/day that represented a contamination level of 21.7 to 47.9 mg FB/kg, respectively.

### 1.2 A study using naturally contaminated maize.

A study conducted in the USA pursued the evidence provided by the toxicological studies with strain MRC 826 in experimental animal that differences exist with respect to the target organ affected in various animals species (Wilson *et al.*, 1985). Following a natural outbreak of equine leukoencephalomalacia (ELEM) on a farm in southeastern Pennsylvania, a maize sample which was fed during the epizootic of

ELEM, was recovered and fed to male Fisher 344 rats over a period of 5-6 months. Despite the many limitations regarding the short duration of the study due to the lack of sufficient material as well as the lack of controls for the nutritionally deficient maize-only diet, the lesions induced were remarkably similar to those reported by Marasas *et al.* (1984a) and Jaskiewicz *et al.* (1987a) with culture material of MRC 826 in BD IX rats. These included multiple hepatic nodules, large areas of adenofibrosis and cholangiocarcinomas that distorted the lobular structure of the liver. The role of the dietary deficiencies, especially those related to methionine and choline, were discussed in detail and their importance in the acceleration and/or promoting the formation of these lesions was recognised. However, the fact that culture material of MRC 826 induced similar lesions using a balanced diet as well as a diet marginally deficient in vitamins and minerals further supported the hypothesis developed by Kriek *et al.* (1981a) and Marasas *et al.* (1984b) that *F. verticillioides* produces toxic principles that are responsible for both ELEM in horses and different toxicological effects in male BD IX rats and, by inference, also in male Fisher 344 rats.

Retrospective analyses of the unique naturally contaminated maize sample used in the Wilson rat study (Wilson *et al.*, 1985) study indicated that it contains a total FB level of 33.1 mg/kg and only trace amounts of aflatoxin B<sub>1</sub> and B<sub>2</sub> (Gelderblom *et al.*, 2004). The same sample was previously reported to contain moniliformin and fusarin C, but none of the major trichothecene mycotoxins T2-toxin and diacetoxyscirpinol (Thiel *et al.*, 1986). The role of moniliformin and fusarin C in the toxicological effects of the fungus has been debated (Thiel *et al.*, 1986; Jaskiewicz *et al.*, 1987a). Moniliformin is apparently not involved in either of the syndromes induced in horses and rats, as MRC 826, which is known to cause both mycotoxicoses, does not produce it. The role of the mutagenic fusarin C has also be questioned in the hepatocarcinogenicity of the fungus (Jaskiewicz *et al.*, 1987a) although very little is known about the synergistic interactions between different mycotoxins and certain dietary deficiencies.

### *1.3 Short-term hepatocarcinogenesis studies related to cancer initiation and promotion.*

#### 1.3.1 Carcinogenicity of fusarin C

The mutagen fusarin C was purified from culture material of *F. verticillioides* MRC 826 and shown to occur naturally in maize (Gelderblom *et al.*, 1983, 1984a, b; Cheng *et al.*, 1985). As fusarin C, a type II substrate for cytochrome P450 (Gelderblom *et al.*,

1988a), exhibited comparable mutagenic activities to aflatoxin B<sub>1</sub> in the presence of metabolic activation a possible role in the carcinogenicity of the fungus was suggested (Gelderblom et al., 1984b). However, short-term carcinogenesis studies utilizing two stage cancer initiation/promotion models in mouse skin and rat liver failed to indicate any cancer initiating properties for fusarin C. As fusarin C is heat labile and rapidly conjugated to glutathione, either chemically and enzymatically via the glutathione-S-transferase (Gelderblom et al., 1988b) and by a microsomal esterase (Gelderblom et al., 1988c), a possible role in the carcinogenicity of the fungus has been abandoned. This notion was further strengthened by the fact that long-term studies using *F. verticillioides* strain MRC1069, containing high levels of fusarin C as compared to the carcinogenic strain MRC 826, failed to induce hepatocarcinogenesis (Jaskiewicz et al., 1987).

### 1.3.2 Cancer promotion by *F. verticillioides*

The cancer promoting activity of culture material of *F. verticillioides* strain MRC 826 in a short-term cancer initiation/promotion liver model in male BD IX rats indicated that both initiation and promotion could be effected (Gelderblom et al., 1988a; 1988b). Subsequent studies showed that other strains of the fungus (MRC 4315, 4319 and 4321) also exhibited cancer promotion activity that, as discussed above for cancer induction, was also associated with a hepatotoxic effect. One of the major findings during these early stages of the characterisation of the active cancer promoters was that several strains of *F. verticillioides* isolated from maize intended for human consumption exhibited this property. In addition *F. verticillioides* strain MRC 826 effected cancer promotion in the absence of DEN initiation suggesting that the carcinogenic principle may effect both phases of cancer induction. This implied that these carcinogenic principles, occurring naturally in maize infected by the fungus, could pose a health risk to humans.

## 1.4 Perspectives

The role of fungal contamination of maize in human and animal diseases has been described since the turn of the 20<sup>th</sup> century. The toxicological effects of the fungus *F. verticillioides* became of interest following reports of the induction of ELEM in horse and donkeys experimentally with maize cultures of the fungus during the 1970's (Wislon, 1971; Kellerman et al., 1972; Marasas et al., 1976). Subsequent studies provided evidence regarding the diversity of toxicological lesions induced in different animals, which include the induction of pulmonary edema in pigs. As *F. verticillioides* is a natural contaminant on maize and plays a role in several animal diseases raise some concerns about a possible role in human diseases. Interest in the possible role

in human disease was pioneered with the finding that the presence of the fungus on maize, a major dietary staple, was correlated with the high incidence rates of OC in rural areas of the former Transkei region of the Eastern Cape Province. These concerns were further strengthened by the finding that the fungus produced mutagenic and carcinogenic principles, which stimulated research to characterise these unknown fungal metabolites. Studies on the mutagenic properties, resulting in the purification and identification of fusarin C, failed to clarify the carcinogenic properties of the fungus. Renewed attempts focused on the characterisation of the hepatotoxic principles in rats by developing a suitable biological model to screen different fractions of the maize cultures of the fungus. During the studies evaluating the carcinogenic effects of fusarin C a cancer initiating/promotion model was established in the liver using diethylnitrosamine (DEN) as cancer initiator. This model addressed both the hepatotoxic and hepatocarcinogenic properties of the fungus in rats and eventually led to the characterization of the major toxic and carcinogenic principles of the fungus, ie the fumonisins.

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## Investigations on the carcinogenicity of fusarin C — a mutagenic metabolite of *Fusarium moniliforme*

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The cancer initiating potential of fusarin C, a mutagen produced by *Fusarium moniliforme* strain MRC 826 was investigated on mouse skin using 12-*O*-tetradecanoyl-phorbol-13-acetate as promoter and in rat liver using phenobarbital as promoter. In neither of these models did fusarin C act as a cancer initiator. Culture material of strain MRC 826, which previously was found to be hepatocarcinogenic in rats, exhibited cancer promoting activity in rat liver using diethylnitrosamine (DEN) as initiator and the induction of gamma glutamyltranspeptidase (GGT)-positive foci as endpoint. The culture material of this fungus could also induce the formation of GGT positive foci without DEN initiation. These results seem to indicate that fusarin C is not involved in the hepatocarcinogenic activity of *F. moniliforme* strain MRC 826.

### Introduction

*Fusarium moniliforme* Sheldon is one of the most prevalent fungi associated with corn intended for human and animal consumption in many countries (1). The incidence of *F. moniliforme* in corn kernels is correlated with human esophageal cancer rates in Transkei, southern Africa (2) and in Henan Province, China (3,4). The actual involvement of this fungus in the development of esophageal cancer has, however, not been proven.

Culture material of *F. moniliforme* strain MRC 826, isolated from home-grown corn in a high risk area of esophageal cancer in Transkei, is highly hepatocarcinogenic in rats (5,6). This material also causes esophageal basal cell hyperplasia (5,6) and a significant enhancement of nitrosamine-induced esophageal carcinoma in rats (7). The chemical nature of the active metabolite(s) produced by this fungus is unknown, but *F. moniliforme* strain MRC 826 is known to produce the potent mutagen fusarin C (8-10). Fusarin C is also produced by other strains of *F. moniliforme* from southern Africa (10), China (11), and the United States (12,13).

Fusarin C is highly mutagenic to bacteria (8,10-12) and also induces clastogenic effects in mammalian cells (11). These findings imply that fusarin C may be carcinogenic. Thus it is important to evaluate the carcinogenicity of fusarin C, particularly in view of the fact that it occurs naturally in corn in Transkei (10), China (11) and the United States (14).

This paper reports on results of experiments used to determine the cancer initiating activity of fusarin C on mouse skin and in a rat liver (15) initiation/promotion model for carcinogenesis. Data are also presented on the cancer promoting activity of

culture material of *F. moniliforme* strain MRC 826 in the rat liver model.

### Materials and methods

#### Chemicals

12-*O*-tetradecanoyl-phorbol-13-acetate (TPA\*), 7,12-dimethylbenz[*a*]anthracene (DMBA) and diethylnitrosamine (DEN) were purchased from Sigma Chemical Co, St Louis, MO. Phenobarbital (PB) was obtained from BDH Chemicals Ltd., Poole, UK. Fusarin C was purified from maize cultures of *F. moniliforme* strain MRC 712 as previously described (10).

#### Culture of fungi

Corn cultures of *F. moniliforme* strains MRC 826 and MRC 712 were prepared as described before (10) and incubated in the dark at 25°C for 2 weeks followed by 2 weeks at 15°C. Wet corn cultures of strain MRC 712 were used directly for the purification of fusarin C. Corn cultures of strain MRC 826 were lyophilized, ground to a fine meal and stored in air-tight containers in the dark at 4°C.

#### Animals

Seven-week-old female ICR/Ha mice, male BD IX rats (~150 g) and female Wistar rats (~150 g) were used. Animals were housed in a controlled environment at 23°C and 50% humidity with a 12 h artificial light cycle. Food and water were available *ad libitum*. Mice were fed rat cubes (Epol Ltd, Cape Town) while rats received a purified diet (16) or duckling mash (Epol Ltd., Cape Town) as indicated in the text.

#### Initiation on mouse skin

Mice were shaved on their backs after most of the hair had been clipped. Only those mice which showed no hair regrowth after 2 days were used. Mice were randomly divided into five groups (8-10 mice per group) and housed five per cage (Table I). All the compounds were applied to the shaved back skin in 0.1 ml acetone as described below.

Group 1 was initiated with DMBA (50 µg/mouse) and after 7 days treated with the promoter, TPA (2 µg/mouse), twice weekly. Group 2 and 3 served as controls and received an equal amount of solvent (0.1 ml acetone), respectively substituting the initiating and promoting treatments of group 1. Groups 4 and 5 were 'initiated' with fusarin C (220 µg and 500 µg per mouse, respectively) under subdued light followed by the promoting treatment with TPA. The incidence of papillomas was recorded weekly from 8 to 16 weeks after commencing the promoting treatment.

#### Initiation in rat liver

The initiation/promotion system developed by Pitot *et al.* (15) for rat liver was utilized.

Rats were fed a synthetic diet (16) and subjected to two-thirds partial hepatectomies (17). The drinking water was supplemented with 5% glucose for the first 24 h post-surgery. The initiating agent dissolved in dimethylsulphoxide (DMSO) was administered (0.1 ml/100 g bodyweight) i.p. 20 h after surgery and promotion commenced 7 days after initiation by supplementing the drinking water with 0.05% PB. After 14 weeks of promotion the gamma glutamyltranspeptidase (GGT) activity was determined histochemically in the livers as described below.

Both the BD IX and Wistar rats were divided into five groups of 5-7 animals per group (Table II). The male BD IX rats were treated as follows. Rats of group 1 were initiated with DEN (30 mg/kg) followed by the PB promoting treatment. Groups 2 and 3 which served as controls, respectively lacked the promoting and initiating treatments of group 1. Groups 4 and 5 were 'initiated' with fusarin C at a dosage of 50 mg/kg and 100 mg/kg, respectively, followed by the PB promoting treatment. The same experimental design was used for female Wistar rats (groups 6,7,8,9 and 10).

#### Promotion in rat liver

The cancer promoting potential of culture material of *F. moniliforme* strain MRC 826 was also determined by employing the protocol suggested by Pitot *et al.* (15). Hepatectomized male BD IX rats were divided into three groups of 5-10 animals per group (Table III). Rats were initiated with DEN (30 mg/kg, i.p.) and were subjected to the 'promoting treatment' 1 week thereafter which consisted of supplementing the synthetic diet (16) with 2% (group 1) culture material of strain MRC 826. Control groups of hepatectomized rats received either the initiation

\*Abbreviations: TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; DMBA, 7,12-dimethylbenz[*a*]anthracene; DEN, diethylnitrosamine; PB, phenobarbital; GGT, gamma glutamyltranspeptidase; DMSO, dimethylsulphoxide.

treatment with DEN (group 2) without promotion or the promotion treatment without initiation (group 3). The promoting potential was also monitored in non-hepatectomized male BD IX rats (3–4 animals per group) on a diet of duckling mash (Table III). The rats in group 4 were initiated with DEN (200 mg/kg, i.p.) and after 7 days the 'promoting treatment' (5% culture of strain MRC 826 in duckling mash) was commenced. Group 5, receiving only DEN (200 mg/kg, i.p.) without promotion and group 6 receiving only the 'promoting treatment' without initiation, served as control groups.

The effects of these treatments were monitored after 14 weeks of promotion by determining hepatic GGT activity histochemically.

#### Histochemical procedures

After 14 weeks of the promoting treatment rats were sacrificed by decapitation and livers were immediately excised. Two tissue blocks (10 × 10 × 2 mm) were cut from the right lateral liver lobe and frozen in OCT compound (Miles Laboratories, Inc., IL) on dry ice and stored at -80°C. Cryostat sections were prepared at -25°C, stained for GGT activity (18) and counter-stained with hematoxylin. The number of GGT-positive foci were scored under a light microscope and only foci containing 10 or more cells were counted. At least 2 cm<sup>2</sup> of tissue were scanned and the number of foci were calculated per cm<sup>2</sup>.

## Results

### Initiation by fusarin C

The first papillomas on DMBA-treated mice appeared 8 weeks after commencement of the promoting treatment with TPA. After 16 weeks all of these mice developed papillomas at an average of four papillomas per mouse (Table I). One mouse in group 4, initiated with 220 µg fusarin C and promoted with TPA developed two papillomas. None of the other mice in either the fusarin C-treated or the control groups developed papillomas.

In the rat liver model using male BD IX rats, DEN initiation followed by PB promotion (group 1) significantly ( $P < 0.01$ ) induced the formation of GGT-positive foci when compared with the control groups 2 and 3 (Table II). A similar result was obtained with female Wistar rats. However, in contrast to when using male BD IX rats, DEN initiation alone (group 7) significantly ( $P < 0.01$ ) induced GGT-positive foci in female Wistar rats when compared with the group that only received the promoting treatment (group 8). In neither of the two rat strains did fusarin C significantly induce the formation of GGT-positive foci. A dosage of 100 mg fusarin C/kg was lethal to all five female Wistar rats whereas two out of five male BD IX rats that received this dosage level died.

### Promotion by *F. moniliforme* strain MRC 826

Culture material of strain MRC 826, incorporated at a level of 2% into the diet, showed cancer promoting potential as it significantly ( $P < 0.01$ ) increased the formation of GGT-positive foci in hepatectomized rats initiated with DEN (group 1, Table III) when compared with rats treated only with DEN (group 2). No effects was obtained without DEN initiation (group 3). However, using non-hepatectomized rats without the initiating treatment, strain MRC 826 at a level of 5% in the diet (group 6) significantly ( $P < 0.05$ ) increased GGT-positive foci when compared with the group that received only the DEN (200 mg/kg) treatment (group 5). DEN treatment followed by the 'promoting' treatment with 5% culture material of strain MRC 826 (group 4) did however cause a larger increase ( $P < 0.025$ ) in the formation of GGT-positive foci.

## Discussion

The chemical nature of the active metabolite(s) responsible for the hepatocarcinogenicity (5,6) of *F. moniliforme* MRC 826 in rats is unknown. The isolation of the potent mutagen fusarin C from culture material of this strain (8–10) suggested that this compound might be involved, particularly since fusarin C has also been reported to be clastogenic in mammalian cells (11).

Table I. Cancer initiation potential of fusarin C on mouse skin

| Group | Initiation             | Promotion        | No. of mice | No. of mice with papillomas <sup>a</sup> | Total no. of papillomas <sup>a</sup> |
|-------|------------------------|------------------|-------------|--|--------------------------------------|
| 1     | DMBA <sup>b</sup>      | TPA <sup>c</sup> | 9           | 9  | 34                                   |
| 2     | DMBA                   | Acetone          | 8           | —  | —                                    |
| 3     | Acetone                | TPA              | 10          | —  | —                                    |
| 4     | Fusarin C <sup>d</sup> | TPA              | 10          | 1  | 2                                    |
| 5     | Fusarin C <sup>e</sup> | TPA              | 10          | —  | —                                    |

<sup>a</sup> After 16 weeks of promotion.

<sup>b</sup> Applied once only (50 µg in 0.1 ml acetone/mouse).

<sup>c</sup> Applied twice weekly (2.0 µg in 0.1 ml acetone/mouse).

<sup>d</sup> Applied once only (220 µg in 0.1 ml acetone/mouse).

<sup>e</sup> Applied once only (500 µg in 0.1 ml acetone/mouse).

Table II. Cancer initiation potential of fusarin C in rat liver

| Group                     | Initiation             | Promotion       | No. of rats    | GGT <sup>a</sup><br>mean no. of foci/cm <sup>2</sup> |
|---------------------------|------------------------|-----------------|----------------|--|
| <i>Male BD IX rats</i>    |                        |                 |                |  |
| 1                         | DEN <sup>c</sup>       | PB <sup>d</sup> | 7              | 7.54 ± 3.41 <sup>b</sup> (A)                         |
| 2                         | DEN                    | —               | 7              | 0.44 ± 0.73 (b)                                      |
| 3                         | DMSO                   | PB              | 7              | 0.46 ± 0.81 (b)                                      |
| 4                         | Fusarin C <sup>e</sup> | PB              | 5              | 0.20 ± 0.45 (b)                                      |
| 5                         | Fusarin C <sup>f</sup> | PB              | 5 <sup>g</sup> | 0.15 ± 0.31 (b)                                      |
| <i>Female Wistar rats</i> |                        |                 |                |  |
| 6                         | DEN <sup>c</sup>       | PB              | 5              | 12.20 ± 1.30 (C)                                     |
| 7                         | DEN <sup>c</sup>       | —               | 4              | 6.92 ± 3.58 (D)                                      |
| 8                         | DMSO                   | PB              | 4              | 0.33 ± 0.58 (e)                                      |
| 9                         | Fusarin C <sup>e</sup> | PB              | 5              | 0.40 ± 0.80 (e)                                      |
| 10                        | Fusarin C <sup>f</sup> | PB              | 5 <sup>h</sup> | —  |

<sup>a</sup> Means of male BD IX and female Wistar rats were not statistically compared. Means followed by the same letter do not differ significantly. If the letters differ, but the cases do not, then  $P < 0.025$ ; if the letters and cases differ, then  $P < 0.01$ .

<sup>b</sup> Values represent the mean ± SD.

<sup>c</sup> Administered once only in DMSO at 30 mg/kg.

<sup>d</sup> Administered at 0.05% in drinking water for 14 weeks.

<sup>e</sup> Administered once only in DMSO at 50 mg/kg.

<sup>f</sup> Administered once only in DMSO at 100 mg/kg.

<sup>g</sup> Two out of five rats died after fusarin C administration.

<sup>h</sup> All rats died after fusarin C administration.

Table III. Cancer promotion potential of culture material of *F. moniliforme* strain MRC 826 in rat liver

| Group                          | Initiation<br>DEN (mg/kg) <sup>c</sup> | Promotion<br>strain MRC 826<br>(% in diet) <sup>d</sup> | No. of rats | GGT <sup>a</sup><br>mean no. of foci/cm <sup>2</sup> |
|--------------------------------|--|---|-------------|--|
| <i>Hepatectomized rats</i>     |  |   |             |  |
| 1                              | 30                                     | 2   | 5           | 3.40 ± 2.30 <sup>b</sup> (A)                         |
| 2                              | 30                                     | —   | 10          | 0.44 ± 0.73 (b)                                      |
| 3                              | —                                      | 2   | 5           | 0.20 ± 0.45 (b)                                      |
| <i>Non-hepatectomized rats</i> |  |   |             |  |
| 4                              | 200                                    | 5   | 4           | 18.25 ± 3.50 (C)                                     |
| 5                              | 200                                    | —   | 3           | 2.33 ± 0.45 (d)                                      |
| 6                              | —                                      | 5   | 3           | 5.25 ± 1.71 (e)                                      |

<sup>a</sup> Means of hepatectomized and non-hepatectomized rats were not statistically compared. Hepatectomized rats: means followed by the same letter do not differ significantly. If the letters and the cases differ, then  $P < 0.01$ . Non-hepatectomized rats: when letters and cases differ, then  $P < 0.025$ ; if letters differ but not the cases, then  $P < 0.05$ .

<sup>b</sup> Values represent the mean ± SD.

<sup>c</sup> Administered once only.

<sup>d</sup> Culture material fed in the diet for 14 weeks.

However, results from a previous study (6) as well as those obtained during this investigation tend not to support this postulate. In the previous investigation (6) rats were fed diets containing culture material of either *F. moniliforme* strain MRC 826 or strain MRC 1069. The diet incorporating MRC 826 contained 0.5 mg fusarin C/kg whilst that incorporating strain MRC 1069 contained 18.2 mg/kg fusarin C. After prolonged exposure, only those rats receiving culture material of strain MRC 826 developed neoplastic lesions in the liver even though they ingested less fusarin C. In addition, liver sections obtained from the group treated with strain MRC 826 stained strongly positive for GGT (25–30 foci/cm<sup>2</sup> of liver tissue), whereas no positive foci were present in rats fed culture material of strain MRC 1069 (6).

There is more than one possible explanation for these observations. Firstly it is possible that cells initiated by fusarin C can be promoted by an active cancer promoter present in the culture material of strain MRC 826. The lack of carcinogenic activity of culture material of *F. moniliforme* strain MRC 1069 (6), even though it contains more fusarin C than strain MRC 826, may be due to the absence of such a promoter. The data of the present investigation has demonstrated promoting activity in the culture material of strain MRC 826 in the rat liver model using DEN (30 mg/kg) as initiator. However, no initiating activity could be demonstrated when fusarin C was tested in either the mouse skin or the rat liver models.

A second possibility is that fusarin C is not involved in the carcinogenic activity of culture material of strain MRC 826. This strain may produce a 'complete' carcinogen that can induce liver cancer upon feeding to rats. In a previous experiment (5) strain MRC 826 caused hepatocellular carcinoma in 80% and ductular carcinoma in 63% of the rats fed a diet containing between 2 and 4% of the culture material. When culture material was incorporated at a level of 0.5% into the diet, the incidence of liver cancer was much lower, (hepatocellular carcinoma in 10% and ductular carcinoma in 38% of the rats) although liver sections stained strongly positive for GGT (6). In the rat liver initiation/promotion model using non-hepatectomized rats without DEN initiation, strain MRC 826 (5% in the diet) induced GGT-positive foci over a period of 14 weeks. These findings imply that *F. moniliforme* strain MRC 826 produces an unidentified hepatocarcinogen that is capable of initiating and promoting liver cancer in rats.

In conclusion, our data do not indicate a positive role for fusarin C in the hepatocarcinogenicity of *F. moniliforme* strain MRC 826. It is possible that another compound(s) which is either not mutagenic or cannot be detected by the *Salmonella* mutagenicity test is involved in the carcinogenic activity of strain MRC 826. Attempts to isolate and characterize such a compound(s) are being continued. Although fusarin C does not appear to be hepatocarcinogenic in rats, indications have been found that it is acutely toxic to rats at high dosage levels. The effects of chronic exposure of rats and other animal species to fusarin C remain to be determined.

### Acknowledgements

The authors wish to thank C.W. Woodroof for tissue preparations and P.W. de Lange for the preparation of the diets.

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Received on 16 June 1986; accepted on 1 August 1986

## Cancer promoting potential of different strains of *Fusarium moniliforme* in a short-term cancer initiation/promotion assay

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A short-term cancer initiation/promotion bioassay was established to screen 10 toxic strains of *Fusarium moniliforme* for their cancer promoting activity in rats. The assay consisted of a four week 'promoting' treatment, effected by incorporating culture material (5%) of each strain into the diet, commencing one week after an initiation treatment with diethylnitrosamine (DEN, 200 mg/kg). The appearance of  $\gamma$ -glutamyltranspeptidase-positive (GGT<sup>+</sup>) foci was used as an indication of promoting activity. Three out of 10 strains of *F.moniliforme* obtained from corn from a high risk area for esophageal cancer in Transkei, southern Africa, had significant cancer promoting activity. A highly significant correlation was found between toxicity expressed as reduction in body weight gain and cancer promoting activity. This finding suggests that the compounds responsible for the hepatotoxicity and hepatocarcinogenicity of *F.moniliforme* could be identical.

### Introduction

A number of short-term *in vivo* cancer initiation/promotion assays have been developed in rats for quantification of the promoting potential of different chemicals (1-3). These test systems utilize a brief exposure of the animal to the initiating carcinogen, usually diethylnitrosamine (DEN\*) followed by the promoting treatment which often includes a mitotic stimulus, to selectively enhance the outgrowth of altered cells resulting from the initiating treatment. An increase in the number or area of hyperplastic nodules or the induction of  $\gamma$ -glutamyltranspeptidase-positive (GGT<sup>+</sup>) liver cell foci is normally used as an indication of the promoting potential of the test substance.

Many investigations have been carried out on the toxic properties of different strains of *Fusarium moniliforme* Sheldon, a common fungal contaminant of corn (4). An association between the incidence of *F.moniliforme* in corn and human esophageal cancer risk has been well established in the Republic of Transkei, southern Africa (5,6). The carcinogenic potential of this fungus was recently recognized when a highly toxic strain *F.moniliforme* MRC 826, isolated from corn in a high risk area for esophageal cancer in Transkei, was found to be hepatocarcinogenic in rats (7,8). Apart from the cholangiocarcinomas and hepatocellular carcinomas that developed almost all the rats treated over the 2 year period had bile duct proliferation, adenofibrosis, hyperplastic nodules, GGT<sup>+</sup> foci and esophageal basal cell hyperplasia. Esophageal carcinoma has, however, not been induced in animals with cultures of *F.moniliforme* MRC

\*Abbreviations: DMSO, dimethylsulfoxide; DEN, diethylnitrosamine; GGT<sup>+</sup>,  $\gamma$ -glutamyltranspeptidase-positive; PB, phenobarbital; SAS, statistical analyses system.

826 and consequently there is no experimental proof of a causative relationship between the fungus and human esophageal cancer.

More recently culture material of *F.moniliforme* MRC 826 was found to exhibit cancer promoting activity in a rat liver initiation/promotion assay (9). This assay was based on a model developed by Pitot *et al.* (10) utilizing hepatectomized rats, DEN (30 mg/kg) as an initiator and culture material, incorporated at a level of 2% in the diet over a period of 14 weeks as the promoter. The induction of GGT<sup>+</sup> foci was used as endpoint and the promoter phenobarbital (PB) incorporated as a positive control. Strain MRC 826 also exhibited cancer promoting activity in non-hepatectomized rats when incorporated at a level of 5% in the diet for 14 weeks following an initiating treatment of 200 mg DEN/kg (9). A far less pronounced effect was, however, obtained without the initiation treatment.

None of the mycotoxins known to be produced by *F.moniliforme* strain MRC 826 including the potent mutagen fusarin C, appear to be involved in its carcinogenic effects in rats (9). It is not known whether other strains of *F.moniliforme* also exhibit carcinogenic activity. This paper reports on the establishment of a short-term cancer initiation/promotion bioassay to investigate the promoting potential of toxic strains of *F.moniliforme* in rat liver. In addition any possible relationship between toxicity and cancer promoting activity was investigated.

### Materials and methods

#### Chemicals

DEN, glycylglycine, L- $\gamma$ -glutamyl-p-nitroanilide and L- $\gamma$ -glutamyl-4-methoxy- $\beta$ -naphthylamide were purchased from Sigma Chemical Company, St Louis, MO.

#### Fungal cultures

During 1985, samples of home-grown corn were collected from 10 households with one or more occupants showing esophageal cytological abnormalities in Kentani, an area in Transkei, southern Africa with a high esophageal cancer rate (5). The incidence of *F.moniliforme* in each corn sample was determined (6) and 10 strains of the fungus from each sample were isolated in pure culture. Cultures on corn of the 100 strains of *F.moniliforme* obtained in this way were screened for toxicity to ducklings as previously described (11). One toxic strain from each of the 10 households was single-spored, lyophilized and deposited in the culture collection of the South African Medical Research Council (MRC).

Lyophilized cultures of 10 Transkeian strains of *F.moniliforme* (MRC 4315-4324) obtained as described above were used to inoculate autoclaved corn (11). Corn cultures were incubated in the dark at 25°C for 2 weeks followed by 2 weeks at 15°C, harvested, lyophilized, stored in air-tight containers at 4°C and ground prior to use. The lyophilized corn cultures were tested for toxicity to ducklings (11), analyzed chemically for moniliformin and fusarin C, and tested for cancer promoting activity in rats as described below.

Strain MRC 826 of *F.moniliforme* was isolated from corn in Kentani, Transkei during 1975 (12). Lyophilized corn cultures of this strain were prepared as described above and compared with the 10 newly isolated Transkeian strains.

#### Chemical analyses

Moniliformin and fusarin C production of the different isolates were monitored according to the methods of Thiel *et al.* (13) and Gelderblom *et al.* (14), respectively. The latter method was modified as follows: after CHCl<sub>3</sub> : IPA (1 : 1, v/v) extraction, an aliquot of the extract representing 10 g of the starting material (50 g) was adsorbed on silica gel (5 g) by evaporation at 40°C. The sample, transferred quantitatively to a silica gel column, was fractionated using MeOH : CHCl<sub>3</sub> (5 : 95) as eluent as described (14).

**Table I.** Incidence and toxicity to ducklings of *Fusarium moniliforme* in home-grown corn from households with occupants showing esophageal cytological abnormalities in a high risk area for esophageal cancer in Transkei

| Household No. | Incidence of <i>F.moniliforme</i> in corn (% kernels infected) | Toxicity of <i>F.moniliforme</i> strains to ducklings (No. strains toxic/10 tested) <sup>a</sup> |
|---------------|--|--|
| 1             | 51   | 7  |
| 2             | 94   | 9  |
| 3             | 66   | 7  |
| 4             | 89   | 7  |
| 5             | 51   | 9  |
| 6             | 34   | 10   |
| 7             | 72   | 9  |
| 8             | 61   | 9  |
| 9             | 76   | 10   |
| 10            | 38   | 10   |
| Overall (%)   | 63   | 87   |

<sup>a</sup>Strains that caused the death of 4/4 ducklings within 14 days were considered to be toxic.

**Table II.** Toxicity to ducklings and fusarin C production by 10 strains of *Fusarium moniliforme* from Transkeian corn<sup>a</sup>

| Strain no. (MRC) | Toxicity to ducklings <sup>b</sup> |                       | Fusarin C concentration <sup>c</sup> (mg/kg $\pm$ SD) |
|------------------|------------------------------------|-----------------------|---|
|                  | Mean day of death (days)           | Total feed intake (g) |   |
| 4315             | 5                                  | 20                    | 120.5 $\pm$ 4.5                                       |
| 4316             | 6                                  | 30                    | 97.8 $\pm$ 9.2  |
| 4317             | 5                                  | 52                    | 296.0 $\pm$ 9.0                                       |
| 4318             | 5                                  | 28                    | 120.3 $\pm$ 5.3                                       |
| 4319             | 5                                  | 18                    | 87.1 $\pm$ 0.8  |
| 4320             | 5                                  | 130                   | 254.4 $\pm$ 10.4                                      |
| 4321             | 5                                  | 12                    | 96.6 $\pm$ 5.4  |
| 4322             | 5                                  | 22                    | 273.0 $\pm$ 5.0                                       |
| 4323             | 4                                  | 40                    | 199.3 $\pm$ 17.7                                      |
| 4324             | 4                                  | 36                    | 201.0 $\pm$ 5.0                                       |
| Control          | —                                  | 2400                  | —   |

<sup>a</sup>None of the strains produced moniliformin (detection limit 10 mg/kg).

<sup>b</sup>All strains caused the death of 4/4 ducklings.

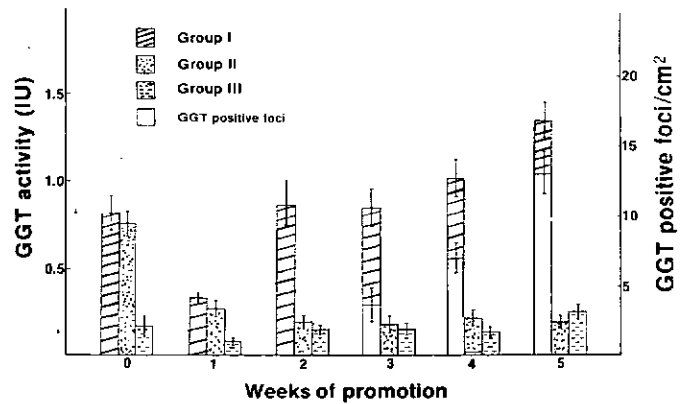
<sup>c</sup>Values represent mean  $\pm$  SD.

#### Rat experiments

Male BD IX rats of ~150 g body weight were used. Animals were kept individually in a controlled environment (23°C and 50% humidity) with a 12 h artificial light cycle. They were fed on rat mash, prepared from rat cubes (Epol Ltd, Johannesburg) and had free access to drinking water.

**Protocol 1.** The time dependent induction of GGT<sup>+</sup> foci by strain MRC 826 was investigated in three groups of 18 rats each. Rats of group I were initiated with DEN (200 mg/kg; i.p.) and after 1 week the promoting treatment (5% culture material in rat mash) was commenced. Rats of group II received the initiating treatment only and those of group III received DMSO instead of the initiating treatment. Three rats from each group were killed each week following the first week of the initiating treatment and GGT levels were determined in the livers both histochemically and spectroscopically (see below).

**Protocol 2.** The cancer promoting activities of culture material of the 11 strains of *F.moniliforme*, including MRC 826, were determined in 12 randomized groups of five rats each. The initiating treatment consisted of one injection of DEN (200 mg/kg, i.p.). The 'promoting' treatment commenced 1 week after initiation. The latter was obtained by incorporating culture material of each strain into the diet (rat mash) at a level of 5% (referred to as type 2 treatment). Due to a toxic effect, culture material of strains MRC 4315, 4319, 4321 and 826 were incorporated in the diet at a level of 2.5% for 1 week after the first week of the promoting treatment (referred to as type 3 treatment). The control group, initiated with DEN,



**Fig. 1.** Time dependent induction of GGT in male BD IX rats by culture material of *F.moniliforme* strain MRC 286. Treatments consisted of DEN initiation followed by the rat mash diet containing 5% of the culture material one week later (Group I), control groups receiving DEN (Group II) and DMSO (Group III) and fed on rat mash only.

received only rat mash (referred to as type 1 treatment). Rats were weighed twice weekly and the experiment terminated after 4 weeks of the 'promoting' treatment. The livers were subjected to GGT analyses as described below.

#### GGT analyses

Rats were sacrificed by decapitation and the liver excised. A tissue block (10 × 10 × 2 mm) was cut from the right lateral liver lobe and frozen on dry ice for histochemical determination of GGT. Cryostat sections (5 μm) were prepared at -20°C and stained for GGT according to the method of Rutenberg *et al.* (15). At least 2 cm<sup>2</sup> of tissue was scanned and the number of GGT<sup>+</sup> foci containing at least 10 cells were calculated per cm<sup>2</sup> of the tissue preparation.

The remaining right lateral liver lobe was used for the spectroscopical determination of GGT. Enzyme activity was determined by the method of Cameron *et al.* (16) utilizing L-γ-glutamyl-p-nitroanilide as substrate and glycylglycine as the γ-glutamyl acceptor. Specific activity (IU) was expressed as μmol substrate transformed per min per mg protein at 37°C using a molar extinction coefficient for p-nitroaniline of 10<sup>10</sup> M<sup>-1</sup> cm<sup>-1</sup>. Protein concentrations were determined according to the method of Lowry *et al.* (17).

#### Statistical methods

All the analyses were performed using the statistical analyses system (SAS) program package. The Student–Newman–Keuls multiple comparison method was used to test for significant differences between the means.

## Results

### Incidence of *F.moniliforme* and duckling toxicity

The incidence of *F.moniliforme* was very high in the 10 samples of home-grown corn from Kentani and the percentage kernels infected ranged from 34 to 94% (Table I).

The 100 strains of *F.moniliforme* obtained from these corn samples exhibited a high degree of toxicity to ducklings in the preliminary screening and 87 caused the death of 4/4 ducklings (Table I). Lyophilized corn cultures of 10 toxic strains selected from these 87 strains were also highly toxic and all caused the death of 4/4 ducklings within 6 days (Table II).

### Chemical analyses

None of the 10 toxic strains produced detectable amounts of moniliformin when analyzed using the analytical method with a detection limit of 10 mg/kg. Lyophilized culture material of all the strains contained high levels of fusarin C ranging from 87.1 to 296.0 mg/kg (Table II). The modified method for the analysis of fusarin C gave a higher percentage recovery (100%) than the previous method (80%) (14). *F.moniliforme* strain MRC 826 has previously been reported to produce fusarin C at a concentration of 83 mg/kg (14) but no moniliformin could be detected (12).

**Table III.** Toxicity and cancer promoting activity in DEN initiated rats of 11 strains of *Fusarium moniliforme* from Transkeian corn

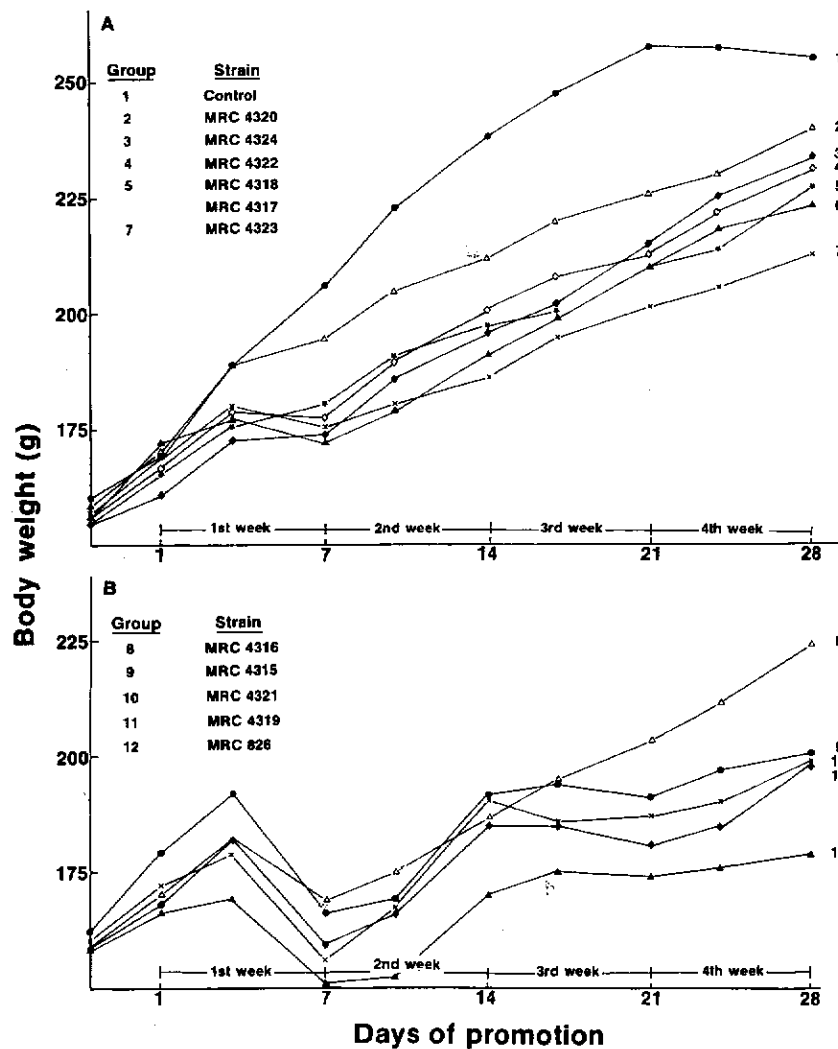
| Strain no.<br>(MRC) | Treatment <sup>a</sup><br>(Type) | Weight gain/loss during promotion <sup>b</sup> |                       | GGT activity <sup>b</sup>            |   |
|---------------------|----------------------------------|--|-----------------------|--------------------------------------|---|
|                     |                                  | 1st week <sup>c</sup>                          | 4th week <sup>c</sup> | Spectroscopical <sup>c</sup><br>(IU) | Histochemical <sup>c</sup><br>(foci/cm <sup>2</sup> ) |
| 4315                | 3                                | -13 ± 3.46 a                                   | 38.8 ± 19.54 ac       | 0.71 ± 0.04 A                        | 10.42 ± 5.51 A  |
| 4316                | 2                                | -0.8 ± 13.14 b                                 | 66.4 ± 21.76 bc       | 0.22 ± 0.21 BC                       | 0.62 ± 0.32 B   |
| 4317                | 2                                | 14.8 ± 4.71 b                                  | 68.0 ± 15.86 bc       | 0.08 ± 0.03 B                        | - B   |
| 4318                | 2                                | 11 ± 4.18 bc                                   | 71.8 ± 14.67 bc       | 0.14 ± 0.02 B                        | - B   |
| 4319                | 3                                | -13.8 ± 12.99 a                                | 39.6 ± 9.10 ac        | 1.12 ± 0.19 D                        | 19.96 ± 4.68 C  |
| 4320                | 2                                | 24.6 ± 3.78 d                                  | 79.6 ± 14.40 bc       | 0.08 ± 0.05 B                        | - C   |
| 4321                | 3                                | -15.4 ± 7.50 a                                 | 39.8 ± 12.48 ac       | 0.49 ± 0.12 B                        | 9.22 ± 2.65 A   |
| 4322                | 2                                | -0.4 ± 2.70 b                                  | 76.6 ± 16.70 bc       | 0.12 ± 0.02 B                        | - B   |
| 4323                | 2                                | 5.6 ± 5.03 bc                                  | 55.4 ± 19.67 bc       | 0.19 ± 0.06 BC                       | - B   |
| 4324                | 2                                | 13.4 ± 5.32 b                                  | 79.2 ± 15.56 bc       | 0.09 ± 0.01 B                        | - B   |
| 826                 | 3                                | -19.2 ± 5.45 a                                 | 7.4 ± 4.51 a          | 1.37 ± 0.35 E                        | 26.32 ± 6.01 D  |
| Control             | 1                                | 36.2 ± 6.06 e                                  | 91.4 ± 45.14 b        | 0.15 ± 0.03 B                        | - B   |

<sup>a</sup>Type 1, normal diet (rat mash); Type 2, culture material (5%) in diet for 4 weeks; Type 3, culture material (2.5%) for 1 week after the 1st week of promotion, 5% otherwise.

<sup>b</sup>Values represent means ± SD.

<sup>c</sup>Means in a column followed by the same letter do not differ significantly.

When small letters are assigned then  $P < 0.05$ , while for capital letters  $P < 0.01$ .



**Fig. 2.** Mean body weights of rats over the 'promoting' period of 4 weeks. (A) Culture material of the *F.moniliforme* strains incorporated in the diet at a level of 5% for 4 weeks. (B) Culture material of the strains, except for MRC 4316, incorporated at a level of 2.5% for a week after the first week of the 'promoting' treatment.

**Table IV.** Pearson correlation coefficients between body weight gains of rats and the induction of GGT

| Weight gain of rats        | GGT activity <sup>a</sup>   |                             |
|----------------------------|-----------------------------|-----------------------------|
|                            | Spectroscopical             | Histochemical               |
| First week of 'promotion'  | $r = -0.69$<br>$P < 0.0001$ | $r = -0.67$<br>$P < 0.0001$ |
| Fourth week of 'promotion' | $r = -0.71$<br>$P < 0.0001$ | $r = -0.68$<br>$P < 0.0001$ |

<sup>a</sup>Determined after 4 weeks of 'promoting' treatment.

#### Time dependent induction of GGT<sup>+</sup> foci (Protocol 1)

Three weeks after commencement of the promoting treatment of Group I rats with strain MRC 826, GGT<sup>+</sup> foci could be observed histochemically in the livers (Figure 1). The number of foci increased up to the fifth week of promotion when the experiment was terminated. No GGT<sup>+</sup> foci were observed in livers of the control groups.

An increase in GGT activity was determined spectroscopically in the livers of the DEN initiated rats (Groups I and II) on commencing with the promoting treatment one week after the initiating treatment. After the first week of the promoting treatment levels in both groups decreased and remained constant throughout the remainder of the experiment in rats from Group II. A marked increase in the spectroscopically determined GGT activity occurred in the Group I animals after the second week of the promoting treatment without any histochemically visible GGT<sup>+</sup> foci. From the third week of the promoting treatment and onwards the increased GGT activity coincided with the appearance of GGT<sup>+</sup> foci. No changes were detected in GGT activity of Group III rats.

#### Promoting activity of *F.moniliforme* strains (Protocol 2)

In addition to MRC 826, three out of 10 strains of *F.moniliforme* (MRC 4315, 4319 and 4321) significantly ( $P < 0.05$ ) induced the formation of GGT<sup>+</sup> foci after four weeks of the promoting treatment (Table III). These three strains all exhibited a significantly ( $P < 0.01$ ) lower promoting activity than strain MRC 826. A fourth strain (MRC 4316), slightly but not significantly induced GGT activity, whereas no effect was obtained with the remaining six strains. A highly significant correlation ( $r = 0.97$ ;  $P < 0.0001$ ) was found between the spectroscopical determination of GGT activity and the histochemical detection of GGT<sup>+</sup> foci.

The mean body weights of the rats during the four week promoting period are illustrated in Figure 2A and B. Culture material of all 11 strains of *F.moniliforme* significantly ( $P < 0.05$ ) reduced rat weight gains compared to the controls after the first week of the 'promoting' treatment (Table III).

Strains MRC 4315, 4319, 4321 and 826 caused significantly ( $P < 0.05$ ) greater reductions in weight gain than the other strains (Table III). The decrease in body weight caused by these four strains was overcome by reducing the amount of culture material in the diet to 2.5% for 1 week during the second week of the promoting treatment (Figure 2B). Rats receiving the culture material of the other seven strains recovered satisfactorily during the second week of the 'promoting' treatment at a dietary level of 5% (Figure 2A). After the fourth week of the 'promoting' treatment, body weights of rats in these groups were not significantly lower than the control group (Table III). Despite the reduction of the culture material of the other four strains to 2.5% in the diet for one week, the mean weight gains of rats

in these groups were still significantly ( $P < 0.05$ ) lower than those of the controls after 4 weeks.

Highly significant ( $P < 0.0001$ ) negative correlations were found between mean body weight gains of rats after the first and fourth week of the 'promoting' treatment and the induction of GGT determined both spectroscopically and histochemically (Table IV).

#### Discussion

The inability to characterize the toxic and carcinogenic compounds produced by *F.moniliforme* (7,8) has necessitated the development of a short-term bioassay in order to monitor their presence in biological samples. This has become even more important since a recent study indicated that these compounds are produced under natural conditions (18).

A number of short-term cancer initiation/promotion bioassays have been successfully used to assess the carcinogenic activity of different chemicals (1,3,19,20). A similar assay was established to monitor the carcinogenic potential of different toxic strains of *F.moniliforme* based on a model previously applied to determine the cancer promoting activity of strain MRC 826 (9).

The present study has demonstrated the induction of GGT<sup>+</sup> foci 3 weeks after DEN initiation (200 mg/kg) by culture material of strain MRC 826 at a dietary level of 5%. Although no GGT<sup>+</sup> foci could be observed after the second week of the 'promoting' treatment, an increase in the activity of the enzyme could be detected spectroscopically at that time. Histochemical examination of these liver sections indicated that the enzyme stained mainly in the periportal areas. Although some positive staining was also noticed in the focal areas, the latter were too small to be regarded as foci since they comprised < 10 cells. The induction of GGT in the periportal areas was also observed after the first week of DEN treatment. Since the induction of GGT in the periportal areas could occur under a variety of conditions not related to carcinogenesis (21), the above-mentioned periportal changes were probably related to toxic effects of DEN and culture material of strain MRC 826. For this reason the histochemical determination of GGT would be more valid for assessing cancer promoting activity. In the application of the assay it was therefore important that toxic effects should be reduced to a minimum by manipulation of the dietary levels of the culture material.

The present study, however, indicates a highly significant correlation between toxicity, expressed as a reduction in rat weight gain, and cancer promoting activity. Only the four strains that caused a significant ( $P < 0.05$ ) reduction in weight gain at the end of the promoting treatment, despite the fact that the level of the culture material was reduced in the diet, exhibited cancer promoting activity. It seems likely that the other strains tested could also exhibit cancer promoting activity at dietary levels higher than 5%.

Our results are in agreement with a postulate from a previous investigation (8) that a relationship exists between the toxicity and carcinogenicity of different strains of *F.moniliforme*. Data presented also suggest that the carcinogenicity of *F.moniliforme* may be mediated by a hepatotoxin acting either as a complete carcinogen, or as a cancer promoter which selectively enhances the outgrowth of initiated cells. This growth selection of initiated cells in the presence of the hepatotoxin could proceed via a mechanism similar to the induction of resistant hepatocytes according to the 'resistant hepatocyte model' (22). Initiated cells could result from the action of a cancer initiator produced by the fungus. Although the mutagen fusarin C is produced by



*F.moniliforme*, this compound exhibited no cancer initiating activity in rat liver (9). No relationship between the fusarin C production and the cancer promoting activity of the different strains was evident which is in agreement with previous results indicating that fusarin C is not carcinogenic (8,9).

The role of the cancer promoting principle(s) in the carcinogenicity of culture material of *F.moniliforme* can only be clarified once these compounds are chemically characterized and tested in long-term experiments in rats. The cancer promoting activity exhibited by the strains of *F.moniliforme* obtained from households of individuals at risk for esophageal cancer further emphasizes the importance of characterizing these compounds. However, at present the relevance of the carcinogenic effects of *F.moniliforme* in rats with regard to the development of human esophageal cancer is not known. The short-term model described in this paper is currently being used as a bioassay in the isolation and purification of the cancer promoting principle(s) from *F.moniliforme* strain MRC 826.

#### Acknowledgements

The authors wish to thank C.Woodroof for tissue preparations, P.W.de Lange and H.J.B.Joubert for conducting the rat experiments, Mrs M.Schlechter for culturing the fungal isolates and Mr E.Sydenham for the moniliformin analyses.

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Received on December 9, 1987; revised on February 22, 1988; accepted on April 25, 1988

# Toxicity of Culture Material of *Fusarium verticillioides* Strain MRC 826 to Nonhuman Primates

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We conducted a chronic feeding study in vervet monkeys (*Cercopithecus aethiops*) over 13.5 years. The experimental design consisted of two dietary treatment groups, each including males and females, fed varying levels of culture material of *Fusarium verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* Sheldon) strain MRC 826 mixed into their daily food ration. Two females were included as treatment controls. We conducted blood chemical analyses bimonthly and recorded all clinical signs during the course of the experiment. We took liver biopsies at various stages during the initial phase of the experiment. Several monkeys were terminated *in extremis* during the experiment. Detailed feed intake profiles were determined 5 years after the experiment began, and the fumonisin B (FB) mycotoxin content of the feed was determined during the final stages of the experiment. The apparent FB consumption patterns were related to changes observed in the biochemical parameters in the blood and urine, including the liver function enzymes and creatinine clearance as well as differential blood counts and sphingolipid levels in the serum and urine. An apparent no-effect threshold for kidney and liver damage is estimated to be between 0.11 and 0.18 mg FB/kg body weight (bw)/day, which corresponds to a feed contamination level of between 8.21 and 13.25 mg FB/kg bw diet. Apart from the effects on the liver and kidney, a wide variety of parameters, including cholesterol and creatine kinase, were also adversely affected. Several blood parameters, including white and red blood cells, also significantly decreased in the treated animals. The serum sphinganine level and the sphingosine/sphinganine ratio, monitored toward the end of the experiment, significantly increased in both the low-dose and high-dose animals. The present study provides important information about the diversity of lesions induced by culture material of *F. verticillioides* in vervet monkeys and the dosage levels of fumonisins to be used in long-term studies in nonhuman primates. **Key words:** culture material, fumonisins, *Fusarium verticillioides*, hepatotoxicity, nonhuman primates. — *Environ Health Perspect* 109(suppl 2):267–276 (2001). <http://ehpnet1.niehs.nih.gov/docs/2001/suppl-2/267-276gelderblom/abstract.html>

Toxicologic studies with culture material of *Fusarium verticillioides* (= *F. moniliforme*) grown on corn have been performed in a variety of experimental animals, including pigs, sheep, horses, baboons, and rats (1). The scientific importance of this fungal species, however, was first recognized with the finding that contamination of feedstuff, particularly corn, with *F. verticillioides* was responsible for natural outbreaks of the mycotoxicosis equine leukoencephalomalacia (ELEM) (2,3). This implied that the causative principle(s) occur(s) under natural conditions and could affect the health of livestock and also humans. The latter was of particular interest because many human populations in southern Africa consume corn as the major dietary staple (4). Efforts were launched in South Africa and elsewhere to develop sensitive screening methods to aid in the chemical isolation of the active compound(s). A duckling toxicity assay was designed whereby numerous isolates of the *F. verticillioides* were screened for the presence of toxic secondary metabolites produced by the fungus (5). Several isolates with different degrees of duckling toxicity were identified and formed the basis of further toxicologic studies in various animal species.

One of these, designated *F. verticillioides* strain MRC 826, was identified as one of the most toxic isolates from corn. This strain caused many toxic effects in experimental animals, such as ELEM in horses, pulmonary edema in pigs, acute nephrosis and hepatosis in sheep, and cirrhosis, intraventricular cardiac thrombosis, and nephrosis in rats (1). A less toxic strain, *F. verticillioides* MRC 602, also induced acute congested heart failure in baboons. A remarkable finding of these early experiments was that the main target organ differed in each animal species although certain organs, including the liver and kidneys, were consistently affected. It was suggested that rats provided the best screening system able to mimic biologic effects of the fungus in the various mammalian species (5). Toxicologic studies in rats eventually formed the basis for evaluating the carcinogenic effects of the potent mutagen fusarin C and subsequently for isolating the fumonisin (F)B mycotoxins from culture material of *F. verticillioides* MRC 826 (6,7).

Studies in nonhuman primates were initially performed with baboons (1). A prominent lesion was acute congestive heart failure, and liver cirrhosis was observed as a principal lesion in another baboon. The latter

study was performed between 5 months and 2 years at varying dietary levels of the culture material of *F. verticillioides* strain MRC 602. Subsequent studies were conducted in rats to evaluate the long-term toxicologic effects of different isolates of *F. verticillioides* including strain MRC 826 (8,9). At the same time, a long-term toxicity study was initiated at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council (MRC), Tygerberg, South Africa, in vervet monkeys (10,11) by feeding different dietary levels of *F. verticillioides* strain MRC 826. In this article we describe the clinical and biochemical data and dose–response effects in relation to the FB exposure levels during the course of the experiment (approximately 13.5 years).

## Materials and Methods

### Animals and Diets

Twelve vervet monkeys (*Cercopithecus aethiops*), consisting of eight females and four males weighing between 1 and 2 kg, were selected from the breeding colony of the MRC Primate Unit of the Experimental Biology Programme of the Medical Research Council, Tygerberg, South Africa. They were caged singly in a closed environment (26 ± 1°C) with a 12-hr photoperiod, 50% humidity, and 20 air changes/hr. The vervet monkeys were divided into three groups consisting of six animals (four females and two males) in the high-dose group, four animals (two males and two females) in the low-dose group, and two animals (females) in the control group. The diet consisted of 57 g of commercial pre-cooked corn meal, 1 g of an in-house vitamin mixture, and 10 g of a protein–vitamin–mineral supplement (PVM Product, Pretoria, South Africa). Culture material of *F. verticillioides* MRC 826 was incorporated at different

This article is based on a presentation at the International Conference on the Toxicology of Fumonisin held 28–30 June 1999 in Arlington, Virginia, USA.

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We thank N.P.J. Kriek for the preliminary histopathologic analyses and J.E. Fincham for his constructive criticism and involvement throughout this study.

Received 25 April 2000; accepted 27 September 2000.

concentrations during a pilot experiment to avoid excessive toxicity (Table 1). The control diet contained 0.5% autoclaved corn meal. All diets were prepared in bulk (approximately 9.5 kg) and stored at 4°C and from 1992 they were routinely analyzed for aflatoxins and FB mycotoxins. The mixed diet was prepared as a porridge after which vitamins D<sub>3</sub> (200 I.U.) and C (40 mg) were added before feeding. The estimated energy and nutrients supplied per vervet monkey per day have been calculated and published in detail elsewhere (11). The diet has been used to sustain successful in-house breeding over several generations with satisfactory development of the offspring (12). The diet was supplemented further with 35.4 ± 4.1 g of whole-wheat brown bread and a slice of apple (70.5 ± 6.6 g) in the afternoon. Feed wastage was monitored starting 4 years after the experiment began. This was accomplished by first weighing the dry and wet food as well as the wet wastage collected from underneath the cage. The dry wastage was extrapolated from the wet:dry ratio of the food. Housing the vervet monkeys in pairs after 5 years circumvented abnormal social behavior caused by solitary confinement. They also had access to large exercise cages for 24 hr/week.

One vervet monkey (female 681) of Group 1 was used in a pilot study (approximately 3 months) to evaluate early signs of toxicity from ingestion of culture material. As the experiment progressed, the level of the culture material incorporated in the diet was altered accordingly (Table 1) to avoid excessive toxicity. At that stage (1984/1985) the FB mycotoxins had not been characterized, and detailed chemical analyses of the diet and

culture material became possible only about 8 years after the experiment began (1991/1992). Subsamples of the different fungal culture batches used during the course of the experiment were retained and subsequently analyzed for fumonisins in order to calculate exposure levels. The different culture batches were prepared according to standardized procedures using autoclaved yellow corn kernels inoculated with single-conidial isolates of *F. verticillioides* strain MRC 826 and were incubated at 25°C for 2 weeks and then for 2 weeks at 15°C (8). Three different culture batches designated batch B (BB), batch 55 (B55), and batch 57 (B57) were used during the experiment. Batch BB was freeze dried; B55 and B57 were oven-dried at 45–50°C. The dry fungal cultures were ground to a fine meal and stored in airtight containers at 4°C.

### Clinical Monitoring

Appetite and habitus were observed daily; body weights, heart rates, and respiration rates were recorded at monthly intervals. Blood samples for measurement of clinical biochemical parameters were collected every 2 months throughout the experiment. These parameters included aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), gamma glutamyltransferase (GGT), alkaline phosphatase (ALP), conjugated, unconjugated, and total bilirubin, glucose, urea, creatinine, sodium, potassium, chloride, calcium, phosphorous, and magnesium. These parameters reflect liver, kidney, muscle, respiratory, intestinal, and bone functions. Full and differential counts of venous blood, collected in potassium EDTA tubes, were obtained with a

Technicon H 6010 C cytochemical flow through cell counter (Beckman/Coulter, Cape Town, South Africa). Plasma lipids and lipoproteins were analyzed at the time the final liver biopsies were taken and when the animals were sacrificed.

Urinary creatinine clearance was monitored when increased serum urea and creatinine became evident. A 24-hr urine sample of each vervet was collected into a plastic container with the aid of a stainless steel funnel (13). The funnels drained the entire cage floor and a strainer prevented contamination with feces. Clearance of creatinine from the serum was expressed as milliliters per kilogram of body weight per minute.

### Disruption of Sphingolipid Metabolism

It has been demonstrated in a variety of animal species that exposure to fumonisin mycotoxins altered sphingolipid metabolism and that FB<sub>1</sub> and FB<sub>2</sub> inhibit the enzyme ceramide synthase in the sphingolipid biosynthetic pathway (14). At the cellular level this leads to an accumulation of sphinganine, which is then manifested in changes in the circulating levels of this base. During the course of the experiment, serum levels of the sphingoid bases sphingosine and sphinganine (and hence their ratio) were monitored over 60 weeks from January 1994 to March 1995 (15). By this time, the experimental groups (controls, low-dose and high-dose groups) consisted of two females each. Serum samples were drawn at regular intervals during this period to yield 10 sets of results. In addition, 24-hr urine samples were collected at the same time as the last two blood samples.

**Table 1.** Summary of the duration, dietary level, and daily intake profiles of different *F. verticillioides* MRC 826 culture batches used during the chronic feeding study in vervet monkeys.<sup>a</sup>

| Group                           | No. of<br>vervet (sex)                              | Feeding regimens              |      |                         |                           |                    |                              |          | Terminated |
|---------------------------------|---|-------------------------------|------|-------------------------|---------------------------|--------------------|------------------------------|----------|------------|
|                                 |   | FCM <sup>b</sup><br>(MRC 826) | (%)  | Commenced<br>(dd/mm/yy) | Termination<br>(dd/mm/yy) | Duration<br>(days) | FMC intake<br>(mg/kg bw/day) |          |            |
| Group 1<br>High dose<br>(trial) | 681(F)  | BB                            | 5    | 13/12/84                | 03/01/85                  | 21                 | 705.8                        | 30/06/87 |            |
|                                 |   | BB                            | 1    | 04/01/85                | 18/01/85                  | 14                 | 141.2                        |          |            |
|                                 |   | BB                            | 0.25 | 19/01/85                | 29/01/86                  | 375                | 35.3                         |          |            |
|                                 |   | B55                           | 0.50 | 30/01/86                | 31/03/87                  | 425                | 70.6                         |          |            |
| Group 1<br>High dose            | 688 (F)<br>702 (M)<br>705 (M)<br>709 (F)<br>712 (F) | BB                            | 1    | 14/03/85                | 23/04/85                  | 40                 | 141.40                       | 03/11/92 |            |
|                                 |   | BB                            | 0.25 | 24/04/85                | 29/01/86                  | 280                | 35.30                        | 30/08/89 |            |
|                                 |   | BB                            | 0.50 | 30/01/86                | 31/03/87                  | 425                | 70.60                        | 24/07/92 |            |
|                                 |   | B55                           | 0.50 | 01/04/87                | 24/10/88                  | 572                | 70.60                        | 14/09/95 |            |
|                                 |   | B57                           | 0.50 | 25/10/88                | 05/06/91                  | 953                | 72.8 ± 17.9 <sup>c</sup>     | 11/11/98 |            |
|                                 |   | B57                           | 1    | 06/09/91                | 11/11/98                  | 2623               | 151.0 ± 36.8 <sup>c</sup>    |          |            |
| Group 2<br>Low dose             | 696 (M)<br>700 (M)<br>710 (F)<br>711 (F)            | BB                            | 0.25 | 14/03/85                | 23/04/85                  | 40                 | 34.0                         | 14/01/94 |            |
|                                 |   | BB                            | 0.1  | 24/04/85                | 29/01/86                  | 280                | 14.0                         | 30/10/92 |            |
|                                 |   | BB                            | 0.25 | 30/01/86                | 31/03/87                  | 425                | 34.0                         | 30/07/97 |            |
|                                 |   | B55                           | 0.25 | 01/04/87                | 24/10/88                  | 572                | 34.0                         | 11/11/98 |            |
|                                 |   | B57                           | 0.25 | 25/10/88                | 05/06/91                  | 953                | 35.2 ± 8.2 <sup>c</sup>      |          |            |
|                                 |   | B57                           | 0.5  | 06/09/91                | 11/11/98                  | 2623               | 73.6 ± 17.4 <sup>c</sup>     |          |            |
| Group 3<br>Control              | 707 (F)<br>708 (F)                                  | Corn meal                     | 0.5% | 14/03/85                | 11/11/98                  | 4993               | 84.5 ± 15.7 <sup>c</sup>     | 11/11/98 |            |
|                                 |   | Corn meal                     | 0.5% | 14/03/85                | 11/11/98                  | 4993               | 84.5 ± 15.7 <sup>c</sup>     | 11/11/98 |            |

<sup>a</sup>Values are means ± SD of at least 20 determinations per group of the surviving vervets. <sup>b</sup>Total fumonisin concentration: MRC 826 BB = 5.31 mg FB/kg, MRC 826 B55 = 2.61 mg FB/kg, and MRC 826 B57 = 3.43 mg FB/kg. <sup>c</sup>Calculated from feed intake data monitored 3–6 times per year from 1989.

## Biopsy and Necropsy Procedures

The toxic effects of the different feeding regimens were evaluated in liver biopsies taken at different time intervals during the course of the experiment. Subsequent investigations regarding histopathologic changes in the liver and kidneys of the animals terminated during the experiment and those sacrificed at the end of the experiment are currently in progress and will be reported elsewhere. Initial biopsies were taken on alternative monkeys of each group on days 2 and 6 of the experiment, and all individuals were subjected to biopsies at 180, 300, 520, 890, and 1,631 days after commencement. Wedge liver biopsies were taken by laparotomy under halothane general anesthesia. For the histopathologic evaluation of these biopsies, we used different staining procedures, including orcin stain to detect bile acids, cholesterol, and copper in hepatocytes; standard hematoxylin and eosin; periodic acid–Schiff; and reticulin stains (10,11).

Necropsy procedures in monkeys that were terminated *in extremis* and at the end of the experiment were performed after perfusion fixation under surgical anesthesia. For this fixation, 4% buffered formalin (pH 7.2) was perfused into the left ventricle at physiologic pressure (100 mm Hg) and flow. Before the perfusion, blood samples and fresh biopsies were collected from the liver and kidneys

for electron microscopy and biochemical analyses. Cutting the femoral and jugular veins as well as cutting deeply into the kidneys and liver ensured free flow of fixative. Immersion of the organs in the buffered formalin, at which time the organ weights were recorded, completed fixation.

## Data Analysis

All statistical analyses and graphical summaries were performed with the Number Cruncher Statistical System (NCSS 2000), Statistical System for Windows (16). The correlation coefficients reported are the partial coefficients—the correlation between two variables, with the effect of other possible confounders excluded. The *p* values reported are the partial values obtained from the multiple regression analyses—the relationship between the dependent and the independent variables after the effect of the other independent variables has been taken into account. The plots used the spline smoother of the scatterplot option of the program to draw the curves.

## Results

### Clinical Observations, Biopsies, Necropsies, and Termination

The biopsies, which were taken at stipulated time points, resulted in the death of one

female. The histopathologic evaluations of these biopsies have been reported elsewhere and suggested an active chronic toxic hepatitis (10) at a very early stage, whereas mild portal-to-portal fibrosis was reported in some animals of the high-dose and low-dose groups (11). Clinical observations indicated that the females were in better condition than the males. A number of vervet monkeys of the low-dose and high-dose groups were terminated during the course of the experiment because of general poor health (Table 2). These conditions of cachexia include ataxia, poor food consumption, and weight loss. The experiment was terminated 4,990 days after commencement. At that stage the two controls (females 707 and 708) and only one each of the low-dose (female 711) and high-dose (female 712) groups survived. The livers of all the animals killed during treatment and at termination showed signs of chronic toxic hepatitis. Some animals (females 711 and 712) showed patchy atheromatous plaques in the aorta. One animal (male 702) died from brain hemorrhages with lesions in the cerebellum, midbrain, and the right cortex. The kidneys were also affected, although some animals, such as male 702, showed no specific lesions. In one of the animals (male 705) terminated after 2,689 days of treatment, only microscopic cytoplasmic vacuolization of

**Table 2.** Clinical symptoms and lesions observed at different times during the experiment as well as the major macroscopic changes noticed at termination.<sup>a</sup>

| Number | Sex | Dosage group | Days after commencement | Symptoms and/or lesions   | Necropsy (macroscopic observations)   |
|--------|-----|--------------|-------------------------|---|---|
| 681    | F   | H            | 929                     | Hemorrhage in abdomen (terminated)  | Pale liver with irregular surface   |
| 688    | F   | H            | 2,791                   | Alopecia on trunk and thighs  | Fibrotic, atrophic liver (chronic toxic hepatitis)  |
| 702    | M   | H            | 1,630                   | Cachexic, ataxic, difficulty in handling and eating food                          | Hemorrhages of about 1 cm in grey matter of midbrain; most recent and largest lesions found in the cerebellum, midbrain, and right cortex of the midbrain         |
| 705    | M   | H            | 2,689                   | Focal hemorrhages in the skin   | Necrosis of the distal and proximal rectum; fibrotic liver (chronic toxic hepatitis); hard kidneys with streaks of blood from the corticomedullary junction       |
| 709    | F   | H            | 2,497                   | Weight loss, yellow discoloration of the periorbital skin, blood in feces         | Fibrotic, atrophic liver (chronic toxic hepatitis)  |
|        |     |              | 3,836                   | Yellow discoloration of the skin  |   |
| 712    | F   | H            | 4,990                   | Termination of experiment   | Liver atrophic accentuated lobulation (chronic toxic hepatitis); multifocal to confluent atheromatous plaques throughout the aorta                                |
| 696    | M   | L            | 2,414                   | Torsion of the colon  | Small, fibrotic liver (chronic toxic hepatitis); colon and stomach contained hemorrhagic ulcers   |
|        |     |              | 3,228                   | Torsion of the colon  |   |
| 700    | M   | L            | 896                     | Mild subcutaneous hemorrhage in the groin area                                    | Fibrotic liver (chronic toxic hepatitis); hemorrhagic diathesis; mild icterus   |
|        |     |              | 1,762                   | Erythema on right thigh and left abdomen  |   |
|        |     |              | 2,723                   | Mild alopecia on left arm, blood in feces   |   |
|        |     |              | 2,747                   | Vomition, reluctant to move   |   |
| 710    | F   | L            | 2,650                   | Starring coat   | Hard fibrotic liver (chronic toxic hepatitis); kidneys showed petechial hemorrhages in cortex; enlarged lymph nodes and inflamed mesenteries                      |
|        |     |              | 2,738                   | Blood in feces  |   |
|        |     |              | 2,812                   | Yellowish discoloration of skin   |   |
|        |     |              | 4,521                   | Cachexia, vomition  |   |
| 711    | F   | L            | 896                     | Focal hemorrhages in the skin of abdomen and left leg (disappeared after 30 days) | Atrophic liver with nodular appearance and accentuated lobulation and cobblestone effect; chronic toxic hepatitis; a few patchy atheromatous plaques in the aorta |
|        |     |              | 3,707                   | Consistently poor feed consumption  |   |
|        |     |              | 4,990                   | Terminated experiment   |   |
| 707    | F   | Controls     | 4,990                   | Termination of experiment   | No clinical signs   |
| 708    | F   | Controls     | 4,990                   | Termination of experiment   | No clinical signs   |

<sup>a</sup>All cases were terminated *in extremis* except females 707, 708, 711, and 712.

epithelial cells of convoluted tubules was present. The other cases that were terminated demonstrated mild to severe cortical nephrosis manifested as toxic atrophy of the epithelia of the convoluted tubules and probably the glomeruli. No acute or chronic inflammation was present. Final body and organ weights (Table 3) indicated that the relative liver weights of the high-dose group appeared to be slightly lower than that of the low-dose group, but the low number of animals and differences in sex meant that statistical analyses could not be performed. However, despite the toxic effects encountered in the liver and kidneys, multiple regression analyses showed a positive correlation ( $r = 0.47$ ;  $p < 0.0001$ ) of fungal culture material (FCM) intake and body weight. It should be noted that the higher dose levels were administered from 1992 onward during the stage of the experiment where the vervets had established their adult weight. The loss in body weight in those vervet monkeys sacrificed *in extremis* was encountered only a few months before

termination. Therefore the dietary levels of FCM used apparently did not affect the normal body weight gain of the vervet monkeys with age.

### Feed and Fumonisin Intake Parameters

Feed intake profiles were conducted in detail about 4 years after commencement of the experiment. Discovery of the fumonisins (7) and the development of the relevant analytic methodology (17) resulted in routine analyses of the maintenance diet for the fumonisins, including retrospective analyses of the different culture batches used. Given these data, the FB intake during the earlier part of the experiment could be estimated fairly accurately (Table 4). However, several variables complicate the calculation of accurate exposure data, expressed as mean intake per kilogram body weight per day for the duration of the experiment. These variables included the varying levels of the fungal material in the diet as well as the use of different culture batches (Table 1) containing different levels

of total FB (FB<sub>1</sub>, FB<sub>2</sub>, and FB<sub>3</sub>), namely MRC 826 BB (5.38 mg FB/kg), MRC 826 B55 (2.61 mg/FB/kg), and MRC BB57 (3.73 mg FB/kg). During the last 8 years only one culture batch (BB57) was used, although the dietary level of the culture material was raised from 0.25 to 0.5% in the low-dose group and from 0.5 to 1% in the high-dose group. The mean total FB intake of the vervet monkeys in the different groups, as a function of the various cultures and dietary levels used during the experimental period, is illustrated in Figure 1.

### Clinical Pathology

For the purpose of this article, the parameters used to assess liver function are presented separately from those associated with kidney function.

**Serum enzymes associated with liver function. General patterns.** Multiple regression analyses of the serum enzyme indicators associated with hepatocellular damage (AST, ALT, LDH, and GGT) indicated that males

**Table 3.** Comparison of body and organ weights of the vervet monkeys fed high and low doses of *F. verticillioides* culture material for different time periods.

| Vervet    | Terminated (dd/mm/yy) | Body weight |       | Liver (g) |       | Left kidney (g) |       | Right kidney (g) |       | Total kidney (g) |       | Heart (g) |       | Brain (g) |  |
|-----------|-----------------------|-------------|-------|-----------|-------|-----------------|-------|------------------|-------|------------------|-------|-----------|-------|-----------|--|
|           |                       | (kg)        | %     | %         | %     | %               | %     | %                | %     | %                | %     | %         | %     |           |  |
| High dose |                       |             |       |           |       |                 |       |                  |       |                  |       |           |       |           |  |
| 681 (F)   | 30/06/87              | 3.67        | 55    | 1.50      | 7.564 | 0.21            | 7.352 | 0.20             | 14.92 | 0.41             | 20.5  | 0.56      | 71    | 1.93      |  |
| 702 (M)   | 30/08/89              | 4.29        | 64.4  | 1.50      | 6.84  | 0.16            | 7.45  | 0.17             | 14.29 | 0.33             | 24.7  | 0.58      | 69.82 | 1.63      |  |
| 705 (M)   | 24/07/92              | 4.79        | 69.2  | 1.44      | 8.35  | 0.17            | 8.31  | 0.17             | 16.66 | 0.35             | 36    | 0.75      | 63.9  | 1.33      |  |
| 688 (F)   | 03/11/92              | 2.70        | 53.5  | 1.98      | 8.885 | 0.33            | 9.884 | 0.37             | 18.77 | 0.70             | 16.9  | 0.63      | 62    | 2.30      |  |
| 709 (F)   | 14/09/95              | 3.38        | 43.62 | 1.29      | 8.91  | 0.26            | 9.7   | 0.29             | 18.61 | 0.55             | 21.59 | 0.64      | 63.3  | 1.87      |  |
| 712 (F)   | 11/11/98              | 5.71        | 79.49 | 1.39      | 8.94  | 0.16            | 8.11  | 0.14             | 17.05 | 0.30             | 49.23 | 0.86      | 71.96 | 1.26      |  |
| Low dose  |                       |             |       |           |       |                 |       |                  |       |                  |       |           |       |           |  |
| 700 (M)   | 30/10/92              | 4.94        | 69.5  | 1.41      | 10.2  | 0.21            | 9.9   | 0.20             | 20.10 | 0.41             | 28.2  | 0.57      | 65.5  | 1.33      |  |
| 696 (M)   | 14/01/94              | 5.71        | 86.96 | 1.52      | 10.66 | 0.19            | 14.95 | 0.26             | 25.61 | 0.45             | 39.78 | 0.70      | 66.4  | 1.16      |  |
| 710 (F)   | 30/07/97              | 3.24        | 82.1  | 2.53      | 7.66  | 0.24            | 7.37  | 0.23             | 15.03 | 0.46             | 22    | 0.68      | 68    | 2.10      |  |
| 711 (F)   | 11/11/98              | 4.48        | 75.78 | 1.69      | 9.32  | 0.21            | 9.28  | 0.21             | 18.60 | 0.42             | 31.7  | 0.71      | 70.54 | 1.57      |  |
| Controls  |                       |             |       |           |       |                 |       |                  |       |                  |       |           |       |           |  |
| 707 (F)   | 11/11/98              | 3.23        | 70.1  | 2.17      | ND    |                 | ND    |                  | ND    |                  | ND    |           | 59.88 | 1.85      |  |
| 708 (F)   | 11/11/98              | 3.69        | 66.56 | 1.80      | 8.43  | 0.23            | 8.79  | 0.24             | 17.22 | 0.47             | 23.9  | 0.65      | 57.57 | 1.56      |  |

ND, not determined.

**Table 4.** Summary of the body weight in relation to the average feed, *F. verticillioides* culture material, and the calculated and estimated fumonisin intake during the experiment.

| Vervet    | Duration (days) | Initial | Body weight (kg)   |                  | Average feed intake (g/kg bw/day) | Mean FCM/kg bw/day (mg) | FB/kg bw/day (mg)        |                         | Total FB (mg) estimated |
|-----------|-----------------|---------|--------------------|------------------|-----------------------------------|-------------------------|--------------------------|-------------------------|-------------------------|
|           |                 |         | Maximum (dd/mm/yy) | Final (dd/mm/yy) |                                   |                         | Estimated <sup>a</sup>   | Calculated <sup>b</sup> |                         |
| High dose |                 |         |                    |                  |                                   |                         |                          |                         |                         |
| 681 (F)   | 929             | 2.57    | –                  | 3.07 (30/06/87)  | 14.12 ± 2.14                      | 80.03 ± 44.59           | 0.38 ± 0.27 <sup>c</sup> | –                       | 330.4                   |
| 688 (F)   | 2,791           | 2.42    | 4.05 (24/07/91)    | 2.70 (03/11/92)  | 15.45 ± 1.79                      | 90.4 ± 40.9             | 0.39 ± 0.22              | –                       | 861.2                   |
| 702 (M)   | 1,630           | 1.75    | 4.45 (14/02/89)    | 4.29 (30/08/89)  | 14.12 ± 2.14                      | 77.6 ± 38.7             | 0.36 ± 0.24              | –                       | 431.6                   |
| 705 (M)   | 2,689           | 1.76    | 5.20 (15/03/91)    | 4.79 (24/07/92)  | 15.45 ± 1.79                      | 89.5 ± 44.7             | 0.39 ± 0.22              | –                       | 832.7                   |
| 709 (F)   | 3,836           | 1.50    | –                  | 3.38 (14/09/95)  | 16.10 ± 1.55                      | 98.5 ± 55.5             | 0.42 ± 0.25              | –                       | 1714.6                  |
| 712 (F)   | 4,990           | 1.50    | 3.92 (27/04/95)    | 3.70 (11/11/98)  | 16.11 ± 1.50                      | 80.1 ± 35.56            | 0.35 ± 0.21              | 0.49 ± 0.20             | 2147.2                  |
| Low dose  |                 |         |                    |                  |                                   |                         |                          |                         |                         |
| 696 (M)   | 3,228           | 2.09    | 6.03 (26/08/93)    | 5.71 (14/01/94)  | 14.91 ± 1.21                      | 36.89 ± 18.69           | 0.15 ± 0.07              | –                       | 1304.08                 |
| 700 (M)   | 2,747           | 2.25    | 6.03 (15/01/91)    | 4.94 (30/10/92)  | 14.23 ± 1.46                      | 36.89 ± 18.69           | 0.15 ± 0.07              | –                       | 1022.98                 |
| 710 (F)   | 4,527           | 1.61    | 3.65 (24/07/91)    | 3.24 (30/07/97)  | 15.1 ± 1.08                       | 37.78 ± 20.68           | 0.16 ± 0.08              | –                       | 1846.20                 |
| 711 (F)   | 4,990           | 1.62    | –                  | 4.48 (11/11/98)  | 15.1 ± 1.08                       | 37.78 ± 20.68           | 0.16 ± 0.08              | 0.17 ± 0.09             | 2037.97                 |
| Control   |                 |         |                    |                  |                                   |                         |                          |                         |                         |
| 707 (F)   | 4,990           | 1.58    | 3.38 (25/06/96)    | 3.23 (10/11/98)  | 17.74 ± 1.67                      | 88.72 ± 8.34            | 0.01 ± 0.006             | –                       | –                       |
| 708 (F)   | 4,990           | 1.45    | 3.80 (05/08/98)    | 3.69 (10/11/98)  | 17.74 ± 1.67                      | 88.72 ± 8.34            | 0.01 ± 0.006             | 0.01 ± 0.01             | 48.94                   |

<sup>a</sup>Estimated from the feed intake profiles. Levels that were estimated before 1989 were based on the feed intake profiles that were measured during 1989. <sup>b</sup>Dosage was calculated from routine analyses of the stock feed samples since 1992. <sup>c</sup>Exposure of female 681 to 5% for 40 days was not included in calculating the average fumonisin intake profile. The average intake during that period was 3.79 mg FB/kg/day.

showed higher levels of LDH and GGT and females had higher levels of ALT; there was no difference between them with respect to AST serum levels (Table 5; Figure 2). AST and GGT increased significantly as a function of time, whereas ALT and LDH decreased. Females exhibited a higher cholesterol level; males had higher levels of total bilirubin. Overall, the total bilirubin increased as a function of time, but cholesterol was not affected. All the markers mentioned above were significantly ( $p < 0.0001$ ) positively correlated with the levels of the FCM and hence the levels of total FB. Glucose and ALP, serum markers not necessarily associated with liver function, showed a negative ( $r = -0.48$ ;  $p < 0.0001$ ) and a positive ( $r = 0.40$ ;  $p < 0.0001$ ) correlation, respectively, with FCM (Table 5; Figure 2). Glucose tended to increase with time, but

there were no differences between males and females. ALP decreased significantly with age, with males exhibiting higher levels than females. Creatine kinase, measured only from 1991 onward, increased with time and was also positively correlated ( $r = 0.57$ ;  $p < 0.0001$ ) with FCM (Table 5).

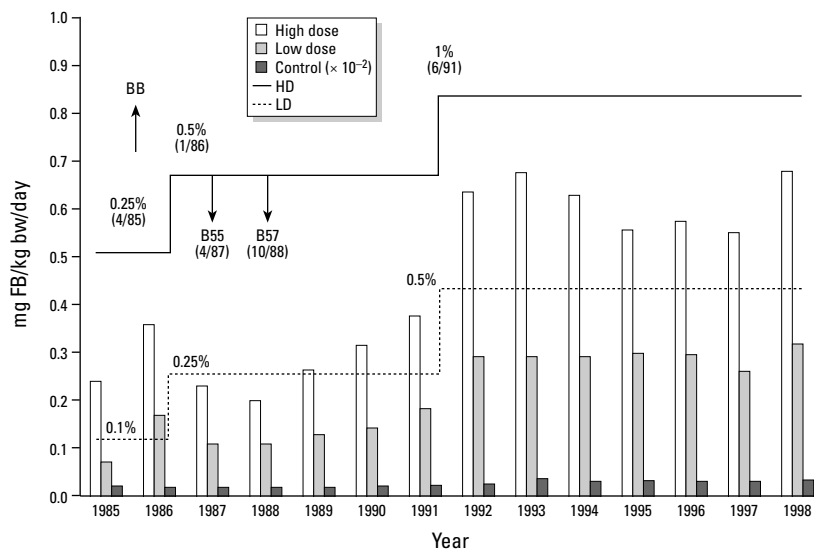
**Dose-response effects.** When considering the above results, two critical time periods exist (Figure 1): when the FB intake was reduced as a result of the use of different culture batches between 1986 and 1990; and when the FB intake profiles were increased due to a doubling of the dietary level of the culture material between 1991 and 1992.

During 1987 a different culture batch of the fungus (B55) replaced the original batch (BB), decreasing the FB intake profile. Both culture batches were included at a dietary

level of 0.5% (high dose) and 0.25% (low dose). The total FB concentrations were 5.38 mg/kg and 2.61 mg/kg culture material for BB and B55, respectively. The FB intake profiles decreased from 0.18 to 0.11 mg/kg bw/day in the low-dose group and from 0.38 to 0.18 mg/kg bw/day in the high-dose group. Toward the end of 1988 another culture batch, B57 (3.73 mg FB/kg), was introduced into the experiment and slightly increased the FB intake profiles to  $0.13 \pm 0.03$  and  $0.29 \pm 0.04$  mg/kg bw/day during 1989 and 1990 for the low-dose and high-dose groups, respectively (Figure 1).

A downward trend occurred in the liver function enzymes, AST and ALT, during the 1986–1988/1989 period in the high-dose group (Figure 2). In the low-dose group, the activities of the enzymes were very similar to those of the control females. No differences were observed between the dosage groups with respect to the total bilirubin and blood glucose levels. However, serum cholesterol levels showed a downward trend in the high-dose group similar to that mentioned for AST and ALT. The levels of ALP tended to decrease with time in the controls and the low-dose group, but this decrease was markedly delayed in the high-dose group. When the dietary level of the culture material (B57) was increased 2-fold during the end of 1991, AST, ALT, and cholesterol levels increased markedly during 1992 in both the high-dose and low-dose groups compared to the control group. There were no clear differences between the low-dose and high-dose groups toward the end of the experiment, although a higher trend was observed initially in AST and cholesterol levels (Figure 2). The same holds for total serum bilirubin and blood glucose, which, respectively, increased and decreased markedly in the high-dose group. The low-dose group also showed marginal effects with levels just above those of the control females. Creatine kinase, monitored from 1991 onward, also showed a marked dose-response effect with no clear-cut difference between the high-dose and low-dose groups (data not shown).

**Serum enzymes associated with kidney function. General patterns.** Serum urea ( $r = 0.41$ ;  $p < 0.0001$ ) and creatinine ( $r = 0.74$ ;  $p < 0.0001$ ) were positively correlated with the dietary levels of FCM and increased significantly compared to the controls (Table 4). In both cases males exhibited higher levels than females; urea decreased and creatinine increased as a function of time. Multiple regression analyses indicated that FCM was negatively correlated ( $r = -0.47$ ;  $p < 0.0001$ ) with a decrease in creatinine clearance. The latter also tended to decrease as a function of time; males had lower clearance values than females.



**Figure 1.** Daily fumonisin intake data of the low-dose (dotted line) and high-dose (solid line) groups of vervet monkeys as a function of the different culture batches of *Fusarium verticillioides* strain MRC 826 (BB, B55, B57) used during the course of the experiment.

**Table 5.** Partial correlation coefficients from multiple regression analyses of different serum parameters associated with liver and kidney function. The correlation factor for FCM was corrected for age and sex.

| Dependent                           | Sex     |                        | Age   |                        | FCM   |                        | $R^2$  |
|-------------------------------------|---------|------------------------|-------|------------------------|-------|------------------------|--------|
|                                     | $r^a$   | $p$ Level <sup>b</sup> | $r$   | $p$ Level <sup>b</sup> | $r$   | $p$ Level <sup>b</sup> |        |
| <b>Serum</b>                        |         |                        |       |                        |       |                        |        |
| Urea (log)                          | -0.11   | 0.0035                 | -0.27 | < 0.0001               | 0.41  | < 0.0001               | 0.2191 |
| Creatinine (log)                    | -0.40   | < 0.0001               | 0.28  | < 0.0001               | 0.56  | < 0.0001               | 0.3629 |
| Glucose (log)                       | 0.08    | 0.0744                 | 0.21  | < 0.0001               | -0.48 | < 0.0001               | 0.2624 |
| Cholesterol (log)                   | 0.17    | < 0.0001               | -0.05 | 0.1177                 | 0.40  | < 0.0001               | 0.1856 |
| Total bilirubin (log)               | -0.20   | < 0.0001               | 0.27  | < 0.0001               | 0.37  | < 0.0001               | 0.2599 |
| AST (log)                           | 0.00    | 0.9597                 | -0.13 | 0.0001                 | 0.67  | < 0.0001               | 0.4545 |
| ALT (log)                           | 0.24    | < 0.0001               | -0.19 | < 0.0001               | 0.42  | < 0.0001               | 0.2067 |
| LDH (log)                           | 0.02    | 0.6535                 | -0.28 | < 0.0001               | 0.47  | < 0.0001               | 0.2366 |
| GGT (log)                           | -0.13   | 0.0002                 | 0.10  | 0.0055                 | 0.53  | < 0.0001               | 0.3296 |
| ALP (log)                           | 0.14    | < 0.0001               | -0.72 | < 0.0001               | 0.40  | < 0.0001               | 0.5207 |
| Creatinine kinase (log)             | 0.00    | 0.9742                 | 0.29  | < 0.0001               | 0.57  | < 0.0001               | 0.3536 |
| <b>Urine</b>                        |         |                        |       |                        |       |                        |        |
| Serum creatinine (log) <sup>c</sup> | -0.0125 | 0.0691                 | -0.15 | 0.0371                 | 0.74  | < 0.0001               | 0.5443 |
| Creatinine clearance                | -0.0109 | 0.0308                 | -0.22 | 0.0018                 | -0.47 | < 0.0001               | 0.2541 |
| Urine (log)                         | -0.0332 | 0.8618                 | 0.02  | 0.7994                 | 0.07  | 0.3917                 | 0.0056 |

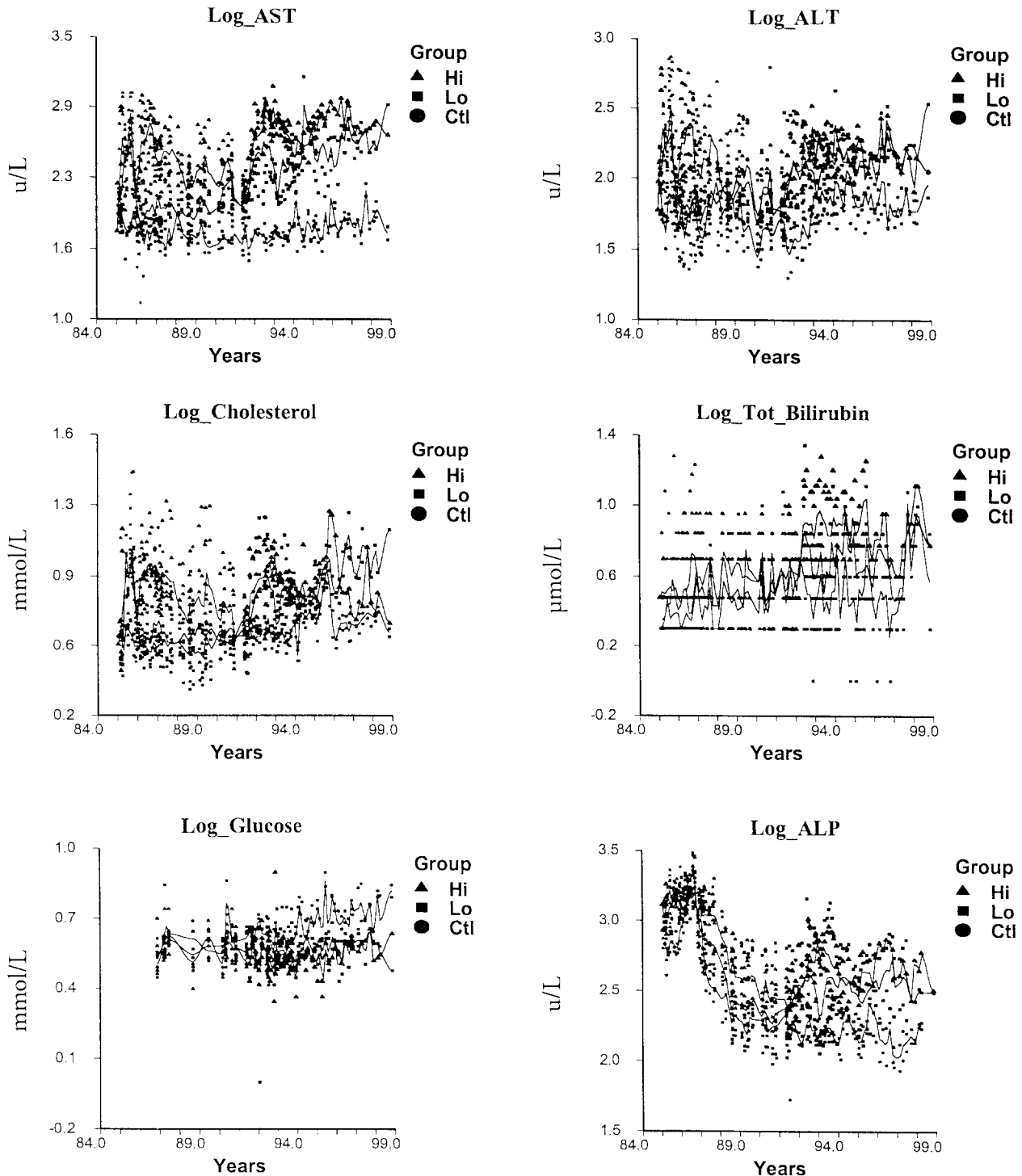
<sup>a</sup>A positive correlation indicates females higher than males. <sup>b</sup>Significance level (on multiple regression analysis). <sup>c</sup>Creatinine clearance was measured from 1991.

**Dose–response effects.** As discussed for the liver function enzymes, two time points were selected: between 1986 and 1988 and between 1991 and 1993 time periods. From 1986 to 1988, no decreases in the serum urea and creatinine levels were noticed in either of the treated groups (Figure 3). During the 1991–1993 dose increase, the serum urea level of the high dose slowly increased until

1995, after which there was a clear distinction between the high-dose and the low-dose and control groups. Creatinine clearance was assessed only from 1992 on, and both the high-dose and low-dose groups showed a reduced capacity (Figure 3). However, the low-dose group tended to revert to control values after about 4 years. The high-dose animals showed a consistently lower creatinine

clearance compared to the control females, and the reduced creatinine clearance capacity relates well with the increased urea and creatinine levels in the serum.

**Differential blood counts.** Differential blood counts were performed from 1991 onward. The white blood cell (WBC) counts were negatively correlated ( $r = -0.49$ ;  $p < 0.0001$ ) with the FCM levels in the diet



**Figure 2.** Multiple regression analyses of selected serum parameters associated with liver function and other important serum components during the experimental period. Hi, high dose; Lo, low dose; Ctl, control; Tot\_Bilirubin, total bilirubin.

(Table 6). In general WBC counts tended to decrease with time, although females exhibited higher WBCs. With respect to the relative contribution to the WBC count, eosinophils, monocytes, and basophils increased as a result of the FCM dietary treatment; the lymphocytes decreased. These parameters were also age dependent; neutrophils and eosinophils tended to decrease and monocytes increased as a function of time. Except for the monocytes, which were higher in males, there were no sex differences in the other WBC components. From the spline smooth (Figure 4), both the low-dose and high-dose groups showed a marked decrease in the WBC count, which tended to approximate values of the controls toward the end of the experiment.

The red blood cell (RBC) count was lower in females ( $r = -0.48$ ;  $p < 0.0001$ ), increased with age ( $r = 0.11$ ;  $p < 0.04$ ), and was negatively correlated ( $r = -0.45$ ;  $p < 0.0001$ ) with FCM dietary levels (Table 6). A similar effect was noticed for hemoglobin (Hb) and the hematocrit (HCT), except that both parameters also increased with age. The red cell distribution width (RDW) positively correlated ( $r = 0.18$ ;  $p < 0.0001$ ) with FCM treatment despite the fact that it generally decreased with age. The graphical plots (spline smooth) indicated that these parameters were affected in both the high-dose and low-dose groups, whereas the low-dose group tended to approximate the control values toward the end of the experiment (Figure 4).

The mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH), and the mean corpuscular hemoglobin concentration (MCHC) were not affected by FCM, whereas the MCV and MCHC were higher in males and tended to increase with age.

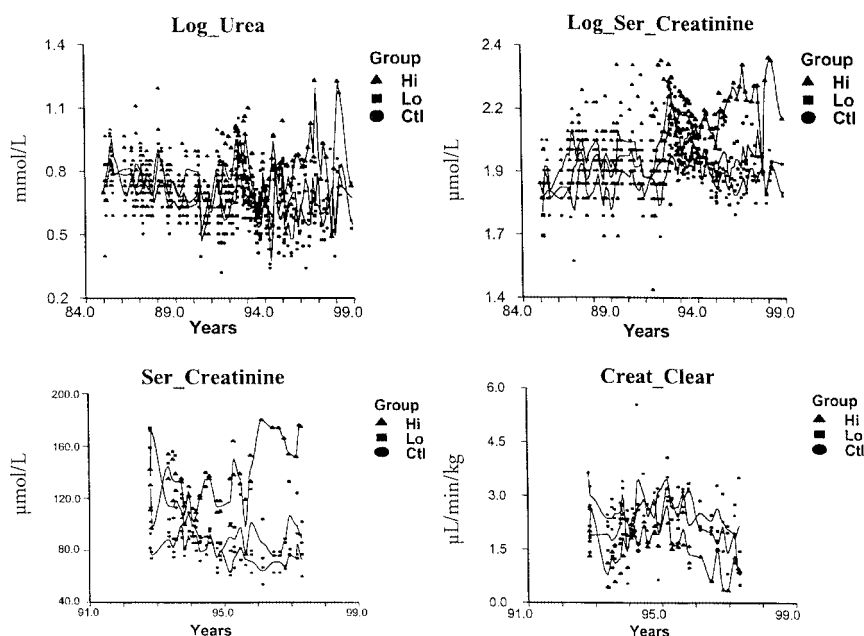
Blood platelet (PL) counts and the mean platelet volume (MPV) significantly ( $p < 0.0001$ ) decreased as a result of FCM treatment despite the fact that PL decreased and MPV increased, respectively, as a function of age. Females had higher platelet counts than males, whereas the MPV tended to be lower in females. The spline smooth graphical plot showed some lowering effects on PL by both the low-dose and high-dose groups throughout the experiment (Figure 4). The MPV clearly showed a decrease in the high-dose group; the low-dose group tended to exhibit similar values toward the end of the experiment.

Two males, one of the high-dose group (male 705) and one of the low-dose group (male 700), were terminated during the second year after the dose increase, followed by the last male (low-dose; male 696) during the third year. One high-dose female (female 688) died within 2 years of the dosage increase.

### Alteration of Sphingolipid Metabolism

During the monitoring period for sphingolipid basis, mean sphingosine levels were not significantly different between any of the experimental groups, whereas sphinganine levels were significantly ( $p < 0.05$ ) elevated in both the low-dose and high-dose groups consuming the FCM (15) as compared to the controls. However, although the high-dose group had a numerically higher sphinganine concentration, the individual variability observed both between and within vervet monkeys in each group caused the 95% confidence limits to be wide. Consequently, this difference between the low-dose and high-dose groups was not statistically significant ( $p > 0.05$ ). During this period, the sphinganine/sphingosine (Sa/So) ratio in the serum increased from 0.43 in the controls to 1.72 in the low-dose and 2.57 in the high-dose groups, with the latter two means significantly elevated over the control ( $p < 0.05$ ) but not significantly different from each other.

During the monitoring period, only a few samples of urine were analyzed. The sphingolipid base levels in urine have been linked to its cellular content because of the presence of exfoliated cells (18); hence, the levels of the individual bases will vary accordingly, making the ratio potentially a more reliable measure of disruption in sphingolipid metabolism. Although the results indicated a numeric increase in the Sa/So ratio for the two experimental groups, once again there was considerable variation and the values were not statistically significantly different.



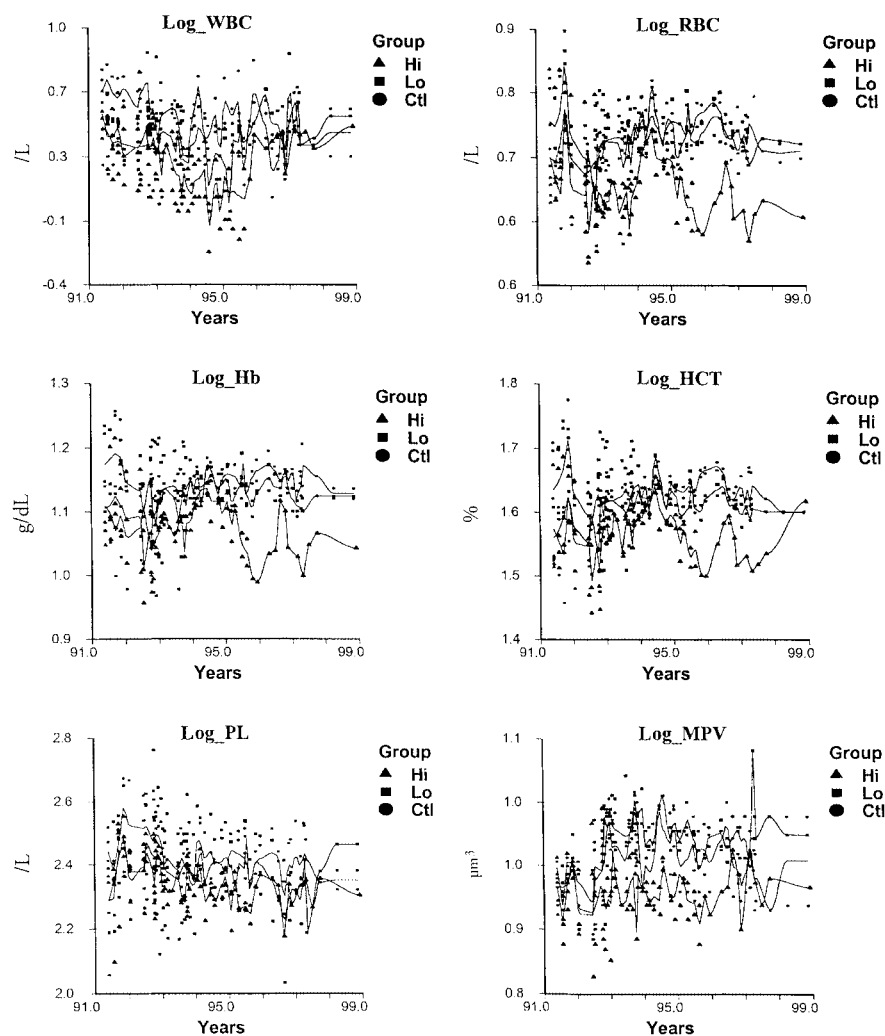
**Figure 3.** Graphical illustration of the effect of the low and high dose of FCM on clinical parameters associated with kidney function. Hi, high dose; Lo, low dose; Ctl, control; Ser\_Creatinine, serum creatinine; Creat\_Clear, creatinine clearance. Creatinine clearance was measured only from 1992 and onward. Serum creatinine measurements were performed during the course of the experiment as well as during the creatinine clearance determinations.

**Table 6.** Partial correlation coefficients from multiple regression analyses of blood parameters measured as a function of sex, year, and FCM. The correlation factor for FCM was corrected for age and sex.

| Dependent         | Sex   |                        | Age   |                        | FCM   |                        | $R^2$  |
|-------------------|-------|------------------------|-------|------------------------|-------|------------------------|--------|
|                   | $r^a$ | $p$ Level <sup>b</sup> | $r$   | $p$ Level <sup>b</sup> | $r$   | $p$ Level <sup>b</sup> |        |
| Blood parameters  |       |                        |       |                        |       |                        |        |
| WBC (log)         | 0.28  | < 0.0001               | -0.23 | < 0.0001               | -0.49 | < 0.0001               | 0.2878 |
| Lymphocytes       | -0.03 | 0.5937                 | 0.06  | 0.2857                 | -0.11 | 0.0435                 | 0.0200 |
| Neutrophils (log) | 0.08  | 0.1941                 | -0.17 | 0.0036                 | -0.08 | 0.1593                 | 0.0333 |
| Eosinophils (log) | -0.02 | 0.7484                 | -0.14 | 0.0243                 | 0.14  | 0.0229                 | 0.0469 |
| Basophils (log)   | -0.10 | 0.1213                 | -0.06 | 0.3057                 | 0.28  | < 0.0001               | 0.0874 |
| Monocytes         | -0.14 | 0.0216                 | 0.38  | < 0.0001               | 0.44  | < 0.0001               | 0.2725 |
| RBC               | -0.48 | < 0.0001               | 0.11  | 0.0446                 | -0.45 | < 0.0001               | 0.3603 |
| Hemoglobin        | -0.61 | < 0.0001               | 0.20  | 0.0003                 | -0.45 | < 0.0001               | 0.4616 |
| RDW (log)         | -0.02 | 0.6688                 | -0.43 | < 0.0001               | 0.18  | < 0.0001               | 0.2443 |
| MCV (log)         | -0.20 | 0.0003                 | 0.13  | 0.0232                 | 0.08  | 0.1606                 | 0.0497 |
| MCH               | -0.22 | < 0.0001               | 0.13  | 0.0216                 | 0.09  | 0.1224                 | 0.0569 |
| MCHC              | -0.05 | 0.3295                 | 0.02  | 0.6751                 | 0.04  | 0.5027                 | 0.0045 |
| PL (log)          | 0.53  | < 0.0001               | -0.40 | < 0.0001               | -0.33 | < 0.0001               | 0.3584 |
| MPV               | -0.13 | 0.0234                 | 0.17  | 0.0029                 | -0.35 | < 0.0001               | 0.1631 |

<sup>a</sup>A positive correlation indicates females higher than males. <sup>b</sup>Significance level (on multiple regression analysis).





**Figure 4.** Dose–response effects of FCM on selected blood parameters from 1991 to 1989. Hi, high dose; Lo, low dose; Ctl, control.

### Histopathology

The liver biopsies, taken at regular intervals up to the middle of 1990, revealed lesions that are characteristic of a chronic toxic hepatitis and that varied from subacute to acute effects in the vervet monkeys receiving the high dose of the culture material (10,11). These changes included perlobular fibrosis, nodular hyperplasia, apoptosis, distortion of reticulin staining pattern, and mild to scant bile duct proliferation, which distorted the normal architecture of the liver. Hepatocyte nodules were uneven in size and showed increased mitosis, uneven nuclear size, and single-cell necrosis (apoptosis). Bi-nucleated cells, fatty changes, and in some cases (female 681) influx of inflammatory cells were noticed randomly throughout the liver. Similar changes were observed in the livers of the vervet monkeys terminated during the experiment. The livers of terminated animals were markedly smaller, with portal-to-portal fibrosis and nodular hyperplasia as constant features (Table 5). In the low-dose group no

specific changes were apparent except in female 710, where disseminated foci of hepatocellular necrosis and inflammatory response occurred with a scant distortion of the reticular pattern. One of the control females (708) also presented with focally disseminated, mild, eosinophilic hepatitis, with no specific toxic lesions of distortion of the lobular structure except in the areas with hepatitis. This lesion was similar to that observed in the low-dose female 710 and, to some extent, in the high-dose female 688 and was ascribed to chronic parasitic hepatitis.

### Discussion

Previous reports on the effects of culture material of *F. verticillioides* MRC 826 on vervet monkeys used in the same long-term experiment (10,11) indicated that the liver is an important target organ. The levels of major serum enzymes used as markers for liver damage (ALT, AST, GGT, and LDH) were elevated in the high-dose and low-dose groups depending on the dietary level of FCM.

Jaskiewicz et al. (10) reported increased levels of AST, ALT, and LDH in vervet monkeys within the first few days in a pilot trial with culture material of MRC 826. The high-dose group that received 1% showed a marked increase within 14 days and a decrease within 2–3 weeks after the dose was lowered to 0.25%. The increase in hepatocellular enzyme activity in the low-dose group was far slower and first appeared 6–7 weeks after the treatment commenced. Increases in total bilirubin and cholesterol paralleled these early changes in liver enzyme activities. These findings were confirmed with multiple regression analysis of all the data as a function of the FCM dietary levels used for the duration of the experiment. The dose–response effects previously described by Jaskiewicz et al. (10) were also clearly illustrated over the period of decreased FCM intake (1986–1988) and the period of increased FCM intake (1991–1993). However, detailed dose–response effects and statistical evaluation of the data with respect to the biochemical parameters measured are complicated by the fact that low numbers of animals were used in each group, both males and females were used, and some of the vervet monkeys from each group were terminated *in extremis* during the course of the experiment. Despite these complications and that fact that some interindividual variations seem to exist among vervet monkeys, the low-dose groups exhibited levels of AST, ALT, LDH, and GGT similar to those of the controls during 1988. During this treatment period, the total FB level was at a minimum and it would appear that at this exposure level (0.10 mg FB/kg bw/day) very few changes are induced in liver function enzymes. The same is true for serum cholesterol, total bilirubin, glucose, and ALP values, although some individual variation appears to occur. Some animals in the high-dose group showed a marked decrease in the levels of the serum enzymes and closely resembled those of the controls. The minimum dosage level of the high-dose group, reached during 1988, was on the order of 0.18 mg FB/kg bw/day.

Changes in the serum parameters, associated with an increase in the FCM level in the diet, were monitored during 1991–1993. All liver function serum enzymes showed a marked increase from the 1991 background level in the high-dose animals. The increased dietary level of FCM represents an FB exposure level from approximately 0.38 mg/kg bw/day in 1991 to 0.64 mg/kg bw/day in 1992. Serum enzyme levels also increased in the low-dose animals after an increase of the FB intake from approximately 0.18 to 0.28 mg/kg bw/day. In some of the low-dose animals the serum enzyme levels equaled those of the high-dose group. A threshold for liver damage in vervet monkeys ranged between

0.10 mg FB/kg bw/day (low dose 1988) and 0.18 mg FB/kg bw/day (low dose 1991), where most of the vervets exhibited baseline values compared to the control females. In general, it appears that males were more sensitive than females because three males were terminated within 2–3 years after the dietary level of the culture material was doubled. Only one high-dose female died during this period.

Histopathologic changes associated with changes in liver enzyme activities have been described in detail. These changes suggest a chronic toxic hepatitis that simulates acute viral hepatitis, with some similarities to the effects induced by aflatoxins. Mild portal-to-portal fibrosis was also described by Fincham et al. (11) in biopsy specimens of both the high-dose and low-dose groups collected up to 1,631 days (middle 1989) after the experiment began. These biopsies also indicated that consumption of culture material of the fungus (high dose) caused atherogenic plasma lipid profiles to develop. These changes entailed elevations of plasma low-density lipoprotein (LDL)-C and apoprotein B strongly associated with accelerated atherosclerosis in vervet monkeys. It was suggested (11) that increased plasma cholesterol could be related to changes at the hepatocyte membrane leading to a reduced or impaired internalization of LDL into the hepatocytes, and hence the reduced clearance from the plasma. The reduced receptor activity might be caused by cytotoxicity induced by the FCM, an aspect that was clearly indicated by the serum enzyme profiles related to liver function. It was suggested that the synthesis of cholesterol was not affected, although in rats cholesterol accumulates both in the plasma and the liver (19). Plaque formation in the aorta of some of the animals sacrificed (Table 3) is therefore of interest in this regard. Additional studies must be conducted to evaluate the role of FB on cholesterol metabolism because it can effect changes in lipid metabolism in rat liver at different levels (19).

We also monitored the effects of the 1986–1988 and 1991–1992 changes in dietary levels of FCM on renal function. The low-dose group did not show any marked changes in serum urea or creatinine during the 1986–1988 culture batch dietary change. However, most of the high-dose animals showed elevated levels and therefore did not reflect the decrease in FB levels from 0.35 mg FB/kg bw/day in 1986 to 0.18 mg/kg bw/day in 1988. The 1991–1992 dosage increase markedly increased the serum creatinine levels in all high-dose vervet monkeys and in most of the animals of the low-dose group. The results of these two time points suggest that a threshold for changes in these two renal parameters exists in the region of 0.18–0.2 mg FB/kg bw/day as discussed for liver function. Creatinine clearance was monitored only

1 year after the dosage increase and showed markedly lower values during 1992 compared to the controls. All females (both high-dose and low-dose groups) showed a remarkable recovery phase over the first 3 years, whereafter creatinine clearance decreased to values similar to those of the control females. Although creatinine clearance is widely interpreted as a measure of glomerular filtration rate (GFR) and therefore renal function, the serum creatinine levels can be affected by many variables apart from glomerular filtration (20). These variables include the reabsorption and secretion of creatinine by renal tubules, which could over- or underestimate the GFR. In the present study, some nephrotoxic effects were noticed in the proximal tubular epithelium that could have decreased the tubular excretion of creatinine and therefore reduced the urinary levels of creatinine and indirectly affected the measured GFR.

Of interest were changes in the level of creatine kinase, which were markedly increased in all the high-dose animals in a dose-dependent manner. Monitoring total creatine kinase activity, measurement normally reflects the muscle isoenzyme because the heart and brain isoenzymes occur at far lower levels (21). As discussed for creatine kinase, care must be taken when interpreting the various clinical biochemical serum parameters. Some of the parameters, such as LDH and ALP, consist of many isoenzymes, each associated with specific tissue compartments, and electrophoretic enzyme characterization is required for identification (20). Bone origin of ALP elevations is quite easy to distinguish from hepatobiliary conditions in the absence of increased levels of AST, ALT, and GGT. AST and ALT have no specific isoenzymes, but AST is quite widely distributed in skeletal and cardiac muscle, liver, and erythrocytes. GGT occurs mainly in the liver and kidneys, but clinically it is confined to liver conditions; in small animals it roughly parallels ALT. Increases in bilirubin can also result from many abnormal physiologic conditions, including liver failure and obstructive biliary disease. Although Jaskiewicz et al. (10) reported cholestasis in the high-dose group, this could not be confirmed in subsequently biopsied animals that received decreased dosage levels of the culture material. It would appear that chronic liver injury was responsible for increased levels of total plasma bilirubin. As discussed for bilirubin, an increase in plasma cholesterol could be related to a number of causes, including diabetes mellitus, liver or biliary disease, or a fatty meal (21). It was suggested that in the absence of cholestasis, a reduced uptake of LDL cholesterol, caused by changes to the hepatocyte membranes, could be an important reason for the increased serum cholesterol (11). Of interest is

the finding of Fincham et al. (11) that culture material caused atherogenic effects in the high-dose vervet monkeys in this experiment.

This study provides interesting exposure parameters to be considered regarding dose–response effects of FB contaminated feeds and/or foods. The no-observed-effect level, with respect to the total fumonisin levels in the corn culture material, for liver and kidney damage in vervet monkeys can be estimated at between 0.11 and 0.18 mg FB/kg bw/day. This level is slightly below the values proposed to effect natural outbreaks of ELEM (0.6–2.1 mg/kg bw/day) (22). The probable daily intake (PDI) calculated for humans consuming corn as the staple diet, such as in the Transkei region of the Eastern Cape Province of South Africa, is 0.36 and 0.047 mg FB/kg bw/day when consuming “moldy” and “healthy” corn, respectively (23). These PDI values decreased considerably when calculated for people consuming corn exported from South Africa (0.002 mg FB/kg bw/day) or from the United States (0.007 mg FB/kg bw/day). Based on the FB-intake profiles of the vervet monkeys of 0.11 to 0.18 mg FB/kg bw/day and an average feed intake profile of  $16.0 \pm 1.6$  mg/kg bw/day, fumonisin contamination levels of corn between 8.21 and 13.25 mg FB/kg diet can be calculated. These values appear not to affect liver and kidney function in the vervet monkey but were suggested to be dangerous to horses, and 10-fold higher values could induce pulmonary edema syndrome in pigs (21). Changes in sphingolipid metabolism, specifically in the sphinganine concentration and the Sa/So ratio, were effected in the serum and also to some extent in the urine of the vervet monkeys at dietary levels of 0.29–0.64 mg FB/kg bw/day. These intake levels represent, as calculated above, contamination levels in corn of 21.7–47.8 mg FB/kg. The vervet monkey appears to be a promising model to study the use of the Sa/So ratio as a biomarker to determine fumonisin exposure in human populations.

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# Carcinogenicity of *Fusarium moniliforme* Culture Material in Rats<sup>1</sup>

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**ABSTRACT**—Two isolates of *Fusarium moniliforme* from corn were used in a chronic study with groups of 30 inbred male BD IX rats fed a semipurified diet that was marginally adequate nutritionally. Group 1 served as the controls and received the semipurified diet containing 5% cornmeal, group 2 received 5% of strain MRC 1069 culture material that was nontoxic to rats, and group 3 received 0.5% of strain MRC 826 culture material that was highly toxic to rats. The amount of the mutagen fusarin C detected in the culture material of strains MRC 826 and MRC 1069 was 104 and 364 mg/kg, respectively. Survival up to 2 years was good in all groups. Pathologic examination showed that many rats in group 2 had mild ductular cell hyperplasia. Almost all rats in group 3 had neoplastic nodules,  $\gamma$ -glutamyltransferase-positive foci, adenofibrosis, and esophageal basal cell hyperplasia. Whereas no tumors were induced in groups 1 and 2, the 21 long-term survivors in group 3 developed 8 cholangiocarcinomas, 2 hepatocellular carcinomas, 4 carcinomas of the forestomach epithelium, and 1 esophageal papilloma. Since neoplastic lesions were confined to rats in group 3 and the diet of these rats contained much less fusarin C than that of group 2, it is highly unlikely that fusarin C was responsible for the carcinogenicity of the MRC 826 culture material. It appears that the toxicity of *F. moniliforme* strains may be related to their carcinogenicity, but the chemical nature of the toxic and carcinogenic metabolite(s) produced by *F. moniliforme* MRC 826 remains unknown.—JNCI 1987; 78:321–325.

Food produced and prepared as was customary by Africans having a high risk for esophageal cancer induced epithelial basal cell hyperplasia and occasional papillomas in the esophagus and proliferative lesions of the liver in rats (1). The most common fungal contaminant of this food, *F. moniliforme* Sheldon, was subsequently shown capable of inducing esophageal epithelial hyperplasia and similar hepatic lesions, many of which progressed to carcinomas (2).

The incidence of *F. moniliforme* in corn (*Zea mays* L.) kernels is well correlated with the incidence of human esophageal cancer rates in Africa (3–5) and in China (6, 7).

African communities having a high risk for esophageal cancer all use corn as a dietary staple (8). Rats fed an uncontaminated corn diet that was marginally deficient in many nutrients were found to have an elevated susceptibility to an esophageal carcinogen; the predisposition could be abolished by the addition of vitamins and minerals (9). Corn, therefore, may play a dual role in precipitating the disease by acting as a carrier for carcinogens and by association with nutritional deficiencies that enhance the early stages of carcinogenesis.

The failure of esophageal basal cell hyperplasia induced by *F. moniliforme* in an earlier study (2) to progress to neoplasia could have been due to the optimal nutritional state of the rats. Furthermore, the high inci-

dence of primary liver cancer also could have been aggravated by the severe cirrhosis that followed exposure to relatively high levels of toxic culture material, as well as by undetected contaminants of the commercial rat feed. The metabolites responsible for pathologic changes are unknown, and it is unclear if carcinogenicity is related to toxicity or to the content of the nontoxic mutagen fusarin C (10).

In the present study, we used a semipurified diet that was likely to have an exceedingly low carcinogen content and that also was low in those nutrients known to have a protective effect against esophageal carcinogenesis. The effects of low levels of culture material of a highly toxic and relatively nontoxic strain of *F. moniliforme*, which has a higher concentration of the mutagen fusarin C, also were compared.

## MATERIALS AND METHODS

**Fungal cultures.**—*F. moniliforme* strains MRC 826 and MRC 1069 were isolated from corn produced in Transkei, South Africa. MRC 826 is a highly toxic strain, whereas MRC 1069 is the least toxic of the 21 isolates tested (11). The latter nontoxic strain is known to produce larger amounts of the mutagen fusarin C in culture than does the toxic MRC 826 (10). Neither strain produces moniliformin (11).

Lyophilized conidia of each strain were used to inoculate autoclaved, moistened, whole yellow corn kernels. Cultures were incubated in the dark at 25°C for 2 weeks, followed by 2 weeks at 15°C (10). The culture material was then lyophilized, ground to a fine meal, and stored in the dark at 0°C until used (2).

**Fusarin C analyses.**—Fusarin C analyses were performed on chloroform-isopropanol extracts of lyophilized culture material of the two strains of *F. moniliforme* (prepurified by solvent partitioning) by normal phase high-performance liquid chromatography (10).

**Rat experiment.**—Ninety inbred male BD IX rats, each weighing approximately 110 g, were randomized

ABBREVIATIONS USED: GGT =  $\gamma$ -glutamyltransferase; H & E = hematoxylin and eosin.

<sup>1</sup> Received February 24, 1986; accepted July 30, 1986.

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<sup>4</sup> We thank Dr. P. G. Thiel for aflatoxin analyses and Mr. P. W. de Lange, Mr. H. J. Joubert, Mrs. C. L. Schreuder, Mr. M. J. van Wyk, Mr. F. S. Venter, and Mr. C. W. Woodroof for competent technical assistance.

TABLE 1.—Diet composition

| Ingredient               | Percent by weight |
|--------------------------|-------------------|
| Cornstarch               | 75.0              |
| Soy protein              | 3.0               |
| Casein                   | 1.0               |
| Egg albumin              | 1.0               |
| Sunflower seed oil       | 3.0               |
| CaCO <sub>3</sub>        | 0.125             |
| NaCl                     | 0.25              |
| ZnCO <sub>3</sub>        | 0.002             |
| Vitamin mix <sup>a</sup> | 0.5 <sup>b</sup>  |
| Sucrose                  | 11.1              |
| Dextrin                  | 5.0               |

<sup>a</sup> Composition of vitamin mix: vitamin K, 50 mg; thiamine hydrochloride, 112 mg; Calcium pantothenate, 600 mg; vitamin B<sub>12</sub>, 1.5 mg; folic acid, 100 mg; biotin, 20 mg; pyridoxine hydrochloride, 121 mg; vitamin A acetate 3.0 g; vitamin D<sub>3</sub>, 50,000 IU; vitamin E (50%), 2.5 g; nicotinamide, 1.0 g; riboflavin, 0.2 g; inositol, 50 g; choline chloride, 50 g; methionine, 25 g; dextrin, 842 g.

<sup>b</sup> Equal to 25% of usual requirement.

into 3 groups and housed individually in plastic cages with stainless steel wire bottoms and tops. They were maintained in a controlled environment at 23°C and 50% humidity, with 22 air changes/hour and a 12-hour light-dark cycle.

A semipurified diet was compounded as shown in table 1. The diet contained marginal amounts of many vitamins and minerals but was not severely deficient in any nutrient, including methionine and choline. Group 1 served as controls and received the semipurified diet containing 5% autoclaved cornmeal. Group 2 received the semipurified diet containing 5% *F. moniliforme* MRC 1069 culture material, and group 3 had 0.5% MRC 826 culture material mixed into the diet. Periodic analyses of the diets for aflatoxin were always negative. The diet and tap water were available ad libitum.

**Observations.**—Each rat was observed daily and was weighed weekly. From each group, 5 rats were sacrificed on the 540th day of the experiment, and the remainder either died or were sacrificed between days 690 and 710. Many were killed when they exhibited signs of illness, discomfort, or progressive weight loss. Rats to be sacrificed were anesthetized with pentobarbital sodium and killed by exsanguination. At autopsy, the esophagus and stomach were opened, macroscopically visible lesions were recorded, and the tissues were pinned on cork prior to fixation and sectioning of each lesion. Specimens of all organs were fixed in 10% buffered Formalin, processed and embedded in wax in the conventional way, stained with H & E, and, where appropriate, with special stains such as for glycogen and reticulin.

Separate samples of each fresh liver were rapidly frozen in OCT Compound (Miles Laboratories, Naperville, IL) in Dry Ice and stored at -20°C. Cryostat-cut sections (4 μm) were stained for GGT activity (12). Only GGT-positive foci containing more than 10 cells were scored. Morphometry was performed on H & E-stained sections to determine the thickness of the esophageal epithelium and the thickness of that part of the epithelium exclusive of the keratin layer.

Pathology terminology has been discussed in previous studies of this fungus (2, 11, 13). In the present study, ductular cell hyperplasia is defined as a mild increase of structures resembling bile ductules, some of which were remotely situated from the portal tracts. Adenofibrosis is a characteristic rat lesion also known as cholangiofibrosis, bile duct adenomatosis, fibroadenoma, and intestinal cell metaplasia. Adenofibrosis is much more advanced than ductular cell hyperplasia, always involving larger ducts with associated fibrosis. Cholangiocarcinomas previously were referred to as ductular carcinomas (2) and were distinguished from advanced adenofibrosis by their larger mass and the presence of irregular mucoid glandular structures, whose epithelial cells exhibit cytoplasmic basophilia, irregular hyperchromatic nuclei, prominent multiple nucleoli, and frequent mitoses. Neoplastic nodules were composed largely of hepatocytes and have also been termed regenerative or hyperplastic nodules. Hepatocellular carcinomas were only diagnosed if all the classical features were present, including infiltrative spread and a larger size than any neoplastic nodules. Esophageal basal cell hyperplasia implied an increase in the usual well-defined single layer of basal epithelial cells to at least 3 layers, with some disturbed polarity in the arrangement of the cells.

## RESULTS

The levels of fusarin C detected in the culture material of *F. moniliforme* strains MRC 1069 and MRC 826 were 364 and 104 mg/kg, respectively.

Control rats (group 1) receiving the semipurified diet had growth curves similar to that of our inbred BD IX rats fed commercial diets. The growth curve of group 2 (5% MRC 1069) usually lagged about 5% behind that of the controls, but ultimately there was little difference (table 2). Group 3 rats (0.5% MRC 826) generally had a mean weight approximately 20% less than that of the controls. Occasional signs of mild poor thrift were apparent in this group, but they appeared to attain normal body size and suffered less from the obesity that normally develops in old rats fed ad libitum. Survival for this type of experiment was good, and over the 27-month study period only 3 rats each from the first 2 groups and 4 from group 3 were discarded due to incidental disease.

Few pathologic changes occurred in the organs of 5 apparently healthy rats of each group sacrificed at 18 months, except in the livers of group 3 (table 2). Lesions known to be induced by high dietary levels of *F. moniliforme*, such as neoplastic nodules and adenofibrosis (2, 11), were already present in some rats, as well as esophageal basal cell hyperplasia.

Lesions in surviving rats that were sacrificed or died 23–27 months after commencement of the experiment are summarized in table 2. Gross changes were most obvious in the livers of rats from group 3; all had an irregular surface with multiple nodules alternating with pale, firm, depressed areas. Microscopic examination confirmed the high frequency of neoplastic nodules,

TABLE 2.—Pathologic changes in rats fed a semipurified (SP) diet containing culture material of 2 *F. moniliforme* strains

| Pathologic change                      | Rats on:       |                    |  |                |                    |            |
|--|----------------|--------------------|--|----------------|--------------------|------------|
|  | SP diet, at:   |                    | SP diet + <i>F. moniliforme</i> strains: |                |                    |            |
|  | 18 mo<br>(n=5) | 23-27 mo<br>(n=22) | 5% MRC 1069, at:                         |                | 0.5% MRC 826, at:  |            |
|  |                | 18 mo<br>(n=5)     | 23-27 mo<br>(n=22)                       | 18 mo<br>(n=5) | 23-27 mo<br>(n=21) |            |
| Mean final body weight, g              | 469            | 517                | 456                                      | 513            | 387                | 416        |
| Esophageal epithelium                  |                |                    |  |                |                    |            |
| Total thickness, $\mu\text{m}$         | 53 $\pm$ 5     | 53 $\pm$ 4         | 51 $\pm$ 3                               | 55 $\pm$ 7     | 61 $\pm$ 2         | 74 $\pm$ 6 |
| Thickness minus keratin, $\mu\text{m}$ | 31 $\pm$ 4     | 32 $\pm$ 2         | 38 $\pm$ 5                               | 36 $\pm$ 6     | 38 $\pm$ 6         | 44 $\pm$ 3 |
| Basal cell hyperplasia                 | 0              | 0                  | 0  | 0              | 1                  | 12         |
| Parakeratosis                          | 0              | 1                  | 0  | 1              | 0                  | 7          |
| Papilloma                              | 0              | 0                  | 0  | 0              | 0                  | 1          |
| Forestomach                            |                |                    |  |                |                    |            |
| Acanthosis                             | 0              | 10                 | 0  | 8              | 2                  | 18         |
| Dysplasia                              | 0              | 2                  | 0  | 2              | 0                  | 11         |
| Papilloma                              | 0              | 5                  | 1  | 3              | 1                  | 13         |
| Carcinoma                              | 0              | 0                  | 0  | 0              | 0                  | 4          |
| Liver                                  |                |                    |  |                |                    |            |
| Fatty change                           | 0              | 2                  | 0  | 11             | 3                  | 21         |
| Neoplastic nodules                     | 0              | 0                  | 0  | 1              | 2                  | 21         |
| GGT-positive foci                      | —              | 0                  | —  | 0              | —                  | 18         |
| Hepatocellular carcinoma               | 0              | 0                  | 0  | 0              | 0                  | 2          |
| Ductular cell hyperplasia              | 0              | 0                  | 1  | 18             | 5                  | 21         |
| Adenofibrosis                          | 0              | 0                  | 0  | 1              | 2                  | 19         |
| Cholangiocarcinoma                     | 0              | 0                  | 0  | 0              | 0                  | 8          |
| Heart                                  |                |                    |  |                |                    |            |
| Disseminated fibrosis                  | 1              | 4                  | 0  | 4              | 1                  | 10         |
| Endocardial fibrosis                   | 0              | 0                  | 0  | 0              | 0                  | 3          |

adenofibrosis, and the presence of 8 cholangiocarcinomas (fig. 1) and 2 hepatocellular carcinomas in the 21 animals that received 0.5% *F. moniliforme* MRC 826 in the diet. Liver sections from 85% of the animals in this group showed between 23 and 36 foci that stained positively for GGT activity (fig. 2). Sections stained with H & E demonstrated occasional single-cell necroses and frequent megalocytosis (11) in hepatocytes trapped between the neoplastic nodules. Hepatocytes within the larger neoplastic nodules were polymorphic, with increased mitoses and clear acidophilic and basophilic cell foci. All of the changes in the larger neoplastic nodules were previously noted to be common following exposure to both relatively high levels of cultured material (2) and corn naturally contaminated with *F. moniliforme* (13). The 2 hepatocellular carcinomas had both metastasized to the lungs (fig. 3).

The esophageal epithelium of rats in group 3 was significantly thickened; the mean measurement was 74  $\mu\text{m}$  as compared to the control value of 53  $\mu\text{m}$ . Distinct basal cell hyperplasia (fig. 4) with mild hyperkeratosis and parakeratosis was evident in most rats of this group. Only 1 rat in group 3, sacrificed on the 720th day of the experiment, had an esophageal papilloma.

The squamous forestomach of rats in group 3 had a high incidence of acanthosis, dysplasia, and papillomas, and 4 carcinomas that had infiltrated the muscular layers were diagnosed.

Myocardial disseminated fibrosis was much more frequent in group 3 rats (table 2), and all 3 cases of endo-

cardial and subendocardial fibrosis also were in this group.

Rats in group 2 fed 5% MRC 1069 had few lesions, and the lesions also were much less severe. The esophagus, forestomach, and heart were unaffected by the treatment. Fatty change in the liver, characterized by very large vacuoles tending to have a centrilobular distribution and similar in nature to that seen in group 3 rats, was present in approximately 50% of the animals. Mild ductular cell hyperplasia was present in the majority of rats, but only 1 rat had a small focus of adenofibrosis. Another rat had a small neoplastic nodule.

The only lesions seen in the control group were those normally occurring spontaneously in the forestomachs of old BD IX rats. Hepatocyte cytoplasm was prominently loaded with glycogen in the controls and in treated animals that were killed while in good condition. This change is likely to be due to the high carbohydrate nature of the diet.

## DISCUSSION

The first study demonstrating the carcinogenicity of *F. moniliforme* (2) was complicated by the toxic effects of relatively high-level exposure (resulting in particularly severe cirrhosis) and the possibility of carcinogen contamination of the commercial diet. Cirrhosis, as well as the severe dietary lipotrope deficiency, as in a later study on naturally contaminated U.S. corn (13), could play major roles in precipitating the development of

hepatic tumors. The present study adequately confirms the hepatocarcinogenicity of *F. moniliforme* MRC 826 culture material in rats maintained on a semipurified diet that was marginally adequate in lipotropic factors. In addition to the 2 hepatocellular carcinomas and 8 cholangiocarcinomas that developed in 21 surviving rats fed this material, GGT-positive foci also were present in 18 of these rats. These foci are widely used as a marker of preneoplastic hepatic lesions during chemical carcinogenesis (14). Furthermore, dietary levels of culture material were very low, and the only distinct sign of any hepatic fibrotic processes in the exposed rats was that associated with adenofibrosis.

Marked effects of *F. moniliforme* MRC 826 on the squamous epithelium of the forestomach, including dysplasia, papilloma, and carcinoma, also were noted in the present experiment. Cancer of the forestomach in rats previously was reported to be caused by a strain of *F. moniliforme* isolated from a high esophageal cancer risk area in China (15, 16).

The previously reported (2) esophageal basal cell hyperplasia caused by *F. moniliforme* MRC 826 in rats has been confirmed, and there can now be no doubt about the specific esophageal hyperplasia-inducing effect of exposure to even very low dietary levels of this fungus.

With the exception of 1 esophageal papilloma in 21 long-term survivors exposed to strain MRC 826, the basal cell hyperplasia induced by *F. moniliforme* failed to progress towards frank neoplasia, even though the dietary level of zinc and other minerals and vitamins known to enhance resistance to esophageal carcinogens was marginal. The only other "spontaneous" papillomas found in many thousands of our BD IX rats examined over the years occurred in 2 of 75 long-term survivors fed naturally *F. moniliforme*-contaminated food (1).

A recent study of children (17) from an African population having a moderately high risk of esophageal cancer shows that within a few years of being weaned onto a staple corn diet, about half have basal cell hyperplasia of the esophagus. Many factors, including nutritional deficiencies, could contribute to the etiology of such lesions. Nevertheless, the role of fungal metabolites in the pathogenesis of esophageal cancer in some communities must be seriously considered.

The nature of the *F. moniliforme* metabolite(s) responsible for the neoplastic effects noted in rats is not known. The potent mutagen fusarin C is known to be produced by *F. moniliforme* isolates from southern Africa (10), China (18), and the United States (19), and also has been shown to occur naturally in corn in high esophageal cancer risk areas in southern Africa (10) and China (18). The two strains used in the present experiment are known to differ in their toxicity to rats (11) and in their potential to produce fusarin C in culture (10), but neither strain produces moniliformin (11). Thus *F. moniliforme* MRC 1069 is less toxic but produces larger amounts of fusarin C than does MRC 826. On the basis of the actual levels of fusarin C detected in the culture material of the 2 strains used in the experiment,

the diet of rats in group 2 fed MRC 1069 contained 18.2 mg/kg fusarin C, whereas the diet of rats in group 3 fed MRC 826 contained only 0.5 mg/kg. Since neoplastic lesions were confined to rats in group 3, it is highly unlikely that fusarin C was responsible for the carcinogenicity of MRC 826 culture material.

In view of the finding that the toxic strain MRC 826 was also carcinogenic and the nontoxic strain MRC 1069 was not, it appears that toxicity is related to carcinogenicity. This relationship suggests that other toxic strains of *F. moniliforme* (11) that cause pathologic changes in rats, similar to those caused by MRC 826, are possibly also carcinogenic. Attempts are being continued to isolate and chemically characterize the toxic and carcinogenic metabolite(s) produced by *F. moniliforme* MRC 826.

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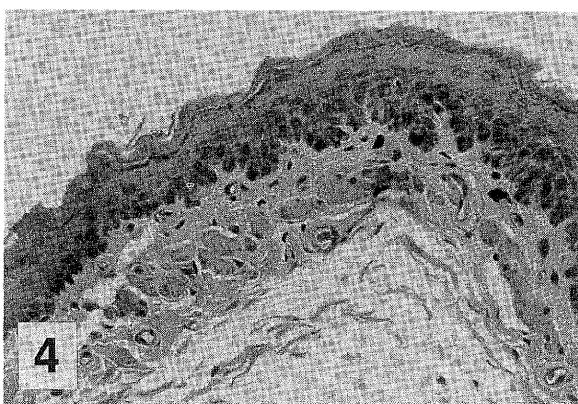
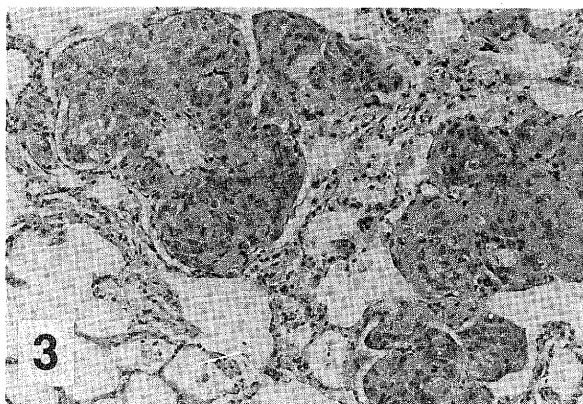
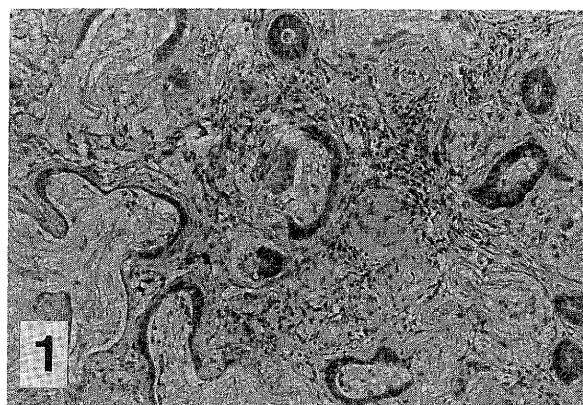


FIGURE 1.—Cholangiocarcinoma of the liver in a rat fed 0.5% *F. moniliforme*-contaminated corn in the diet for 2 years. Note the chaotic proliferation of the incomplete glandular structures that contain pools of mucin and debris, with intervening prominent secondary desmoplasia. H & E.  $\times 120$

FIGURE 2.—Large neoplastic nodules that are strongly positive for GGT in a rat exposed to dietary *F. moniliforme*. GGT.  $\times 60$

FIGURE 3.—Pulmonary metastases were found in 2 rats with hepatocellular carcinomas. H & E.  $\times 135$

FIGURE 4.—Basal cell hyperplasia in the esophageal epithelium of a rat receiving 0.5% *F. moniliforme*-contaminated corn in the diet. H & E.  $\times 240$



## CHAPTER II

### ISOLATION, CHARACTERISATION AND NATURAL OCCURRENCE OF THE FUMONISINS

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## ISOLATION, CHARACTERISATION AND NATURAL OCCURRENCE OF THE FUMONISINS

### 2.1 *Fumonisin purification*

The two-stage cancer initiating promoting model in rat liver, using diethylnitrosamine (DEN) as cancer initiator, was successfully used as a bioassay to purify the cancer promoting compounds, the fumonisins B (FB) mycotoxins from maize culture material of *F.verticillioides* strain MRC 826 (Gelderblom *et al.*, 1988a). The bulk of the fumonisins was recovered in a 75% MeOH/water extract after extraction with ethyl acetate and subsequent partitioning of the aqueous methanol extract with chloroform. The fumonisins eluted from a reversed phase Amberlite XAD-2 column in MeOH and further fractionated on silica gel using CHCl<sub>3</sub>/MeOH/CH<sub>3</sub>COOH (6:3:1) as the developing solvent. Final purification of the two major compounds FB<sub>1</sub> and FB<sub>2</sub> were achieved on reversed phase C18 column using MeOH/H<sub>2</sub>O as eluting solvents. The major FB containing fractions and the purified FB<sub>1</sub> significantly increased the mean number of liver foci in the DEN-initiated rats during a feeding period of 4 weeks, similarly to the *F. verticillioides* maize culture material. The subacute pathological changes of FB<sub>1</sub>, characteristic of a toxic hepatitis, were similar to that described for the fungus and in agreement with the finding that cancer promotion was associated with a hepatotoxic effect. Similar to the fungal culture material (Gelderblom *et al.*, 1988b), FB<sub>1</sub> induces preneoplastic lesions in the absence of DEN-initiation, suggesting that FB<sub>1</sub> is a complete carcinogen.

The preparative-scale isolation of the fumonisins was undertaken during which two minor constituents, FB<sub>3</sub> and FB<sub>4</sub> were purified (Cawood *et al.*, 1991). Two structural n-acetyl analogues of FB<sub>1</sub> and FB<sub>2</sub>, FA<sub>1</sub> and FA<sub>2</sub>, respectively were also purified for the first time. Structural analogues that resulted as artifacts from the isolation process, the mono- and dimethyl ester derivatives of FB<sub>1</sub> and FB<sub>2</sub> were purified for studies on structural activity. The formation of these analogues, however, decreased the final yield to approximately 40 % for both FB<sub>1</sub> and FB<sub>2</sub>. The quantitative method developed

was applied for the bulk purification of FB<sub>1</sub> and FB<sub>2</sub> to a purity of >90% which was used for biological studies.

## 2.2 Structural elucidation and structural analogues

Chemical structures of the fumonisins and the structural analogues were elucidated by mass spectrometry and <sup>1</sup>H and <sup>13</sup>C nmr spectroscopy (Bezuidenhout *et al.*, 1988). FB<sub>1</sub> and FB<sub>2</sub> are di-esters, at C-14 and C15 hydroxy positions, of propane-1, 2, 3-tricarboxylic acid (TCA) and 2-amino-12, 16-dimethyl-3, 5, 10, 14, 15 pentahydroxyicosane. FB<sub>2</sub> is the C-10 deoxy analogue of FB<sub>1</sub>, while FA<sub>1</sub> and FA<sub>2</sub> are the n-acetyl derivatives of FB<sub>1</sub> and FB<sub>1</sub> respectively. Recently, FB<sub>3</sub> and FB<sub>4</sub> were found to exist as structural epimers, which should be considered during the quantification of FB<sub>3</sub> (Gelderblom *et al.*, 2007).

## 2.3 Natural occurrence

The natural occurrence of mycotoxins in basic dietary staples is of concern, as it is known to adversely effect many aspects of human health. The ubiquitous nature of the fungus, specifically on maize, and the involvement in many natural outbreaks of *F. verticillioides*-associated diseases in animals predicted that the toxic principles could occur naturally. The sample selected to investigate the natural occurrence of FB<sub>1</sub>, was obtained from a rural area in the former Transkei region, shown to be contaminated with other *Fusarium* mycotoxins, including moniliformin, zearalenone, deoxynivalenol and the mutagen fusarin C (Thiel *et al.*, 1982, 1986; Gelderblom *et al.*, 1984a). Two independent HPLC methods utilizing maleyl anhydride and fluorescamine derivitisation of the free amino group and subsequent confirmation with GS-MS provides conclusive evidence of the natural occurrence of FB<sub>1</sub>. Apparent level of FB<sub>1</sub> was considerably higher in the “moldy” and *Fusarium* infected kernels as compared to the “healthy” maize (Sydenham *et al.*, 1990). The study provides proof, not only for the natural occurrence of FB<sub>1</sub>, but for the co-occurrence with other *Fusarium* mycotoxins and the mutagen fusarin C, and evidence that humans may well be exposed. The first report that FB<sub>1</sub> and FB<sub>2</sub> also occur in maize samples associated with leukoencephalomalacia (LEM) in horses suggested that these mycotoxins could be involved in the development

of this syndrome (Voss *et al.*, 1989). Feeding studies utilizing the same material of *F. verticillioides* in rats demonstrated hepato- and nephrotoxicity that, together with the known toxic effects by FB<sub>1</sub> in rats (Gelderblom *et al.*, 1988a) further provides evidence that the fumonisins could be the causative principles for LEM.

#### 2.4 *Fumonisin production and heat stability in maize cultures*

Studies on the toxicological effects of *F. verticillioides* strain MRC 826 were complicated by the variation of the toxigenic properties of different cultured batches. Prior to the discovery of the fumonisins, toxicity was determined by the duckling test (Leslie *et al.*, 1996), while clinical chemical analyses for liver function enzymes were utilised during chronic feeding studies to assess the extent of toxicity in a specific animal species. Variation in the toxicity is likely to be determined by toxin production that is affected by different parameters including temperature, aeration, water activity, growth kinetics and the composition of the growth medium. To ascertain optimal fumonisin production for bulk purification these different parameters had to be optimized and to screen other *Fusarium* spp for their ability to produce these mycotoxins.

A study by Thiel *et al.* (1991) screened 27 taxa in 9 of the sections of *Fusarium*. Maize cultures of the different species were prepared in glass fruit jars (see below) and analysed by HPLC using o-phthaldialdehyde derivatisation with fluorescence detection. Fumonisin production was restricted to *F. verticillioides* (*F. moniliforme*) and *F. proliferatum* with *F. verticillioides* strain MRC 826 found to be the highest producer. Only one other species, *F. nygamai*, isolated from soil was also found to produce FB<sub>1</sub> and FB<sub>2</sub>. Subsequent studies on fumonisin production therefore utilised *F. verticillioides* strain MRC 826

Two different experiments utilizing a) whole yellow maize kernels in 2-liter glass fruit jars (Alberts *et al.*, 1990) and finely ground yellow maize kernels in 100 by 18 mm glass petri dishes (Alberts *et al.*, 1993) were conducted. In the first study, using whole maize kernels, the initial growth rate was higher at 25 °C than 20 °C reaching the stationary phase after 4 to 6 weeks. FB production

commenced after 2 weeks during the active growth phase and started to decrease after 13 weeks. Although the overall maximal yield, ranging from 17.9 to 16.5 g FB<sub>1</sub>/kg dry weight, respectively, did not differ between the 2 temperatures, the average yield was significantly higher at 25 °C (9.5g vs 8.7g). Incubation of the cultures at 30 °C provided very low yields of 0.6g FB<sub>1</sub>/kg dry weight. In order to increase the surface area and aeration a subsequent experiment was conducted using ground yellow maize kernels in the form of a patty contained in a glass petri dish. Maximum FB<sub>1</sub> production (11.6 g/kg dry weight) was achieved after 2 weeks with an apparent maximum yield (P>0.05) of 14.8 g FB<sub>1</sub>/kg dry weight after 3.5 weeks (Alberts *et al.*, 1993). The production of FB<sub>2</sub>, which was approximately 50% lower, followed similar trends. Compared to the first study, FB<sub>1</sub> production in the glass petri dish patty method provides a more convenient procedure for the production of high yields in a short time that facilitates the bulk purification thereof.

The ground maize petri dish patty model was subsequently developed to produce [<sup>14</sup>C] isotope labeled FB<sub>1</sub> using L-[methyl-14C] methionine (Alberts *et al.*, 1993). Optimum incorporation of [<sup>14</sup>C] that occurs at C-21 and C-22 of the molecule was achieved after 9 days in the presence of unlabeled methionine, yielding a specific radio activity of 36 µCi/mmol. Despite the controlled conditions, variation the labeling of FB<sub>1</sub> occurred which was ascribed to differences in the viability of the inocula and variations in the composition of the yellow maize used. A study using liquid cultures was conducted as the composition is less complex, but the FB<sub>1</sub> production was extremely low as compared to the maize patty cultures, making the labeling of FB<sub>1</sub> with a higher specific activity problematic (Alberts *et al.*, 1994).

The stability of the fumonisins was addressed to evaluate the effect of cooking, as maize porridge is the traditional way of preparing maize to be consumed in Africa. Boiling of freeze dried maize culture material in H<sub>2</sub>O (1:1 w/v) for 30 min did not reduce the FB<sub>1</sub> concentration (Alberts *et al.*, 1990). The cancer promoting properties of the boiled culture material were also not

reduced when considering the induction of GGT positive foci in DEN-initiated rats.

### 2.5 *Perspectives*

The characterization of the fumonisins was mainly hampered by the lack of a suitable model that represents the diverse toxicological effects of the fungus in different animal species. It was not known whether a toxic hepatic lesion in rats represents the action of a toxin(s) that is responsible for LEM in horses and pulmonary edema in pigs. The characterization of the fumonisin mycotoxins, therefore, has been an important breakthrough and can be regarded as a turning point that clarified many of the toxicological effects of *F. verticillioides* in animals since the first report that the fungus is a causative agent for ELEM.

The stepwise elucidation of the carcinogenic principles produced by the fungus following the findings of the hepatocarcinogenic effects resulting in the characterization of a potent mutagen fusarin C, were the major initial events in the characterization of the fumonisins. Despite the findings that fusarin C is not the major carcinogenic principle produced by the fungus, the isolation of this mutagen led the way to elucidate the cancer promoting potency of the fungus resulting in the development a short-term cancer initiating/promoting cancer model in the liver. An important aspect for developing the model was the notion that all carcinogens are not mutagens and, that a bioassay based on genotoxicity, such as the Salmonella mutagenicity test, would not necessarily identify the actual carcinogen. The finding that the different cultures of the fungus exhibiting cancer promoting activity in rat liver was associated with a toxic effect, provided the first evidence that the toxic principle(s) is also likely to exhibit cancer promoting activity. This was based on the findings that the hepatocarcinogenic effects of the fungal culture material were associated with a chronic toxic hepatitis with development of hepatocyte nodules, early markers for cancer development in the liver. These events and knowledge that accumulated since the first report on the carcinogenicity of the fungus in 1981 resulted in the development of the liver bioassay and the purification of the fumonisins in a matter of a few months.

Following the discovery of the fumonisins, emphasis was placed on the development of suitable analytical techniques to determine its natural occurrence and studies regarding the production profiles in maize in order to supply ample fumonisin standard stock material for analytical purposes and biological investigations. Optimisation of fumonisin production in maize by developing the maize patty culture technique, using *F. verticillioides* strain MRC 826, led to the bulk purification of large amounts. The maize patty model was also successfully utilized for the production of radio-labeled fumonisins to be utilized in biological investigations. The setting of an international “gold” standard for fumonisins and the infrastructure to produce stock preparations of high purity created the basis for the detailed elucidation of the toxicological effects in different animal species and subsequent investigation of the underlying mechanisms involved.

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## Effects of Temperature and Incubation Period on Production of Fumonisin B<sub>1</sub> by *Fusarium moniliforme*

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Received 15 November 1989/Accepted 11 March 1990

The kinetics of the production of fumonisin B<sub>1</sub> (FB<sub>1</sub>) by *Fusarium moniliforme* MRC 826 in corn cultures was investigated as a function of fungal growth at various incubation temperatures. The growth rate of *F. moniliforme*, as measured by ergosterol concentration, was higher at 25°C than at 20°C, reaching a stationary phase after 4 to 6 weeks in both cases. FB<sub>1</sub> production commenced after 2 weeks during the active growth phase, continued to increase during the stationary phase, and decreased after 13 weeks. The overall maximal yield of FB<sub>1</sub> (17.9 g/kg, dry weight) was obtained in corn cultures incubated at 20°C for 13 weeks, but it was not significantly ( $P > 0.05$ ) higher than the maximum yield (16.5 g/kg, dry weight) obtained at 25°C after 11 weeks. However, a significantly ( $P < 0.05$ ) higher mean yield was detected at 25°C (9.5 g/kg, dry weight) than at 20°C (8.7 g/kg, dry weight). Production reached a plateau after 7 weeks of incubation at 25°C or 9 weeks of incubation at 20°C. The maximal production of FB<sub>1</sub> at 30°C was very low (0.6 g/kg, dry weight). FB<sub>1</sub> was also found to be heat stable, as there was no reduction in the FB<sub>1</sub> concentration after boiling culture material of *F. moniliforme* MRC 826.

*Fusarium moniliforme* Sheldon is one of the most prevalent fungi associated with basic human and animal dietary staples such as corn (10, 13-15). Culture material of *F. moniliforme* MRC 826, isolated from corn intended for human consumption in Transkei, southern Africa, proved to be highly toxic to a variety of experimental animals (6, 8, 9), has been shown to cause leukoencephalomalacia in horses (8) and to be hepatocarcinogenic in rats (7, 12), and has cancer-promoting activity in a short-term cancer initiation-promotion model in rats (3, 4). The latter bioassay was used in the isolation and purification of two new mycotoxins, fumonisins B<sub>1</sub> (FB<sub>1</sub>) and B<sub>2</sub> (FB<sub>2</sub>), from culture material of *F. moniliforme* MRC 826 (2). The structures of FB<sub>1</sub> and FB<sub>2</sub> have recently been elucidated (1), and FB<sub>1</sub> has been shown to have cancer-promoting activity in rats (2) and to cause leukoencephalomalacia in a horse (11).

The fumonisins may be of major importance in the etiology of several animal and human diseases. However, for a proper evaluation of the toxicological effects of these mycotoxins, sufficient amounts have to be purified for a variety of biological studies. At present, no information is available on experimental conditions for the optimal production of the fumonisins in culture. This paper reports the chemical determination of FB<sub>1</sub> in corn cultures of *F. moniliforme* and the influence of temperature and incubation period on the production of FB<sub>1</sub> in these cultures. In addition, the relationship between fungal growth and FB<sub>1</sub> production as well as the heat stability of this mycotoxin were also investigated.

### MATERIALS AND METHODS

**Fungal cultures.** The strain of *F. moniliforme* used in all experiments was originally isolated from corn in Transkei, southern Africa, during 1975 and deposited in the culture collection of the South African Medical Research Council

(MRC) as *F. moniliforme* MRC 826 (14). Lyophilized conidia were used to inoculate autoclaved (1 h at 121°C and 120 kPa on each of two consecutive days) corn (400 g of whole yellow corn kernels and 400 ml of water in 2-liter wide-mouthed glass fruit jars with a diameter of 11 cm and a cotton cover), and cultures (surface/volume ratio, approximately 2:5) were incubated in the dark at 20, 25, and 30°C. Triplicate jars were harvested weekly; their contents were weighed, lyophilized, and ground; and the concentration of FB<sub>1</sub> was determined as described below.

**Chemical analyses.** (i) **Fumonisin B<sub>1</sub> standard.** FB<sub>1</sub> was isolated and purified as described previously (2). For use as an analytical standard, FB<sub>1</sub> was further subjected to two successive column separations on silica gel and reverse phase (C<sub>18</sub>) columns, using CHCl<sub>3</sub>-CH<sub>3</sub>OH-CH<sub>3</sub>COOH (6:3:1) and CH<sub>3</sub>OH-H<sub>2</sub>O (3:1) as eluants, respectively. The purity of FB<sub>1</sub> was verified by nuclear magnetic resonance spectroscopy.

(ii) **Extraction and HPLC quantification.** Culture extracts were prepared and partially purified by the maleyl derivatization method described previously (20). Briefly, culture material (5 g) was extracted with CH<sub>3</sub>OH-H<sub>2</sub>O (3:1; 50 ml). A sample (25 ml) was evaporated to dryness, taken up in an equal volume of CH<sub>3</sub>OH-H<sub>2</sub>O (1:3), and extracted twice with CHCl<sub>3</sub> (50 ml). Final purification of the aqueous phase was accomplished by using a Sep-pak C<sub>18</sub> cartridge, eluting FB<sub>1</sub> in CH<sub>3</sub>OH-H<sub>2</sub>O (3:1; 10 ml). Purified extracts were treated with maleic anhydride and analyzed by high-performance liquid chromatography (HPLC) by the method of Siler and Gilchrist (18). HPLC analyses were performed on a Waters liquid chromatograph (Waters Associates, Milford, Mass.), using a reverse-phase cartridge (C<sub>18</sub>; 10 μm) in a radial compression module (model RCM-100) and 0.05 M KH<sub>2</sub>PO<sub>4</sub>-CH<sub>3</sub>OH (3:7; pH 3.5) as the mobile phase. The eluate was monitored at 230 nm. To determine whether the HPLC technique is quantitative at both low and high FB<sub>1</sub> concentrations, a standard curve was composed by analyzing

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different amounts (1, 2, 3, 4, and 5  $\mu\text{g}$ ) of pure  $\text{FB}_1$  standard after maleylation. For determination of the percent recovery, control corn (5 g) was spiked in triplicate with 1.5 mg of pure  $\text{FB}_1$  standard and analyzed as described above.

(iii) **Ergosterol analyses.** Ergosterol determinations were performed in duplicate on corn cultures of *F. moniliforme* MRC 826 incubated for 0, 2, 4, 6, 8, 10, 13, 15, and 17 weeks at 20, 25, and 30°C. The method of Seitz et al. (17) was used, with minor modifications. Samples (5 g) were extracted with methanol, the methanol extract was saponified for 30 min, and the mixture was extracted with petroleum ether (bp 60 to 80°C). After evaporation of the aqueous phase, the residue was dissolved in 2.5 ml of  $\text{CH}_2\text{Cl}_2$ -isopropanol (98:2) and a 100-fold dilution was used for HPLC analysis on a Waters liquid chromatograph, using a silica gel (Microsil) column (5.0 mm by 30 cm; 7  $\mu\text{m}$ ) and  $\text{CH}_2\text{Cl}_2$ -isopropanol (98:2) as the mobile phase. The eluate was monitored at 280 nm.

**Stability to heat.** Lyophilized culture material of *F. moniliforme* MRC 826 (500 g) was boiled in 500 ml of water for 30 min, after which it was oven dried at 60°C for 24 h. Both boiled and untreated materials were incorporated in rat mash (Epol Ltd., Johannesburg, South Africa) at a dietary level of 5% and fed to diethylnitrosamine-initiated male BD IX rats over a period of 4 weeks (3). Noninitiated rats receiving the untreated (nonheated) culture material (5%) served as the controls. The induction of gamma-glutamyl-transpeptidase-positive foci, a common marker for preneoplastic lesions (5), was used as endpoint for the assay. In addition, the boiled and untreated materials were analyzed for  $\text{FB}_1$  by HPLC as described above.

**Statistical analyses.** All analyses were performed by using the general linear model analysis of variance procedure of the Statistical Analysis System program package. The Student-Newman-Keuls multiple comparison method was used to test for significant differences between main-effect means. Significance tests for differences between the yields at 20 and 25°C for the separate weeks were performed by using a significance level adjusted for the number of tests performed. The method used was Bonferroni inequality; i.e., an observed difference is deemed significant if its  $P$  value is less than  $P' = 1 - (1 - P)^{1/n}$ , where  $P$  is desired overall significance level and  $n$  is the number of weeks for which observations were taken.

## RESULTS AND DISCUSSION

The analytical technique used for the quantification of  $\text{FB}_1$  in culture material of *F. moniliforme* MRC 826 had a recovery of approximately 85%. The detection limit of the method was in the order of 10  $\mu\text{g/g}$ , as reported by Sydenham et al. (20). HPLC chromatograms demonstrating the eluting position of  $\text{FB}_1$  standard and a purified extract of a *F. moniliforme* MRC 826 corn culture are illustrated in Fig. 1. The standard curve indicated that, for the concentration range used, a linear relationship ( $r = 0.9955$ ) exists between the recorder response and the amount of  $\text{FB}_1$  analyzed. The method described is suitable for the determination of  $\text{FB}_1$  in extracts of corn cultures of *F. moniliforme* MRC 826 as a result of the high quantities found to be produced by this strain in culture.

The fumonisins have recently been found to occur naturally in corn in South Africa (20) and the United States (21). These findings emphasize the importance of screening human and animal foodstuffs for the presence of these mycotoxins. However, to assess human exposure, the heat sta-

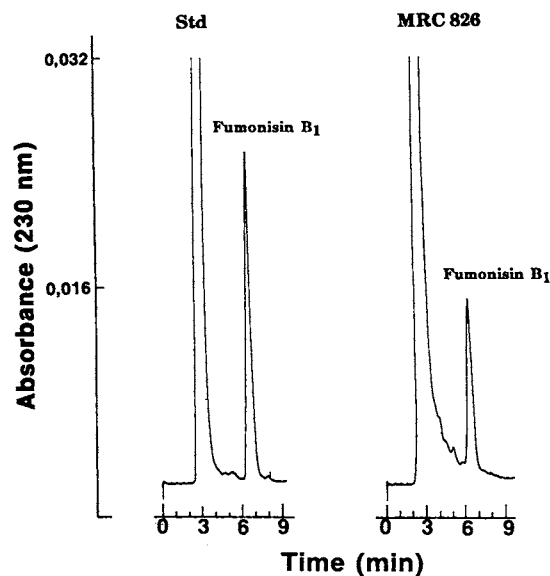


FIG. 1. HPLC-eluting chromatograms of  $\text{FB}_1$  standard (Std) and a purified extract of culture material of *F. moniliforme* MRC 826.

bility of the fumonisins is of major importance. In the present study, no difference in the  $\text{FB}_1$  concentration of boiled and untreated culture materials could be detected by HPLC (Table 1). In addition, culture material also retained its cancer-promoting (gamma-glutamyl-transpeptidase-positive) activity in diethylnitrosamine-initiated rats (Table 1). The data indicate that  $\text{FB}_1$  is not destroyed by cooking and could therefore enter the human food chain.

The optimum incubation temperature for growth of *Fusarium* spp. in culture has been reported to be alternating at 25°C day/20°C night, although they also grow well at constant temperatures between 20 and 25°C (16). As incubation of fungal cultures at alternating temperatures is impractical, we investigated the production of  $\text{FB}_1$  at constant temperatures of 20, 25, and 30°C. The ergosterol content of the fungal cultures was used as an indication of fungal growth, since it is a useful indicator of fungal invasion of grains (17), measuring both viable and nonviable fungal biomass.

Kinetics of the growth of *F. moniliforme* MRC 826 in corn

TABLE 1. Effect of heat treatment on  $\text{FB}_1$  concentration and cancer-promoting activity of corn culture material of *F. moniliforme* MRC 826 in diethylnitrosamine (DEN)-initiated rats

| MRC 826 sample      | $\text{FB}_1$ concn (g/kg, dry wt) <sup>a</sup> | DEN initiation <sup>b</sup> | Mean GGT activity (foci/cm <sup>2</sup> ) <sup>c</sup> |
|---------------------|---|-----------------------------|--|
| Untreated           | 2.3 $\pm$ 0.6                                   | -                           | ND   |
| Untreated           | 2.3 $\pm$ 0.6                                   | +                           | 4.85 $\pm$ 0.92  |
| Boiled <sup>d</sup> | 2.5 $\pm$ 0.2                                   | +                           | 3.45 $\pm$ 0.71  |

<sup>a</sup> Values represent means  $\pm$  standard deviations of triplicate determinations.

<sup>b</sup> Indicates whether or not the rats to be tested for cancer-promoting gamma-glutamyl-transpeptidase activity had been initiated with DEN (200 mg/kg). The promoting treatment commenced 1 week after initiation. Boiled and untreated culture material was incorporated in rat mash at a dietary level of 5% for a period of 4 weeks, five rats per treatment.

<sup>c</sup> Values represent means  $\pm$  standard deviations of five rats. GGT, Gamma-glutamyl-transpeptidase; ND, none detected.

<sup>d</sup> Boiled in water for 30 min and dried at 60°C for 24 h.

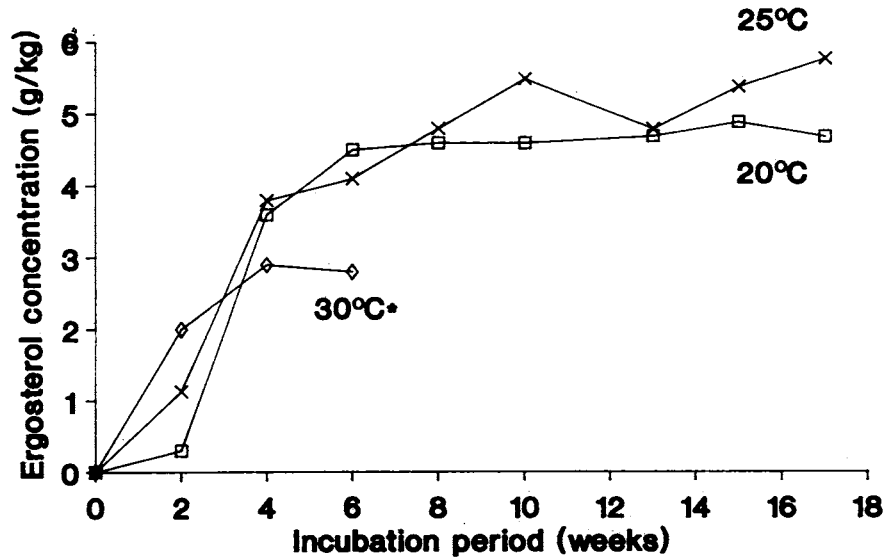


FIG. 2. Time course of growth as measured by ergosterol concentration of *F. moniliforme* MRC 826 in corn cultures at 20, 25, and 30°C. \*Cultures not further analyzed during the remainder of the experiment.

cultures at 20, 25, and 30°C are presented in Fig. 2. During the first 2 weeks of incubation, cultures at 30°C grew significantly better than those at 20°C ( $P < 0.01$ ) and 25°C ( $P < 0.05$ ), while after 4 weeks the cultures incubated at 20 and 25°C had higher ergosterol concentrations than those incubated at 30°C; only that obtained at 25°C, however, was significant ( $P < 0.05$ ). After 6 weeks, significantly ( $P < 0.05$ ) higher levels of ergosterol were detected in cultures incubated at 20 and 25°C than at 30°C, and no further increase occurred in the cultures incubated at 30°C. Although the initial growth rate was higher at 25°C than at 20°C, the maximum growth rates were similar, reaching a stationary phase at 4 to 6 weeks. In this experiment, the optimum

conditions for growth of *F. moniliforme* MRC 826 prevailed in cultures incubated at 20 and 25°C.

As our culture vessels and incubation conditions allowed evaporation, changes in the moisture content of cultures during incubation almost certainly influenced both growth and FB<sub>1</sub> production. This is evident from the data concerning the weight loss (Fig. 3) of the cultures incubated at the different temperatures. From 2 weeks onward, the cultures at 30°C had lost significantly more weight than those at 25°C ( $P < 0.05$ ) and 20°C ( $P < 0.01$ ). The weight loss between cultures incubated at 20 and 25°C also differed significantly ( $P < 0.01$ ) from 2 weeks onward. This difference increased markedly with time, and the weight loss after 4 weeks at

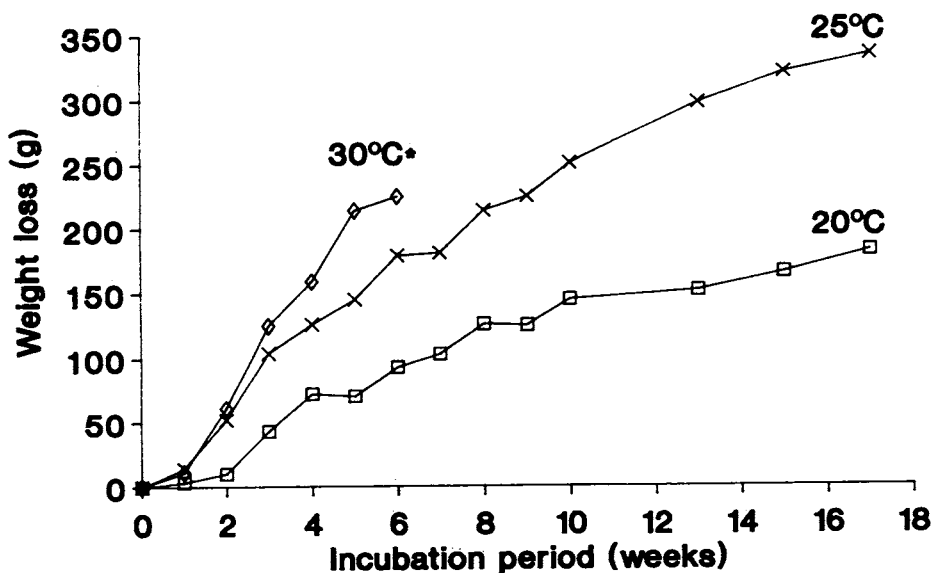


FIG. 3. Time course of weight loss of corn cultures of *F. moniliforme* MRC 826 at 20, 25, and 30°C. \*, Cultures not further analyzed during the remainder of the experiment.

TABLE 2. Time course of FB<sub>1</sub> production by *F. moniliforme* MRC 826 in corn cultures at 20 and 25°C

| Wk   | FB <sub>1</sub> concn (g/kg, dry wt) <sup>a</sup> |                | P <sup>b</sup> |
|------|---|----------------|----------------|
|      | 20°C  | 25°C           |                |
| 0    | ND  | ND             | NS             |
| 1    | ND  | ND             | NS             |
| 2    | 0.4 ± 0.25 a                                      | 1.2 ± 0.38 a   | NS             |
| 3    | 3.5 ± 0.91 ab                                     | 6.7 ± 1.10 b   | <0.05          |
| 4    | 6.1 ± 0.62 bc                                     | 9.3 ± 3.26 bc  | <0.01          |
| 5    | 5.4 ± 1.08 bc                                     | 11.6 ± 1.00 cd | <0.01          |
| 6    | 7.4 ± 3.78 c                                      | 10.3 ± 1.28 c  | <0.05          |
| 7    | 8.9 ± 0.79 cd                                     | 14.6 ± 3.21 de | <0.01          |
| 8    | 11.2 ± 2.10 d                                     | 11.7 ± 1.18 cd | NS             |
| 9    | 15.3 ± 0.85 e                                     | 11.5 ± 1.55 cd | <0.01          |
| 10   | 15.2 ± 1.59 e                                     | 12.7 ± 0.33 cd | NS             |
| 11   | 16.2 ± 0.62 e                                     | 16.5 ± 2.36 e  | NS             |
| 13   | 17.9 ± 1.84 e                                     | 15.4 ± 1.47 de | NS             |
| 15   | 14.3 ± 1.40 e                                     | 12.3 ± 0.76 cd | NS             |
| 17   | 8.1 ± 3.27 cd                                     | 9.1 ± 0.71 bc  | NS             |
| Mean | 8.7   | 9.5            | <0.05          |

<sup>a</sup> Values represent means ± standard deviations of triplicate determinations. Means in a column followed by the same letter do not differ significantly ( $P > 0.05$ ). ND, Not detected.

<sup>b</sup> Denotes significance of differences between means in rows (20 and 25°C). NS, Not significant.

25°C was identical to that of the cultures at 20°C after 8 weeks. The weight loss observed in these cultures cannot be ascribed solely to a loss in moisture content as fungal growth did occur (Fig. 2) and some carbon dioxide must have been produced. However, the weight loss in the cultures at 30°C between 4 and 6 weeks of the incubation treatment can only be ascribed to evaporation as no fungal growth occurred during this period (Fig. 2).

As the moisture content undoubtedly limited both fungal growth and FB<sub>1</sub> production at 30°C, data for FB<sub>1</sub> production at 30°C were not included in the statistical evaluation of the growth-dependent production at different temperatures.

Production of FB<sub>1</sub> at 20 and 25°C commenced at 2 weeks and continued to increase during the stationary phase, confirming the inverse relationship reported previously (19) between the rate of mycelial growth and the biosynthesis of many secondary metabolites of fungi. The concentration of FB<sub>1</sub> started to decrease at both temperatures after 13 weeks. Since FB<sub>1</sub> is heat stable, the decrease in the FB<sub>1</sub> concentration after incubation periods longer than 13 weeks probably stems from enzymatic cleavage of the molecule or a conversion to other related compounds or both.

The initial rate of FB<sub>1</sub> production was faster at 25°C than at 20°C (Table 2), with FB<sub>1</sub> production significantly ( $P < 0.01$  to 0.05) higher at 25°C than at 20°C between weeks 3 and 7. An apparent maximum FB<sub>1</sub> yield (17.9 g/kg, dry weight) was obtained at 13 weeks at 20°C (Table 2), but this was not significantly ( $P > 0.05$ ) higher than the maximum yield obtained after 11 weeks at 25°C (16.5 g/kg, dry weight). However, the mean FB<sub>1</sub> yield over the total incubation period was significantly ( $P < 0.05$ ) higher at 25°C than at 20°C, with production reaching a plateau after 7 weeks of incubation at 25°C in contrast to 9 weeks at 20°C.

It can be concluded that, under the conditions of the present experiment, the optimal incubation regimen for FB<sub>1</sub> production by *F. moniliforme* MRC 826 in cultures on corn, in terms of cost effectiveness, is 7 weeks at 25°C. However, the effect of various moisture levels, at different incubation

temperatures, on the production of FB<sub>1</sub> and other fumonisins needs to be investigated.

#### ACKNOWLEDGMENTS

We thank M. Schlechter and P. Smith of the Research Institute for Nutritional Diseases, Tygerberg, South Africa, for competent assistance.

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## Production of [<sup>14</sup>C]Fumonisin B<sub>1</sub> by *Fusarium moniliforme* MRC 826 in Corn Cultures

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Received 17 March 1993/Accepted 26 May 1993

**Kinetics of growth and fumonisin production by *Fusarium moniliforme* MRC 826 in corn "patty" cultures were investigated, and a technique was developed for the production of [<sup>14</sup>C]fumonisin B<sub>1</sub> ([<sup>14</sup>C]FB<sub>1</sub>) by using L-[methyl-<sup>14</sup>C]methionine as the precursor. A significant (*P* < 0.01) correlation exists between fungal growth and FB<sub>1</sub> (*r* = 0.89) and FB<sub>2</sub> (*r* = 0.87) production in corn patties, beginning after 2 days and reaching the stationary phase after 14 days of incubation. [<sup>14</sup>C]FB<sub>1</sub> was produced by adding L-[methyl-<sup>14</sup>C]methionine daily to cultures during the logarithmic phase of production. Incorporation of the isotope occurred at C-21 and C-22 of the fumonisin molecule and was enhanced in the presence of unlabeled L-methionine. Although the concentration of exogenous unlabeled methionine is critical for incorporation of the <sup>14</sup>C label, optimum incorporation was achieved by adding 50 mg of unlabeled L-methionine and 200 μCi of L-[methyl-<sup>14</sup>C]methionine to a corn patty (30 g) over a period of 9 days, yielding [<sup>14</sup>C]FB<sub>1</sub> with a specific activity of 36 μCi/mmol.**

The fumonisin B mycotoxins are a group of structurally related metabolites (Fig. 1) produced by *Fusarium moniliforme* Sheldon (6), one of the most prevalent fungi associated with corn (*Zea mays* L.), which is used as basic human food and animal feed (18). Fumonisin B<sub>1</sub> (FB<sub>1</sub>), the major fumonisin produced both in culture (2) and under natural conditions (26), is responsible for several toxicological effects in animals. These effects include the neurotoxic syndrome, leukoencephalomalacia in horses (15), pulmonary edema in pigs (13), and hepatocarcinogenic and hepatotoxic effects in rats on chronic feeding over an extended period (9). Apart from two reports on the excretion and tissue distribution of FB<sub>1</sub> in rats (23, 24), little work has been done on the metabolism of the fumonisins in affected animals.

As a result of its high sensitivity, isotopic labeling has greatly facilitated investigations into the metabolism in animals of several mycotoxins such as [<sup>14</sup>C]zearalenone in chickens (8, 19) and mice (3) and [<sup>3</sup>H]T-2 toxin in chickens (7) and a lactating cow (27), as well as studies of the subcellular distribution of [<sup>3</sup>H]T-2 toxin in perfused rat livers (20). The availability of isotopically labeled FB<sub>1</sub> would therefore be useful in research to clarify the mechanisms involved in the above-mentioned toxicological effects of this mycotoxin.

The biosynthesis of isotopically labeled mycotoxins produced by *Fusarium* spp. has been carried out with both solid (11, 12) and liquid (4, 17) media. Although production of mycotoxins is much higher in solid media, isotopic labeling in solid media yields compounds with low specific activities (12). The production of FB<sub>1</sub> in corn cultures (2) begins 14 days after inoculation and reaches a plateau (16.5 g/kg) after 7 weeks, whereas production in a liquid medium begins after 3 days and reaches a maximum (73 μg/ml) after 29 days (14). Since the relatively long period of FB<sub>1</sub> production in both these media would encourage nonspecific incorporation of isotopically labeled precursors into cell components other

than the fumonisins, these media are not ideal for use in isotopic labeling of FB<sub>1</sub>. In the present study we investigated the kinetics of growth and fumonisin production by *F. moniliforme* MRC 826 in corn "patties" to evaluate the culture system required for isotopic labeling experiments. In subsequent experiments attention was directed toward optimizing the conditions for labeling the methyl groups in FB<sub>1</sub> by using L-[methyl-<sup>14</sup>C]methionine as the precursor.

### MATERIALS AND METHODS

**Chemicals.** Amberlite XAD-2 and silica gel 60 (Kieselgel 60; diameter, 0.063 to 0.200 mm) were purchased from Merck SA. The reversed-phase packing material (C<sub>18</sub>; Bondesil; diameter, 40 μm) was obtained from Analytichem International, Harbor City, Calif., or prepared from silica gel 60 by the method of Kingston and Gerhardt (16). Analytical-grade organic solvents were glass distilled prior to use for the final column purification steps of FB<sub>1</sub> on silica gel and RP C<sub>18</sub>. The L-[methyl-<sup>14</sup>C]methionine (specific activity, 56 mCi/mmol) was obtained from Amersham.

**Fumonisin standards.** FB<sub>1</sub>, FB<sub>2</sub>, and FB<sub>3</sub> standards were purified as described by Cawood et al. (6) and had a chemical

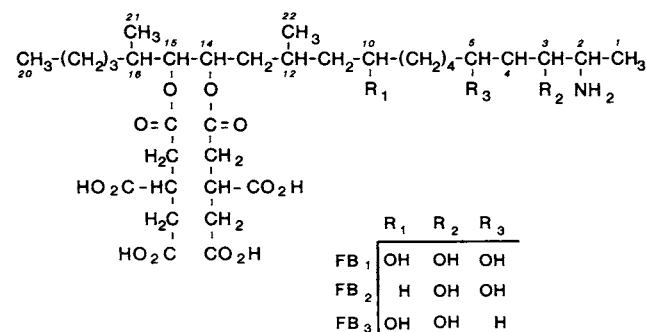


FIG. 1. Chemical structures of FB<sub>1</sub>, FB<sub>2</sub>, and FB<sub>3</sub>.

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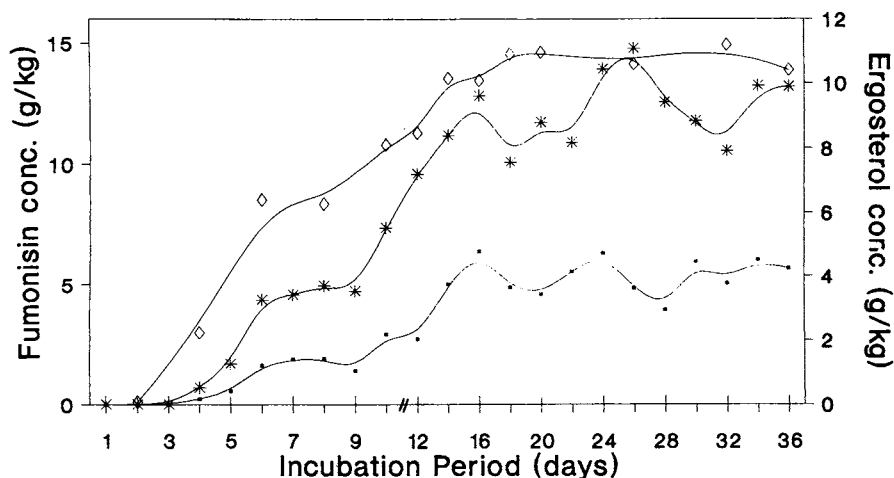


FIG. 2. Time course of growth, as measured by ergosterol concentration (◇) and production of FB<sub>1</sub> (\*) and FB<sub>2</sub> (□) by *F. moniliforme* MRC 826 in corn patty cultures.

purity of greater than 95% as assessed by thin-layer chromatography, high-pressure liquid chromatography (HPLC), and <sup>1</sup>H- and <sup>13</sup>C-nuclear magnetic resonance (NMR) spectroscopy.

**Corn patty cultures.** Yellow corn kernels, ground to fine meal (20 to 30 g) was used to prepare patty media in Pyrex petri dishes (100 by 18 mm) by the addition of water (20 to 30 ml) and then autoclaving (1 h at 121°C and 120 kPa) on each of two consecutive days. In all experiments 1 ml of a suspension of lyophilized conidia of *F. moniliforme* MRC 826 (2) in 100 ml of sterile distilled water was used to inoculate media.

**Analytical methods.** Extraction, purification, and HPLC quantification of FB<sub>1</sub>, FB<sub>2</sub>, and FB<sub>3</sub> were performed by the techniques described by Alberts et al. (1). The ergosterol concentration in corn cultures was determined by the method of Sietz et al. (25) with slight modifications (2).

**Kinetics of fungal growth versus fumonisin production.** Inoculated corn patty cultures were incubated at 25°C in the dark, and cultures were harvested daily in triplicate for 10 days and thereafter on every other day for 36 days (Fig. 2). Cultures were lyophilized and ground, and the fumonisin concentrations were determined. Ergosterol concentrations were determined in duplicate in cultures harvested every other day over the incubation period.

**Production of [<sup>14</sup>C]FB<sub>1</sub>.** Inoculated corn patties (30 g) were incubated at 25°C in the dark for 12 days. An aliquot (1 ml) of a solution of L-[methyl-<sup>14</sup>C]methionine in sterile distilled water (10 ml) was applied topically in small droplets to each culture every 24 h from days 3 to 11 (9 days) during

incubation. The cultures were harvested on day 12 and lyophilized, the fumonisins were quantified as described above, the FB<sub>1</sub> was isolated, and its specific activity (in microcuries per millimole) was determined. The dilution factor was obtained from the equation (specific activity of precursor ÷ specific activity of product) × (number of labeled sites in product ÷ number of labeled sites in precursor).

(i) **Isotopic labeling.** Preliminary experiments (Table 1) were conducted to evaluate L-[methyl-<sup>14</sup>C]methionine as a precursor for the labeling of FB<sub>1</sub>. In the first experiment, 50 μCi of L-[methyl-<sup>14</sup>C]methionine was added to a corn patty (20 g) as described above. The origin of the methyl groups present in the FB<sub>1</sub> molecule was established by using a mixture of L-[methyl-<sup>14</sup>C]methionine and L-[methyl-<sup>13</sup>C]methionine (97 atom% of <sup>13</sup>C) as a precursor and performing <sup>13</sup>C-NMR spectroscopy of the enriched FB<sub>1</sub>. The effect of unlabeled L-methionine (100 mg), which was admixed with the L-[methyl-<sup>14</sup>C]methionine, on the incorporation of the <sup>14</sup>C label was evaluated in a third experiment. The role of acetate as a precursor was also investigated by monitoring the incorporation of sodium [1-<sup>14</sup>C]acetate (125 μCi) in the presence of unlabeled sodium acetate (250 mg) and sodium [1-<sup>13</sup>C]acetate (500 mg).

(ii) **Effect of unlabeled L-methionine on growth, fumonisin production, and isotopic labeling.** The effect of unlabeled L-methionine on fungal growth and fumonisin production was investigated by adding different amounts (0, 50, 100, 150, 200, 250, and 300 mg) of unlabeled L-methionine to cultures over a period of 9 days as described above (Fig. 3).

TABLE 1. Preliminary results for specific activities obtained by using different isotopes of methionine and sodium acetate as precursors in corn patty cultures of *F. moniliforme* MRC 826

| Precursor      | Amt of unlabeled precursor (mg) | Amt of <sup>13</sup> C-labeled precursor (mg) | Amt of <sup>14</sup> C-labeled precursor (μCi) | Amt of corn (g) | Sp act (μCi/mmol) | Dilution factor |
|----------------|---------------------------------|---|--|-----------------|-------------------|-----------------|
| L-Methionine   | 0                               | 0   | 50   | 20              | 4.26              | >100            |
|                | 0                               | 50  | 25   | 30              | 6.03              | 25.0            |
|                | 100                             | 0   | 100  | 30              | 22.07             | 13.7            |
| Sodium acetate | 0                               | 500   | 125  | 30              | 2.274             | 138.5           |
|                | 250                             | 0   | 125  | 30              | 5.577             | 113.0           |



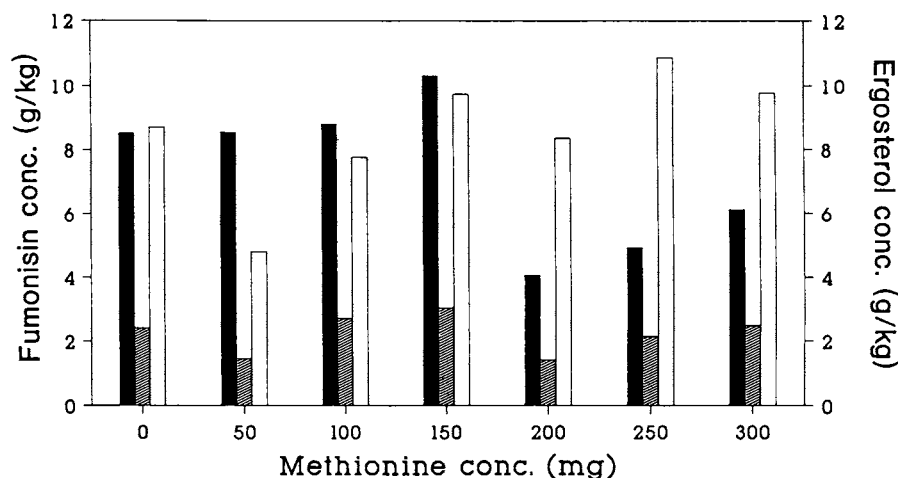


FIG. 3. Effect of L-methionine treatments on growth, as measured by ergosterol concentration (□) and production of FB<sub>1</sub> (■) and FB<sub>2</sub> (▨) by *F. moniliforme* MRC 826 in corn patty cultures.

In a subsequent experiment the effect of different amounts of unlabeled L-methionine (0, 50, 100, 150, 200, 250, and 300 mg) on the incorporation of the <sup>14</sup>C label was investigated by using a fixed amount of L-[methyl-<sup>14</sup>C]methionine (25 μCi) (Table 2).

(iii) **Isotopic labeling using different concentrations of L-[methyl-<sup>14</sup>C]methionine at constant unlabeled L-methionine concentrations.** In three separate experiments, different levels of L-[methyl-<sup>14</sup>C]methionine (50, 100, and 200 μCi) were added during the growth period with fixed amounts (50, 150, and 300 mg) of unlabeled L-methionine (Table 3) in each case.

**Purification of FB<sub>1</sub>.** Extraction and purification of FB<sub>1</sub> were performed essentially as previously described (6). Culture material (20 g [dry weight]) was extracted twice with ethyl acetate (100 ml) by blending (Polytron) and filtering (Whatman no. 4 filter paper). The residue was extracted three times with CH<sub>3</sub>OH-H<sub>2</sub>O (3:1) after the pH had been adjusted to 3.5 with 10 M HCl prior to blending (1). The combined extracts were evaporated to dryness under reduced pressure at 45°C, reconstituted in 50 ml of CH<sub>3</sub>OH-H<sub>2</sub>O (1:3), and extracted three times with 100 ml of CHCl<sub>3</sub>. The aqueous phase was evaporated to dryness under reduced pressure at 45°C. Subsequent purification was performed with Amberlite XAD-2, silica gel 60 and RP C<sub>18</sub> columns as previously described (6).

**NMR spectroscopy.** NMR spectra were recorded for solutions in [<sup>2</sup>H<sub>6</sub>]dimethylsulfoxide on a Bruker AC-300 spectroscope (7.0 T) operating at 300 MHz for <sup>1</sup>H nuclei and 75 MHz for <sup>13</sup>C nuclei. Chemical shifts are reported relative to tetramethylsilane at δ 0.00.

**Liquid scintillation counting.** The liquid scintillation cocktail for aqueous samples (Ready Value) was obtained from Beckman, Fullerton, Calif. The purified [<sup>14</sup>C]FB<sub>1</sub> samples (1 mg) were analyzed by liquid scintillation counting for 10 min in a Packard Tri-carb 460CD instrument. Quenching was corrected by using external standardization for calculations of disintegrations per minute.

**Statistical analyses.** An analysis of variance was carried out and the coefficients of variation were determined on the original data for the experiments relating to (i) fumonisin production and fungal growth and (ii) the influence of methionine on fumonisin production and fungal growth. Tukey's

Studentized range *Q* test (28) was then used to determine whether the means for the incubation period and the methionine treatment were significantly different. In addition, correlation coefficients were determined for (i) fungal growth and fumonisin production and (ii) methionine treatment versus growth and fumonisin production.

## RESULTS AND DISCUSSION

Biosynthetic isotopic labeling of several mycotoxins produced by *Fusarium* spp. has been achieved by addition of isotopically labeled precursors to fungal cultures during the logarithmic growth phase (5, 11). The optimum period for the addition of labeled precursors was determined by the rate of fumonisin production as a function of fungal growth in corn patty cultures of *F. moniliforme* MRC 826 over a period of 36 days. Production of FB<sub>1</sub> and FB<sub>2</sub> in corn patty cultures began 3 days after inoculation (Fig. 2) and increased rapidly between days 4 and 14, whereafter a plateau was reached. An apparent maximum yield of FB<sub>1</sub> (14.8 g/kg [dry weight]) and FB<sub>2</sub> (6.4 g/kg [dry weight]) was observed after 26 and 16 days, respectively, but was not significantly (*P* > 0.05) higher than the maximum yield of FB<sub>1</sub> (11.2 g/kg [dry weight]) and FB<sub>2</sub> (5.0 g/kg [dry weight]) obtained after 14 days of incubation.

A significant (*P* < 0.01) correlation was observed between

TABLE 2. Specific activities of FB<sub>1</sub> obtained following addition of 25 μCi of L-[methyl-<sup>14</sup>C]methionine and different concentrations of unlabeled L-methionine to corn patty cultures of *F. moniliforme* MRC 826

| Amt of L-methionine (mg) | Amt of corn (g) | Amt of L-[methyl- <sup>14</sup> C] methionine (μCi) | Sp act (μCi/mmol) | Dilution factor |
|--------------------------|-----------------|---|-------------------|-----------------|
| 0                        | 30              | 25  | 0.90              | >100            |
| 50                       | 30              | 25  | 3.34              | 45.2            |
| 100                      | 30              | 25  | 2.64              | 28.6            |
| 150                      | 30              | 25  | 2.86              | 17.6            |
| 200                      | 30              | 25  | 2.18              | 17.3            |
| 250                      | 30              | 25  | 2.14              | 14.1            |
| 300                      | 30              | 25  | 2.40              | 10.5            |

TABLE 3. Specific activities of pure FB<sub>1</sub> obtained following the addition of unlabeled L-methionine and different concentrations of L-[methyl-<sup>14</sup>C]methionine to corn patty cultures of *F. moniliforme* MRC 826

| Amt of L-[methyl- <sup>14</sup> C]methionine (μCi) | Amt of corn (g) | Amt of L-methionine (mg) | Fumonisin concn (g/kg [dry wt]) |                 |                 | Sp act (μCi/mmol) | Dilution factor |
|--|-----------------|--------------------------|---------------------------------|-----------------|-----------------|-------------------|-----------------|
|  |                 |                          | FB <sub>1</sub>                 | FB <sub>2</sub> | FB <sub>3</sub> |                   |                 |
| 50   | 30              | 50                       | 13.09                           | 5.03            | 1.28            | 1.82              | 165.8           |
| 100  | 30              | 50                       | 7.08                            | 2.98            | 1.14            | 11.90             | 50.8            |
| 200  | 30              | 50                       | 6.71                            | 2.80            | 0.81            | 36.00             | 33.6            |
| 50   | 30              | 150                      | 13.70                           | 2.62            | 0.68            | 5.71              | 17.7            |
| 100  | 30              | 150                      | 16.11                           | 2.38            | 0.61            | 10.71             | 18.8            |
| 200  | 30              | 150                      | 15.17                           | 3.62            | 0.87            | 18.57             | 21.7            |
| 50   | 30              | 300                      | 3.24                            | 2.22            | 0.87            | 4.75              | 10.6            |
| 100  | 30              | 300                      | 3.18                            | 2.10            | 0.64            | 6.48              | 15.5            |
| 200  | 30              | 300                      | 2.75                            | 1.90            | 0.64            | 10.26             | 19.6            |

fungal growth (as measured by ergosterol concentration) (2) and FB<sub>1</sub> ( $r = 0.89$ ) and FB<sub>2</sub> ( $r = 0.87$ ) production in the corn patty cultures. Although growth and fumonisin production in corn patty cultures are accomplished in a shorter period (stationary phase after 14 days) than in corn jars (stationary phase after 7 weeks) (2), the incorporation of isotopically labeled precursors in cell components other than the fumonisins would also be minimized. The higher growth rate and fumonisin production in corn patty cultures can be attributed to the greater accessibility of nutrients in ground corn as well as the greater availability of oxygen as reflected by the surface/volume ratio of corn patty cultures (1:1) and corn kernels in jars (2:5) (2). Therefore the corn patty cultures were likely to be more suitable than jar cultures for isotopic labeling experiments.

To circumvent any adverse effects of the high levels of added precursor on fungal growth and metabolite production (21), we carried out experiments by the pulsed addition of precursors every 24 h during the active production phase (days 3 to 14) of the fumonisins. In preliminary experiments with unlabeled, 1-<sup>13</sup>C-labeled, and 1-<sup>14</sup>C-labeled sodium acetate, FB<sub>1</sub> was produced with a specific activity of 2.27 and 5.58 μCi/mmol (Table 1). It would appear that with increasing concentrations of acetate, a decrease in the incorporation of <sup>14</sup>C, probably as a result of a dilution effect, was obtained since no enhancement of signals was observed in the <sup>13</sup>C-NMR spectrum of the purified FB<sub>1</sub>. It would appear that, when using corn patty cultures, sodium acetate is not an ideal precursor for the <sup>14</sup>C labeling of FB<sub>1</sub>.

In the first experiment with L-[methyl-<sup>14</sup>C]methionine (50 μCi) as precursor, FB<sub>1</sub> was produced with a specific activity of 4.26 μCi/mmol (Table 1). The location of the isotope label in the FB<sub>1</sub> structure was successfully determined in a subsequent experiment with a mixture of L-[methyl-<sup>14</sup>C]methionine (25 μCi) and L-[methyl-<sup>13</sup>C]methionine (50 mg, 97 atom% of <sup>13</sup>C) as precursors. In the <sup>13</sup>C-NMR spectrum, enhancements were observed for the resonances at δ<sub>C</sub> 15.47 (enrichment factor, 5.2) and 20.16 (enrichment factor, 4.5), which have been assigned to C-21 and C-22, respectively (4). This result is in agreement with the labeling pattern of FB<sub>1</sub> in liquid cultures with L-[methyl-<sup>2</sup>H<sub>3</sub>]methionine as the precursor (22): deuterium was present only in the C-12 and C-16 methyl groups.

Addition of L-[methyl-<sup>13</sup>C]methionine (50 mg) in the second experiment yielded FB<sub>1</sub> with a higher specific activity (6.03 μCi/mmol) than in the first experiment, even though only half the amount of L-[methyl-<sup>14</sup>C]methionine was used

(Table 1). A similar effect was noted in the third experiment: the admixture of 100 mg of unlabeled L-methionine with twice the amount of L-[methyl-<sup>14</sup>C]methionine (100 μCi) used in the first experiment gave FB<sub>1</sub> with ca. 5 times the specific activity (22.07 μCi/mmol). Although incorporation of the <sup>14</sup>C label occurs only if the chemical concentration of the methionine exceeds the  $K_m$  for the relevant transport system, we investigated the effect of added L-methionine on fungal growth and fumonisin production of FB<sub>1</sub> and FB<sub>2</sub>.

Although added L-methionine had no significant ( $P > 0.05$ ) effect either on fungal growth or on FB<sub>2</sub> production, there was a marked effect on FB<sub>1</sub> production (Fig. 3). A significant ( $P < 0.05$ ) increase in FB<sub>1</sub> production was observed up to the level of 150 mg of added L-methionine. At higher levels the added L-methionine had an inhibitory effect and production of FB<sub>1</sub> decreased. This phenomenon was confirmed in a later study, in which the effect of different concentrations of L-[methyl-<sup>14</sup>C]methionine at a constant amount of unlabeled L-methionine was investigated (Table 3). These data are in agreement with the finding of Plattner and Shackelford (22) that the overall yield of FB<sub>1</sub> in liquid cultures of *F. moniliforme* is increased in the presence of L-methionine.

The stimulating effect of added unlabeled L-methionine on incorporation of the <sup>14</sup>C label was confirmed by subsequent labeling experiments (Table 2): a low specific activity (0.90 μCi/mmol) was obtained when unlabeled L-methionine was omitted. It is likely that the small amount of added L-[methyl-<sup>14</sup>C]methionine is diluted with endogenous L-methionine in the cell and is consequently utilized mainly for the normal metabolic processes instead of incorporated into FB<sub>1</sub>. In contrast, at higher levels of added (exogenous) L-methionine, less is directed to metabolic purposes and consequently more of the added material is incorporated into FB<sub>1</sub>. In general, the dilution values obtained (Tables 2 and 3) are determined by the ratio of the added <sup>14</sup>C label to the total amount of unlabeled methionine present (exogenous and endogenous) during FB<sub>1</sub> synthesis. Therefore, above a certain level of unlabeled methionine the specific activity of the FB<sub>1</sub> will decrease with increasing methionine concentrations and the amount of the added L-[methyl-<sup>14</sup>C]methionine incorporated will become progressively smaller. From these studies it can be concluded that the optimum level of exogenous methionine is ca. 50 mg, since this leads to an optimum dilution value of ca. 30 and the highest specific activities (Tables 2 and 3). It should be kept in mind that the natural L-methionine content of the medium is critical in determining the optimum amount of L-methionine to be

added during labeling experiments. Since the L-methionine content of yellow corn is on the order of 1.67 mg/g (10), L-methionine additions to other media containing higher or lower concentrations should be compensated for.

In subsequent experiments the specific activity of the [<sup>14</sup>C]FB<sub>1</sub> was enhanced by increasing the amount of L-[methyl-<sup>14</sup>C]methionine (50, 100, and 200 μCi) but keeping the amount of added unlabeled L-methionine constant at either 50, 150, or 300 mg per patty (Table 3). The results again revealed the same trend in that the highest specific activity was achieved in the presence of 50 mg of unlabeled L-methionine and 200 μCi of L-[methyl-<sup>14</sup>C]methionine and decreased with increasing L-methionine concentrations. The [<sup>14</sup>C]FB<sub>1</sub> with the highest specific activities obtained in the present study (22 to 36 μCi/mmol) has been used effectively in metabolic studies with experimental animals (24).

Variation in the growth and fumonisin production profiles (Fig. 2) will occur between different runs, depending on the composition of the corn and the viability of the inoculum, which could have a profound effect on the radiolabeling of the fumonisins. Although production of most *Fusarium* mycotoxins is much lower in liquid media than in solid media (12, 14, 22), the nutritional composition of liquid media is less complex, can be controlled more accurately, and usually results in higher specific activities of labeled toxins than when solid media are used. Future studies will be directed toward obtaining a suitable liquid medium which supports optimum fumonisin production for the isotopic labeling of the fumonisins.

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## Structure Elucidation of the Fumonisin, Mycotoxins from *Fusarium moniliforme*

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The structures of the fumonisins, a family of structurally related mycotoxins isolated from cultures of *Fusarium moniliforme*, were elucidated by mass spectrometry and <sup>1</sup>H and <sup>13</sup>C n.m.r. spectroscopy as the diester of propane-1,2,3-tricarboxylic acid and either 2-acetylamino- or 2-amino-12,16-dimethyl-3,5,10,14,15-penta-hydroxyicosane as well as in each case the C-10 deoxy analogue; in all cases both the C-14 and C-15 hydroxy groups are involved in ester formation with the terminal carboxy group of propane-1,2,3-tricarboxylic acid.

The fungus *Fusarium moniliforme* Sheldon occurs world-wide on a variety of plant hosts and is one of the most important ear rot pathogens of maize (*Zea mays* L.).<sup>1</sup> An isolate of *F. moniliforme* (strain MRC 826) obtained from maize in an area of the Transkei with a high oesophageal cancer rate<sup>2</sup> was found to be not only highly toxic to experimental animals and able to induce leukoencephalomalacia in horses,<sup>3</sup> but also hepatocarcinogenic to rats,<sup>4</sup> and mutagenic to *Salmonella typhimurium*.<sup>5</sup> Although *F. moniliforme* (strain MRC 826) produces the mutagen fusarin C,<sup>6</sup> this compound is apparently not involved in the carcinogenicity of the fungus. In continuation of our efforts towards solving this important problem, we now report on the isolation and structure elucidation of a family of related mycotoxins, the fumonisins, from *F. moniliforme*.

Extraction of cultures of *F. moniliforme* (strain MRC826)

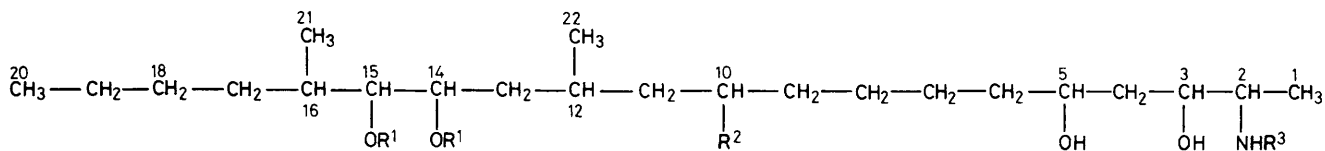
grown on sterilized maize with aqueous methanol gave an extract which was active in rat liver histopathological and cancer initiation/promotion bioassays.<sup>7</sup> Subsequently, extensive fractionation of this extract, using macroreticular polystyrene resin (XAD-2), Sephadex LH-20 and reversed phase silica gel chromatography, and guided by the assays mentioned, resulted in the isolation of a mixture of fumonisin A<sub>1</sub> (1) and A<sub>2</sub> (2), as well as fumonisin B<sub>1</sub> (3) and B<sub>2</sub> (4). Treatment of the mixture of fumonisin A<sub>1</sub> and A<sub>2</sub> with an excess of diazomethane, followed by chromatography on silica gel (chloroform-methanol, 92:8 v/v) gave colourless oils of the tetramethyl ester derivatives (5) and (6), respectively.

Liquid secondary ion mass spectrometry (l.s.i.-m.s.) of tetramethylfumonisin A<sub>1</sub> (5),  $\nu_{\max}$ . (CHCl<sub>3</sub>) 1730 (ester CO) and 1650 (amide CO) cm<sup>-1</sup>, gave the protonated molecular ion as  $m/z$  820 ( $M + H$ )<sup>+</sup>, supported by an ion at  $m/z$  842 ( $M$

+ Na)<sup>+</sup>. The multiplicities of the different resonances in the <sup>13</sup>C n.m.r. spectrum of (5) were determined by generating the proton-decoupled CH, CH<sub>2</sub>, and CH<sub>3</sub> subspectra using the DEPT pulse sequence.<sup>8</sup> These data (see Table 1) suggested the empirical formula C<sub>40</sub>H<sub>69</sub>NO<sub>16</sub> for (5) which is consistent with the accurate mass measurement of the ion of highest mass in the electron impact (e.i.) mass spectrum at *m/z* 770.4321 (C<sub>39</sub>H<sub>64</sub>NO<sub>14</sub>) [*M* - (H<sub>2</sub>O + CH<sub>3</sub>O)<sup>+</sup>]. The presence of three hydroxy groups in (5) was indicated by the formation of a tris(trimethylsilyl) derivative (*M*<sup>+</sup> 1035, C<sub>49</sub>H<sub>93</sub>NO<sub>16</sub>Si<sub>3</sub>) on treatment with *N*-methyl-*N*-trimethylsilyl trifluoroacetamide and a triacetate (*M*<sup>+</sup> 945, C<sub>46</sub>H<sub>75</sub>NO<sub>19</sub>) on acetylation. Basic

hydrolysis with 0.05 M methanolic potassium hydroxide for 16 h at room temperature gave a neutral product (7), C<sub>24</sub>H<sub>49</sub>NO<sub>6</sub>, and a salt which on acidification and methylation with an excess of diazomethane was converted into a product identical (g.c.-m.s.) to trimethylpropane-1,2,3-tricarboxylate.

The proton-proton connectivity pattern of tetramethyl fumonisin A<sub>1</sub> (5) was determined by two-dimensional (2D) (<sup>1</sup>H, <sup>1</sup>H) correlation spectroscopy using the COSY-45 pulse sequence,<sup>9</sup> and defined two distinct structural units, *i.e.* C-1—C-5 and C-10—C-17. The involvement of both the C-14 and C-15 oxygen atoms in ester linkages with the terminal



- (1) R<sup>1</sup> = COCH<sub>2</sub>CH(CO<sub>2</sub>H)CH<sub>2</sub>CO<sub>2</sub>H, R<sup>2</sup> = OH, R<sup>3</sup> = C<sup>23</sup>OC<sup>24</sup>H<sub>3</sub>  
 (2) R<sup>1</sup> = COCH<sub>2</sub>CH(CO<sub>2</sub>H)CH<sub>2</sub>CO<sub>2</sub>H, R<sup>2</sup> = H, R<sup>3</sup> = COCH<sub>3</sub>  
 (3) R<sup>1</sup> = COCH<sub>2</sub>CH(CO<sub>2</sub>H)CH<sub>2</sub>CO<sub>2</sub>H, R<sup>2</sup> = OH, R<sup>3</sup> = H  
 (4) R<sup>1</sup> = COCH<sub>2</sub>CH(CO<sub>2</sub>H)CH<sub>2</sub>CO<sub>2</sub>H, R<sup>2</sup> = R<sup>3</sup> = H  
 (5) R<sup>1</sup> = COCH<sub>2</sub>CH(CO<sub>2</sub>CH<sub>3</sub>)CH<sub>2</sub>CO<sub>2</sub>CH<sub>3</sub>, R<sup>2</sup> = OH, R<sup>3</sup> = COCH<sub>3</sub>  
 (6) R<sup>1</sup> = COCH<sub>2</sub>CH(CO<sub>2</sub>CH<sub>3</sub>)CH<sub>2</sub>CO<sub>2</sub>CH<sub>3</sub>, R<sup>2</sup> = H, R<sup>3</sup> = COCH<sub>3</sub>  
 (7) R<sup>1</sup> = H, R<sup>2</sup> = OH, R<sup>3</sup> = COCH<sub>3</sub>

Table 1. N.m.r. data for tetramethylfumonisin A<sub>1</sub> (5).<sup>a</sup>

| Carbon atom | δ <sub>C</sub> <sup>b</sup> | δ <sub>H</sub> <sup>c,j</sup> | <i>J</i> (HH)/Hz | Carbon atom | δ <sub>C</sub> <sup>b</sup> | δ <sub>H</sub> <sup>c</sup> | <i>J</i> (HH)/Hz |
|-------------|-----------------------------|-------------------------------|------------------|-------------|-----------------------------|-----------------------------|------------------|
| 1           | 18.03 Q                     | 1.139 d                       | 6.8              | 25          | 170.88 S                    | —                           |                  |
| 2           | 49.72 D                     | 3.902 m                       |                  | 26          | 35.27 T <sup>h</sup>        | 2.780 dd <sup>h</sup>       | 16.7, 6.7        |
| 3           | 71.49 D                     | 3.79 m                        |                  | 27          | 37.28 D <sup>f</sup>        | 2.603 dd                    | 16.8, 6.4        |
| 4           | 40.22 T                     | 1.56                          |                  | 28          | 35.10 T <sup>h</sup>        | 3.22 m                      |                  |
| 5           | 68.83 D                     | 3.82                          |                  | 29          | 173.41 S                    | 2.732 dd <sup>h</sup>       | 16.6, 7.1        |
| 6           | 38.55 T <sup>d</sup>        |                               |                  | 30          | 171.69 S <sup>g</sup>       | 2.583 dd                    | 16.7, 6.5        |
| 7           | 25.63 T <sup>e</sup>        |                               |                  | 31          | 52.31 Q <sup>i</sup>        | 3.664 s <sup>i</sup>        |                  |
| 8           | 25.48 T <sup>e</sup>        |                               |                  | 32          | 51.82 Q <sup>i</sup>        | 3.645 s <sup>i</sup>        |                  |
| 9           | 37.22 T <sup>d</sup>        |                               |                  |             |                             |                             |                  |
| 10          | 68.78 D                     | 3.56                          |                  |             |                             |                             |                  |
| 11          | 43.08 T                     | 1.37                          |                  | 33          | 171.42 S                    | —                           |                  |
| 12          | 25.17 D                     | 0.96                          |                  | 34          | 35.10 T <sup>h</sup>        | 2.672 dd                    | 16.8, 8.1        |
| 13          | 35.39 T                     | 1.75                          |                  | 35          | 37.19 D <sup>f</sup>        | 2.400 dd                    | 16.8, 5.9        |
| 14          | 71.24 D                     | 1.64                          |                  | 36          | 34.93 T <sup>h</sup>        | 3.22 m                      |                  |
| 15          | 77.79 D                     | 1.28                          | 11.3, 3.3, 2.3   | 37          | 173.93 S                    | 2.647 dd <sup>h</sup>       | 16.7, 6.5        |
| 16          | 33.64 D                     | 5.137 ddd                     | 8.8, 3.3         | 38          | 171.66 S <sup>g</sup>       | 2.583 dd                    | 16.7, 6.5        |
| 17          | 31.84 T                     | 4.867 dd                      |                  | 39          | 52.15 Q <sup>i</sup>        | 3.645 s <sup>i</sup>        |                  |
| 18          | 28.48 T                     | 1.58                          |                  | 40          | 51.80 Q <sup>i</sup>        | 3.634 s <sup>i</sup>        |                  |
| 19          | 22.73 T                     | 1.31                          |                  |             |                             |                             |                  |
| 20          | 13.92 Q                     | 0.98                          |                  |             |                             |                             |                  |
| 21          | 15.36 Q                     | 1.23                          |                  |             |                             |                             |                  |
| 22          | 20.36 Q                     | 1.08                          |                  |             |                             |                             |                  |
| 23          | 170.48 S                    | ~1.2                          |                  |             |                             |                             |                  |
| 24          | 23.32 Q                     | 0.829 t                       | 7.1              |             |                             |                             |                  |
|             |                             | 0.872 d                       | 6.8              |             |                             |                             |                  |
|             |                             | 0.912 d                       | 6.6              |             |                             |                             |                  |

<sup>a</sup> Recorded on a Bruker WM-500 spectrometer. <sup>b</sup> Relative to CDCl<sub>3</sub> at δ<sub>C</sub> 77.00. Chemical shifts of the proton-bearing carbon atoms were correlated with specific proton resonances.<sup>9,11</sup> <sup>c</sup> Relative to CDCl<sub>3</sub> at δ<sub>H</sub> 7.24. <sup>d-i</sup> May be interchanged. <sup>j</sup> The NH proton appears as a doublet at δ<sub>H</sub> 6.071 [*J*(NH, 2-H) 8.7 Hz].

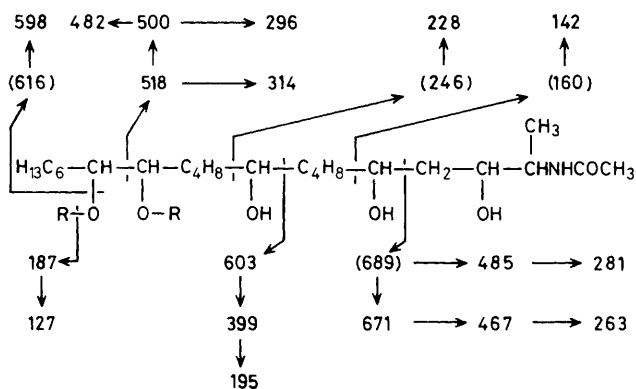


Figure 1. Fragmentation pattern observed in the e.i. mass spectrum of (5).

carboxy groups of the two propane-1,2,3-tricarboxylate moieties (as indicated by the  $^1\text{H}$  and  $^{13}\text{C}$  n.m.r. data)<sup>10</sup> and the location of the methyl groups in (5) followed from the two- and three-bond ( $^{13}\text{C}$ ,  $^1\text{H}$ ) connectivity pattern (Table 2) determined in a chemical shift correlation experiment adjusted to detect correlations *via* long-range ( $^{13}\text{C}$ ,  $^1\text{H}$ ) couplings ( $nJ$  5 Hz).<sup>9,11</sup>

The fragmentation pattern for compound (5) (see Figure 1) in the electron impact mass spectrum was of vital importance in determining the linkage of the C-1—C-5 and C-10—C-17 structural fragments by a  $\text{C}_4\text{H}_8$  group and placing a  $\text{C}_6\text{H}_{13}$  substituent at C-15. The base peak in the mass spectrum occurs at  $m/z$  187 ( $\text{C}_8\text{H}_{11}\text{O}_5$ ) and is associated with the cleavage of the ester moiety as indicated. The loss of 204 mass units from a number of fragment ions throughout the spectrum is due to the loss of a dimethyl propane-1,2,3-tricarboxylate group. The location of the hydroxy groups in (5) as indicated by the n.m.r. data is consistent with the fragment ions at  $m/z$  671, 603, 228, and 142 and was confirmed by the fragmentation pattern of the tris(trimethylsilyl) derivative.

Tetramethylfumonisins  $\text{A}_2$  (6),  $\text{C}_{40}\text{H}_{69}\text{NO}_{15}$ , contains one oxygen atom less than tetramethylfumonisins  $\text{A}_1$  (5). The protonated molecular ion of (6) appears at  $m/z$  804 ( $M + \text{H}^+$ ) in the l.s.i. mass spectrum and the fragment ion which arises from the molecular ion in the e.i. spectrum through loss of ( $\text{H}_2\text{O} + \text{CH}_3\text{O}^+$ ) appears at  $m/z$  754 ( $\text{C}_{39}\text{H}_{64}\text{NO}_{13}$ ). A comparison of the fragmentation patterns of compounds (6) and (5) shows that it is the C-10 hydroxy group of (5) which is replaced by a hydrogen atom in (6) as the bond cleavage which generates the fragment ions  $m/z$  603  $\rightarrow$  399  $\rightarrow$  195 in the spectrum of (5) does not occur. In addition a number of bond cleavages common to both compounds generate fragment ions of 16 mass units less for tetramethylfumonisins  $\text{A}_2$  (6).

L.s.i. mass spectrometry of fumonisins  $\text{B}_1$  (3) ( $\text{C}_{34}\text{H}_{59}\text{NO}_{15}$ ) gave the protonated molecular ion as  $m/z$  722 whereas that of fumonisins  $\text{B}_2$  (4) ( $\text{C}_{34}\text{H}_{59}\text{NO}_{14}$ ) appears at  $m/z$  706. In each case ions are also present at  $m/z$  values corresponding to ( $M + \text{Na}$ ) $^+$ . A comparison of the  $^{13}\text{C}$  n.m.r. data of these two compounds† indicates that once again it is the C-10 hydroxy group of fumonisins  $\text{B}_1$  (3) ( $\delta_{\text{C}}$  67.17) which is replaced by a hydrogen atom in fumonisins  $\text{B}_2$  (4). In addition, the  $^1\text{H}$  and

Table 2. Two- and three-bond ( $^1\text{H}$ ,  $^{13}\text{C}$ ) connectivity pattern for tetramethylfumonisins  $\text{A}_1$  (5).

| $\delta_{\text{H}}$ | Correlation signals $\delta_{\text{C}}$ /p.p.m.    |
|---------------------|--|
| 0.829 (20-H)        | 22.73 T (C-19), 28.48 T (C-18)                     |
| 0.872 (21-H)        | 31.84 T (C-17), 33.64 D (C-16)                     |
|                     | 77.79 D (C-15)                                     |
| 0.912 (22-H)        | 25.17 D (C-12), 35.39 T (C-13)                     |
| 1.139 (1-H)         | 49.72 D (C-2), 71.49 D (C-3)                       |
| 1.949 (24-H)        | 170.48 S (C-23)                                    |
| 4.867 (15-H)        | 33.64 D (C-16), 71.24 D (C-14),<br>170.88 S (C-25) |
| 5.137 (14-H)        | 77.79 D (C-15), 171.42 S (C-33)                    |

$^{13}\text{C}$  resonances of the atoms of the *N*-acetyl group present in the fumonisins A metabolites, e.g. (5):  $\delta_{\text{H}}$  1.949;  $\delta_{\text{C}}$  170.48, and 23.32, are absent from the spectra of the fumonisins B compounds.

It is of interest to note that a related mono-ester of propane-1,2,3-tricarboxylic acid and 1-amino-11,15-dimethyl-2,4,5,13,14-pentahydroxyheptadecane is a host-specific phytotoxin produced by *Alternaria alternata* (Fr.) Keissler f. sp. *lycopersici*, the causal agent of stem canker disease of tomato.<sup>10,12</sup>

We thank the Alexander von Humboldt Stiftung for a research fellowship (to C. P. G-A) and M. Glaessner, University of Bayreuth, for the recording of mass spectra.

Received, 13th October 1987; Com. 1476

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† (3):  $\delta_{\text{C}}$  ( $\text{CD}_3\text{SOCD}_3$ ) 175.91S, 175.60S, 174.31S, 173.97S, 171.45S (C-25), 171.39S (C-33), 76.48D (C-15), 70.35D (C-14), 68.42D (C-13), 67.17D (C-10), 66.01D (C-5), 51.69D (C-2), 43.28T (C-11), 40.72T (C-4), 38.17D (C-27 and C-35), 38.19T, 38.07 ( $2 \times$  T), 37.80T (C-26), 36.54T (C-28), 36.38T (C-34), 35.72T (C-36), 32.96D (C-16), 30.96T (C-17), 28.08T (C-18), 25.43T, 25.28T, 25.20D (C-12), 22.22T (C-19), 20.19Q (C-22), 15.57Q (C-1), 15.51Q (C-21), 13.86Q (C-20).

## Isolation of the Fumonisin Mycotoxins: A Quantitative Approach

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A method for the preparative-scale isolation of the fumonisin B (FB) mycotoxins, from corn cultures of *Fusarium moniliforme*, is described and quantitatively evaluated. Eighty percent of FB<sub>1</sub> and 60% of FB<sub>2</sub> were recovered after extraction with CH<sub>3</sub>OH/H<sub>2</sub>O (3:1). The fumonisins, including the newly discovered FB<sub>3</sub> and FB<sub>4</sub>, were purified using Amberlite XAD-2, silica gel, and reverse-phase C<sub>18</sub> chromatography. The Amberlite XAD-2 purification step proved to be the most effective cleanup procedure, while subsequent chromatography on silica gel and RP C<sub>18</sub> effectively separate the individual fumonisins to a purity of over 90%. The relatively low final yield (40%) of FB<sub>1</sub> and FB<sub>2</sub> may be ascribed to (1) the strong affinity of FB<sub>1</sub> for silica gel, (2) the low initial recovery (60%) of FB<sub>2</sub>, and (3) the formation of monomethyl and dimethyl esters of FB<sub>1</sub> and FB<sub>2</sub>, as well as their interference in the purification of the individual fumonisins. The *N*-acetyl derivatives of FB<sub>1</sub> and FB<sub>2</sub> were also purified and shown to be metabolites of *F. moniliforme*.

### INTRODUCTION

Recent investigations on the mycotoxins produced by *Fusarium moniliforme* Sheldon indicate that the fumonisin mycotoxins could be responsible for the major toxicological and carcinogenic effects caused by corn cultures of the fungus in experimental animals (Gelderblom et al., 1988a,b, 1991; Kellerman et al., 1990; Marasas et al., 1988; Voss et al., 1989). At present little is known about the biological effects of these toxins in different animal species. The need to develop efficient and cost-effective methods for purifying sufficient quantities for biological evaluation is therefore of particular importance since the fumonisin mycotoxins occur under natural conditions (Sydenham et al., 1990a,b; Shephard et al., 1990) and could enter the human and animal food chains.

The first paper on the isolation and purification of fumonisin B<sub>1</sub> (FB<sub>1</sub>) and fumonisin B<sub>2</sub> (FB<sub>2</sub>) dealt mainly with the detection of cancer-promoting compounds from cultures of a carcinogenic strain of *F. moniliforme*; the purification techniques were described only qualitatively (Gelderblom et al., 1988a). Extraction of the fumonisin mycotoxins was achieved with CH<sub>3</sub>OH/H<sub>2</sub>O (3:1) followed by a solvent-partitioning step using CHCl<sub>3</sub>. The subsequent purification of the aqueous phase was effected on Amberlite XAD-2, silica gel, and reverse-phase (C<sub>18</sub>) chromatographic columns yielding FB<sub>1</sub> and FB<sub>2</sub>, both with a purity of approximately 90%.

This paper describes methods for the quantitative purification of FB<sub>1</sub>, FB<sub>2</sub>, and the newly discovered fumonisin B<sub>3</sub> (FB<sub>3</sub>) from corn cultures of *F. moniliforme* strain MRC 826. In addition, several other structurally related compounds, including fumonisin A<sub>1</sub> (FA<sub>1</sub>) and fumonisin A<sub>2</sub> (FA<sub>2</sub>), which were previously observed during the purification of FB<sub>1</sub> and FB<sub>2</sub> (Gelderblom et al., 1988a; Bezuidenhout et al., 1988), and fumonisin B<sub>4</sub> (FB<sub>4</sub>) were purified and their chemical structures determined.

### MATERIALS AND METHODS

**Chemicals.** Amberlite XAD-2 and silica gel 60 (Kieselgel 60, 0.063–0.200 mm) were purchased from Merck SA. The reverse-phase (RP) packing material (C<sub>18</sub>; Bondesil; 40 μm) was obtained from Analytichem International, Harbour City, CA, or prepared from silica gel 60 (0.063–0.200 mm) according to the method of Kingston and Gerhart (1976). All of the organic solvents were of analytical grade and glass-distilled prior to use during the final column purification steps on silica gel and RP C<sub>18</sub>.

**Fungal Cultures.** Corn cultures of *F. moniliforme* strain 826 were prepared as described previously (Alberts et al., 1990) and incubated in the dark for 6 weeks at 25 °C. Cultures were oven-dried at 50 °C, ground, and stored at 4 °C until required.

**Fumonisin Standards.** FB<sub>1</sub>, FB<sub>2</sub>, and FB<sub>3</sub> (Figure 1) were purified according to the method described in the present paper. For use as analytical standards the fumonisins were further subjected to two successive column separations on silica gel (second column) and reverse-phase (C<sub>18</sub>) columns as described below. The purity of both standards was verified by nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC) (Alberts et al., 1990), and silica gel thin-layer chromatography (TLC), and they are considered for the purpose of this study as 100% pure.

**Detection and Quantification of the Fumonisins.** All of the sample extracts and column fractions were analyzed by silica gel TLC for the presence of the different fumonisins using CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O/CH<sub>3</sub>COOH (55:36:8:1) and/or CHCl<sub>3</sub>/CH<sub>3</sub>OH/CH<sub>3</sub>COOH (6:3:1) as developing solvents. The TLC plates were developed by spraying with a *p*-anisaldehyde [0.5 g of *p*-anisaldehyde in CH<sub>3</sub>OH/CH<sub>3</sub>COOH/H<sub>2</sub>SO<sub>4</sub> (8:10:5)] and/or a ninhydrin (0.2% in ethanol) solution and heated at 120 °C until color development. On the basis of the TLC analyses, column fractions were combined in such a manner that the most effective separation between the individual fumonisins was accomplished. Subsamples of the combined column fractions and sample extracts were kept throughout the purification process for the quantification of FB<sub>1</sub>, FB<sub>2</sub>, and FB<sub>3</sub>.

Quantification of the fumonisins in the sample extracts and combined column fractions was achieved by HPLC analyses of the maleyl derivatives as described previously (Alberts et al., 1990). The mobile phases used consisted of (1) CH<sub>3</sub>OH/0.1 M KH<sub>2</sub>PO<sub>4</sub> (7:3; 10 mM MgCl<sub>2</sub>; pH 3.5) for FB<sub>1</sub> analyses and (2) CH<sub>3</sub>OH/0.1 M KH<sub>2</sub>PO<sub>4</sub> (18:7; 10 mM MgCl<sub>2</sub>; pH 3.5) for FB<sub>2</sub> and FB<sub>3</sub> analyses.

**Extraction and Solvent Partitioning.** Culture material (1 kg) was extracted twice with ethyl acetate (1 L) by blending (Waring blender) and filtering (Whatman No. 4). The residue

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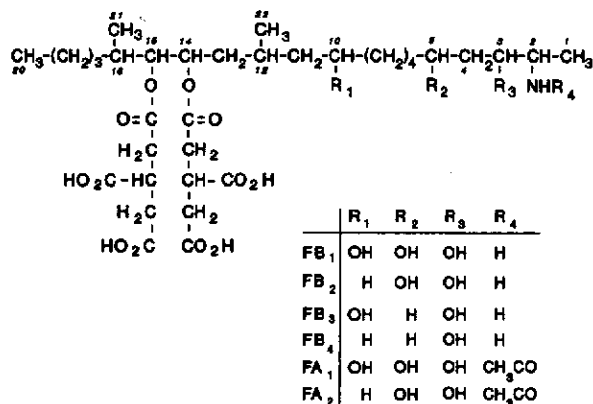
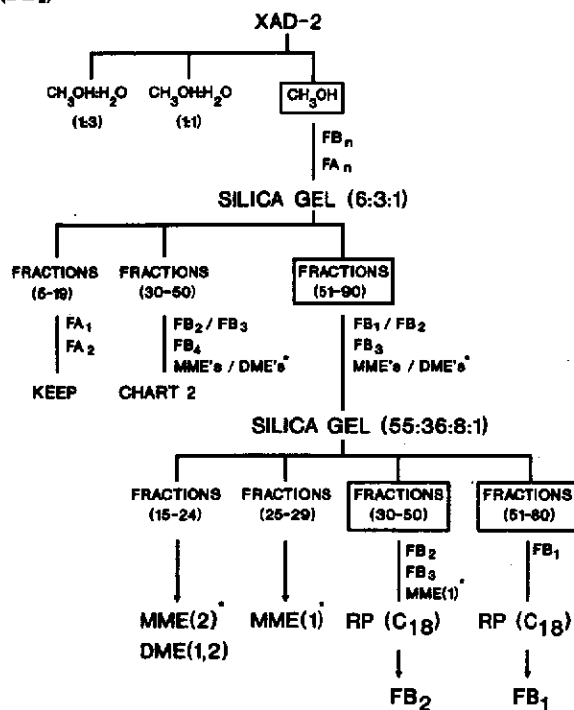


Figure 1. Chemical structures of the different fumonisins produced by *F. moniliforme*.

Chart I. Schematic Representation of the Procedures Used for the Isolation of Fumonisin B<sub>1</sub> (FB<sub>1</sub>) and B<sub>2</sub> (FB<sub>2</sub>)



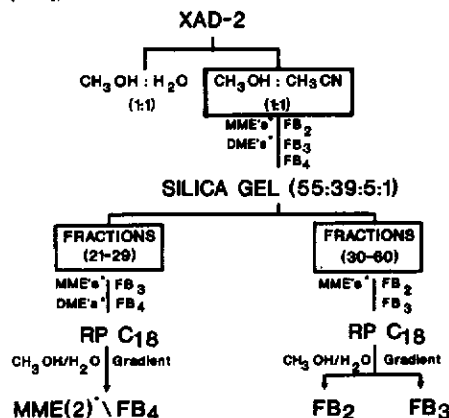
\* MMEs/DMEs, monomethyl esters/dimethyl esters; MME(1), monomethyl ester of FB<sub>1</sub>; MME(2), monomethyl ester of FB<sub>2</sub>; DME(1,2), dimethyl ester of FB<sub>1</sub> and FB<sub>2</sub>, respectively.

was extracted once with 1 L and twice with 1.5 L of CH<sub>3</sub>OH/H<sub>2</sub>O (3:1) as described above. The combined extracts were evaporated to dryness under vacuum at 50 °C; the residual material was dried at 60 °C and stored at 4 °C for FB<sub>1</sub> and FB<sub>2</sub> analyses. The dried culture extract was dissolved in CH<sub>3</sub>OH/H<sub>2</sub>O (1:3, 200 mL) at 50 °C and partitioned with CHCl<sub>3</sub> (3 × 100 mL) as described previously (Gelderblom et al., 1988a). The aqueous phase was evaporated to dryness under vacuum at 50 °C. A schematic diagram of the column chromatographic purification procedures for the different fumonisins is outlined in Charts I and II.

**Purification of FB<sub>1</sub>.** *Amberlite XAD-2.* The above extraction residue (125 ± 32 g, Table I) was dissolved in CH<sub>3</sub>OH/H<sub>2</sub>O (1:3; 100 mL) and applied to an Amberlite XAD-2 column (7 × 87 cm; sample:resin 1:15) previously equilibrated with CH<sub>3</sub>OH/H<sub>2</sub>O (1:3). After the column was washed successively with CH<sub>3</sub>OH/H<sub>2</sub>O (1:3; 1 L) and CH<sub>3</sub>OH/H<sub>2</sub>O (1:1; 1.5 L), the fumonisin mycotoxins were eluted with CH<sub>3</sub>OH (1 L).

*Silica Gel 60. First Column [CHCl<sub>3</sub>/CH<sub>3</sub>OH/CH<sub>3</sub>COOH (6:3:1) as Eluant].* The residue obtained from the methanol fraction

Chart II. Schematic Representation of the Procedures Used for the Isolation of Fumonisin B<sub>2</sub> (FB<sub>2</sub>), B<sub>3</sub> (FB<sub>3</sub>), and B<sub>4</sub> (FB<sub>4</sub>)



\* MMEs, monomethyl esters, DMEs, dimethyl esters; MME(2), monomethyl ester of FB<sub>2</sub>.

Table I. Quantification of the Extraction of FB<sub>1</sub> and FB<sub>2</sub> from Fungal Culture Material

| sample                                    | wt, g        | FB <sub>1</sub> concn, <sup>a</sup> g | FB <sub>2</sub> concn, <sup>a</sup> g |
|---|--------------|---------------------------------------|---------------------------------------|
| corn culture                              | 1000         | 2.24 ± 0.40                           | 1.12 ± 0.33                           |
| ethyl acetate                             | 81.1 ± 3.4   | nd <sup>b</sup>                       | nd <sup>b</sup>                       |
| CH <sub>3</sub> OH/H <sub>2</sub> O (3:1) | 125.2 ± 17.6 | 1.82 ± 0.5 (81.3%) <sup>c</sup>       | 0.67 ± 0.2 (59.8%) <sup>c</sup>       |

<sup>a</sup> Values represent means ± standard deviation of two different samples each done in triplicate. <sup>b</sup> nd, not detected. <sup>c</sup> The percentage of FB<sub>1</sub> and FB<sub>2</sub> recovered in the CH<sub>3</sub>OH/H<sub>2</sub>O (3:1) fraction is given in parentheses.

(10 ± 0.6 g) was first fractionated on a silica gel column (5.5 × 85 cm; 1100 g) using CHCl<sub>3</sub>/CH<sub>3</sub>OH/CH<sub>3</sub>COOH (6:3:1) as the mobile phase. The sample was dissolved in the eluant (70 mL) and applied to the column which contained anhydrous Na<sub>2</sub>SO<sub>4</sub> (ca. 50 g) on top of the silica gel. After elution of 1.3 L of mobile phase at a flow rate of 3.5 mL/min, fractions (50 mL) were collected and analyzed by TLC. Fractions containing FA<sub>1</sub> and FA<sub>2</sub> (5–19), FB<sub>2</sub> and FB<sub>3</sub> (30–50), and FB<sub>1</sub>, FB<sub>2</sub>, and FB<sub>3</sub> (51–90) were combined separately (Chart I) and the solvents evaporated at 50 °C under reduced pressure. The subsequent purification of the FB<sub>2</sub>/FB<sub>3</sub> mixture (fractions 30–50) and the other structurally related compounds is described separately.

*Second Column [CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O/CH<sub>3</sub>COOH (55:36:8:1) as Eluant].* The main FB<sub>1</sub> fraction (51–90; 4.8 ± 0.22 g) was purified by silica gel column chromatography as described above, but without the Na<sub>2</sub>SO<sub>4</sub>, using CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O/CH<sub>3</sub>COOH (55:36:8:1) as eluant. The sample, dissolved in 20 mL of the eluant, was applied to the column and eluted at 2 mL/min. After elution of 1.2 L of mobile phase, the flow rate was changed to 3.5 mL/min while fractions (50 mL) were collected and analyzed by TLC. Fractions 50–80 (2.14 ± 0.32 g) contained only FB<sub>1</sub>. The fractions containing a mixture of FB<sub>2</sub>, FB<sub>3</sub>, and other related compounds, such as monomethyl esters (MMEs) and dimethyl esters (DMEs), were combined separately (Chart I).

*Reverse Phase (C<sub>18</sub>).* The final purification of FB<sub>1</sub> was achieved on a RP C<sub>18</sub> column (1.5 × 50 cm; 200 g) equipped with end fittings and using CH<sub>3</sub>OH/H<sub>2</sub>O (1:1) as eluant. The eluant flow was maintained at 1.5 mL/min with the aid of a peristaltic pump. The pH of the sample, dissolved in CH<sub>3</sub>OH/H<sub>2</sub>O (1:1; 20 mL), was adjusted to 3.5 with 1 N HCl prior to application to the column. Fractionation was effected by running a 800-mL linear gradient from CH<sub>3</sub>OH/H<sub>2</sub>O (1:1) to CH<sub>3</sub>OH/H<sub>2</sub>O (4:1) at a flow rate of 1.5 mL/min. Fractions (15 mL) were collected after 400 mL of the gradient had eluted from the column. The purity of FB<sub>1</sub> (1.33 ± 0.44 g), which eluted as a single peak from the column (fractions 8–15), was determined by HPLC (Alberts et al., 1990).

**Purification of FB<sub>2</sub>.** *Silica Gel.* During the first chromatographic separation on silica gel the FB<sub>3</sub>-containing fractions (30–50) and the FB<sub>1</sub>-containing fractions (51–90) each contained



similar amounts of FB<sub>2</sub>. The purification of FB<sub>2</sub> from the FB<sub>2</sub>/FB<sub>3</sub> sample will be described separately. The FB<sub>2</sub> that coeluted with FB<sub>1</sub> and some FB<sub>3</sub> was fractionated on a silica gel column as described above (second column). As indicated in Chart I, FB<sub>2</sub> eluted first from the column (fractions 30–50) followed by FB<sub>1</sub>, while a small amount of FB<sub>3</sub> coeluted with FB<sub>2</sub>.

**Reverse Phase (C<sub>18</sub>).** The C<sub>18</sub> material, prepared from silica gel as described by Kingston and Gerhart (1976), was equilibrated with CH<sub>3</sub>OH/H<sub>2</sub>O (1:1) and packed in a column (3 × 27 cm) supplied with end fittings. The sample, at a pH of 3.5, was fractionated using a 800-mL linear gradient from CH<sub>3</sub>OH/H<sub>2</sub>O (1:1) to CH<sub>3</sub>OH while the flow was maintained at 1.5 mL/min. Fractions (15 mL) were collected after 300 mL had eluted from the column. FB<sub>2</sub>, with a purity of 90%, was obtained in fractions 17–30. FB<sub>3</sub>, which coeluted with FB<sub>2</sub> during the course of the second silica gel column fractionation, was obtained in fractions 5–14 with a purity of only 20%.

**Purification of FB<sub>3</sub>.** *Amberlite XAD-2.* The material containing FB<sub>2</sub> and FB<sub>3</sub> (fractions 30–50, Chart I), obtained from the first silica gel column purification, was further fractionated on Amberlite XAD-2 (Chart II). The Amberlite XAD-2 column (4.5 × 30 cm) was equilibrated with CH<sub>3</sub>OH/H<sub>2</sub>O (1:1), and the sample (3.24 ± 0.2 g), dissolved in the same solvent, was applied after the pH had been adjusted to 3.5 with 0.1 N HCl. After the column was washed with 400 mL of CH<sub>3</sub>OH/H<sub>2</sub>O (1:1), the fumonisins were eluted with CH<sub>3</sub>OH/CH<sub>3</sub>CN (1:1; 250 mL). Fractions containing both FB<sub>2</sub> and FB<sub>3</sub> were combined and the solvents evaporated in vacuo at 50 °C.

*Silica Gel 60.* A silica gel column (5 × 45 cm; 300 g) was equilibrated with CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O/CH<sub>3</sub>COOH (55:39:5:1). The material obtained from the Amberlite XAD-2 column (2.16 ± 0.20 g) was applied to the column, and after elution of 450 mL (1.0 mL/min), fractions (30 mL) were collected. Fractions 30–60, containing a mixture of FB<sub>2</sub> and FB<sub>3</sub>, were pooled and the solvents evaporated at 50 °C.

**Reverse Phase (C<sub>18</sub>).** Final separation of FB<sub>2</sub> and FB<sub>3</sub> was achieved on the RP C<sub>18</sub> column described above. The column was first regenerated by successive elution with CH<sub>3</sub>OH (250 mL) containing 0.1 MMgCl<sub>2</sub> and H<sub>2</sub>O (250 mL) and subsequently equilibrated with CH<sub>3</sub>OH/H<sub>2</sub>O (1:1). After application of the sample (1.99 ± 0.10 g, pH 3.5), separation was achieved by gradient elution as described earlier using a 800-mL gradient from CH<sub>3</sub>OH/H<sub>2</sub>O (1:1) to CH<sub>3</sub>OH. FB<sub>3</sub> (0.38 ± 0.02 g), which eluted first, chromatographed as a single spot on TLC and was subjected to further analyses (HPLC and NMR) to confirm its purity. FB<sub>2</sub> (0.37 ± 0.01 g) with a purity of 92% was obtained from subsequent fractions.

**Purification of Structurally Related Compounds.** In the course of purifying FB<sub>1</sub>, FB<sub>2</sub>, and FB<sub>3</sub> several other compounds that reacted similarly to the fumonisins with both *p*-anisaldehyde and ninhydrin were observed by silica gel TLC. On the basis of their reaction with ninhydrin these compounds were divided into ninhydrin-positive and ninhydrin-negative compounds.

**Ninhydrin Positive Compounds.** TLC analysis of the CH<sub>3</sub>OH fraction from the Amberlite XAD-2 column and the fractions obtained during its subsequent purification on silica gel (first column) indicated the presence of several spots from compounds with higher *R<sub>f</sub>* values than FB<sub>1</sub> and FB<sub>2</sub> (Table IV). These spots were eventually shown to consist of a mixture of the monomethyl and dimethyl esters of both FB<sub>1</sub> and FB<sub>2</sub> and another fumonisin given the trivial name of FB<sub>4</sub> (Figure 1). The same fractionation steps used during the purification of FB<sub>2</sub> and FB<sub>3</sub> were applied for the isolation of FB<sub>4</sub> (Chart I). Subsequent fractionation of FB<sub>4</sub>, after the RP C<sub>18</sub> purification step, was performed on a silica gel column using CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O/CH<sub>3</sub>COOH (55:36:8:1) as eluant. The FB<sub>4</sub> was finally fractionated through a Sep-Pak C<sub>18</sub> cartridge as described below and subjected to structural analyses.

After the second silica gel column, a pure preparation of a mixture of monomethyl esters (MMEs) of FB<sub>1</sub> containing some inorganic compounds was obtained (Chart I). The fraction containing the mixture of DMEs of FB<sub>1</sub> and FB<sub>2</sub> and the MME of FB<sub>2</sub> was further fractionated on a silica gel column (2 × 60 cm) using CHCl<sub>3</sub>/CH<sub>3</sub>OH/H<sub>2</sub>O/CH<sub>3</sub>COOH (55:36:8:1) as the eluant. This chromatographic system achieved good separation

between the DME of FB<sub>1</sub> and the MME and DME of FB<sub>2</sub>. Final purification of the MMEs and DMEs of FB<sub>1</sub> and FB<sub>2</sub> for structure elucidation by NMR was achieved on a C<sub>18</sub> Sep-Pak cartridge (Waters, Millipore Corp., Milford, MA). The cartridge was washed with CH<sub>3</sub>OH and equilibrated with CH<sub>3</sub>OH/H<sub>2</sub>O (1:1). After application of the sample (pH 3.5), 20 mL of the eluant was passed through the cartridge. The compounds were eluted using CH<sub>3</sub>OH. The solvent was evaporated and the pure compounds subjected to structural analyses.

**Ninhydrin Negative Compounds.** Fractions obtained during chromatography on the first silica gel column revealed two spots on TLC with *R<sub>f</sub>* values of 0.38 and 0.46, respectively, upon spraying with *p*-anisaldehyde (Table IV). These compounds, called FA<sub>1</sub> and FA<sub>2</sub> (Bezuidenhout et al., 1988) (Figure 1), were further purified on an Amberlite XAD-2 column (4.5 × 30 cm) using the same elution procedures as described for the purification of FB<sub>3</sub>. The material, still containing both FA<sub>1</sub> and FA<sub>2</sub>, was subsequently fractionated on a silica gel column (2 × 65 cm) using CHCl<sub>3</sub>/CH<sub>3</sub>OH/CH<sub>3</sub>COOH/H<sub>2</sub>O (65:25:6:4) as eluant. TLC analyses of the fractions indicated that FA<sub>1</sub> (fractions 30–40) and FA<sub>2</sub> (fractions 20–27) were cleanly separated and each combined fraction exhibited a single spot on TLC. The final purification of each compound was achieved on a RP C<sub>18</sub> column (3 × 30 cm) equilibrated with CH<sub>3</sub>OH/H<sub>2</sub>O (1:1). The compounds were eluted in CH<sub>3</sub>OH/H<sub>2</sub>O (3:1) after the column had been washed with the equilibration solvent (250 mL).

The ratio of sample to RP packing material was approximately 1:100, whereas for silica gel it was 1:250. The pH of all the samples was adjusted to 3.5 prior to fractionation on Amberlite XAD-2 and RP C<sub>18</sub> columns. For samples of 50 mg or less final purification was accomplished using Sep-Pak C<sub>18</sub> cartridges.

## RESULTS AND DISCUSSION

In the present study the fundamental extraction and purification steps reported previously (Gelderblom et al., 1988a) were used, with minor modifications, for the isolation of FB<sub>1</sub>, FB<sub>2</sub>, FB<sub>3</sub>, and FB<sub>4</sub> and other structurally related compounds. Chemical structures of the newly discovered fumonisins B<sub>3</sub> and B<sub>4</sub> are given in Figure 1.

Extraction of the fumonisins was carried out using CH<sub>3</sub>OH/H<sub>2</sub>O (3:1) after the culture material had been extracted with ethyl acetate to remove lipid-soluble material. The latter step was very effective in that, although approximately 8% of material was recovered from the ethyl acetate extract, no fumonisin mycotoxins were extracted (Table I). Subsequent extraction with aqueous CH<sub>3</sub>OH resulted in an 81.3% recovery of FB<sub>1</sub> from the culture material (Table I). The recovery of the analytical technique for the quantitation of FB<sub>1</sub> in fungal cultures, using the same extraction solvent, is on the order of 85% (Alberts et al., 1990). In contrast, a far lower yield of FB<sub>2</sub> (59.8%) was obtained which could partly be ascribed to the extraction solvent used, as it was selected for the isolation and quantification of FB<sub>1</sub> (Alberts et al., 1990). From this study it would appear that aqueous methanol is not a good extraction solvent for FB<sub>2</sub>, and further investigation is necessary.

A high-percentage recovery of both FB<sub>1</sub> (98%) and FB<sub>2</sub> (97.5%) was obtained from the aqueous phase after the CHCl<sub>3</sub> partitioning step, while a low (1.1-fold) purification was achieved. The aim of the solvent-partitioning step is, as in the case with the ethyl acetate extraction, to remove lipid-soluble material that could interfere during the Amberlite XAD-2 column purification step.

Chromatographic separation on the Amberlite XAD-2 column was performed by a stepwise increase in the CH<sub>3</sub>OH content of the eluting solvent (Chart I). Fractionation on this column is based on group separation. The bulk (>94%) of the fumonisins was recovered in the final methanol eluate (Tables II and III), while a high degree of purification was achieved (12.1-fold for FB<sub>1</sub>, 8.7-fold

Table II. Quantification of FB<sub>1</sub> and FB<sub>2</sub> during Successive Column Purification Steps

| purification step        | sample wt, <sup>a</sup> g | fumonisin wt, <sup>a</sup> g | recovery, <sup>b</sup> % | fumonisin concn, % | purification <sup>c</sup> |      |
|--------------------------|---------------------------|------------------------------|--------------------------|--------------------|---------------------------|------|
|                          |                           |                              |                          |                    | degree                    | fold |
| solvent partitioning     | 125.0 ± 32.6              | FB <sub>1</sub> 2.64 ± 0.28  | 98                       | 2.1                |                           | 1.1  |
|                          |                           | FB <sub>2</sub> 1.23 ± 0.17  | 97.5                     | 1.0                |                           |      |
| XAD (CH <sub>3</sub> OH) | (i) 10.0 ± 0.6            | FB <sub>1</sub> 2.55 ± 0.18  | 98                       | 25.5               | 12.1                      | 12.5 |
|                          | (ii) 13.3 ± 0.1           | FB <sub>2</sub> 1.16 ± 0.16  | 94                       | 8.7                | 8.7                       | 9.6  |
| silica gel (6:3:1)       | (i) 4.75 ± 0.22           | FB <sub>1</sub> 1.90 ± 0.15  | 74.5                     | 40.0               | 1.6                       | 26   |
|                          | (ii) 5.60 ± 0.22          | FB <sub>2</sub> 0.68 ± 0.03  | 58                       | 12.1               | 1.4                       | 22   |
| silica gel (55:36:8:1)   | (i) 2.14 ± 0.32           | FB <sub>1</sub> 1.30 ± 0.35  | 68                       | 60.7               | 1.5                       | 58   |
|                          | (ii) 1.30 ± 0.07          | FB <sub>2</sub> 0.49 ± 0.14  | 72                       | 37.7               | 3.1                       | 96   |
| RP (C <sub>18</sub> )    | (i) 1.33 ± 0.44           | FB <sub>1</sub> 1.20 ± 0.29  | 91                       | 90.2               | 1.5                       | 94   |
|                          | (ii) 0.50 ± 0.04          | FB <sub>2</sub> 0.46 ± 0.01  | 94                       | 92.0               | 2.4                       | 250  |

<sup>a</sup> All values represent means ± standard deviation calculated from performing each purification in triplicate. The quantitation of the recoveries of FB<sub>1</sub> and FB<sub>2</sub> was done in two separate triplicate series. <sup>b</sup> Percentage fumonisin recovered during each purification step. <sup>c</sup> Degree of purification represents the increase in fumonisin concentration achieved by each purification step, while the fold represents the extent of purification of each fumonisin after the solvent-partitioning step.

Table III. Quantification of FB<sub>2</sub> and FB<sub>3</sub> during Successive Column Purification Steps

| purification step                           | sample wt, <sup>a</sup> g | fumonisin wt, <sup>a</sup> g | recovery, <sup>b</sup> % | fumonisin concn, % | purification <sup>c</sup> |         |
|---|---------------------------|------------------------------|--------------------------|--------------------|---------------------------|---------|
|   |                           |                              |                          |                    | degree                    | fold    |
| solvent partitioning                        | 125 ± 32.6                | FB <sub>2</sub> 1.23 ± 0.17  |                          | 1.0                |                           |         |
|   |                           | FB <sub>3</sub> 0.62 ± 0.08  |                          | 0.5                |                           |         |
| XAD (CH <sub>3</sub> OH)                    | 13.3 ± 0.1                | FB <sub>2</sub> 1.16 ± 0.16  | (a) 94                   | 8.7                | 8.7                       | 9.6     |
|   |                           | FB <sub>3</sub> 0.61 ± 0.08  | (b) 98                   | 4.6                | 9.2                       | 9.6     |
| silica gel (6:3:1)                          | 3.24 ± 0.17               | FB <sub>2</sub> 0.49 ± 0.06  | (a) 42                   | 15.1               | 1.7                       | 39      |
|   |                           | FB <sub>3</sub> 0.52 ± 0.08  | (b) 85                   | 16                 | 3.5                       | 39      |
| XAD (CH <sub>3</sub> OH/CH <sub>3</sub> CN) | 2.16 ± 0.20               | FB <sub>2</sub> 0.46 ± 0.09  | (a) 94                   | 21.3               | 1.4                       | 58      |
|   |                           | FB <sub>3</sub> 0.51 ± 0.08  | (b) 98                   | 23.6               | 1.5                       | 58      |
| silica gel (55:39:5:1)                      | 1.99 ± 0.10               | FB <sub>2</sub> 0.35 ± 0.02  | (a) 76                   | 17.6               | 0.8                       | 63      |
|   |                           | FB <sub>3</sub> 0.36 ± 0.03  | (b) 71                   | 18.1               | 0.8                       | 63      |
| RP (C <sub>18</sub> )                       | (a) 0.37 ± 0.02           | FB <sub>2</sub> 0.33 ± 0.03  | (a) 94                   | 89.2               | 5.1                       | (a) 338 |
|   | (b) 0.38 ± 0.02           | FB <sub>3</sub> 0.36 ± 0.02  | (b) 99                   | 94.7               | 5.2                       | (b) 328 |

<sup>a</sup> All values represent means ± standard deviation calculated from performing each purification step in triplicate. <sup>b</sup> Percentage fumonisin recovered during each purification step. <sup>c</sup> Degree of purification represents the increase in fumonisin concentration achieved by each purification step, while the fold represents the extent of purification of each fumonisin after the solvent-partitioning step. Quantitation of FB<sub>2</sub> and FB<sub>3</sub> eluted from the XAD-2 column was performed on two different CH<sub>3</sub>OH eluates (a and b).

for FB<sub>2</sub>, Table II; 9.2-fold for FB<sub>3</sub>, Table III). This chromatographic procedure is therefore by far the most effective purification step for removing residual material other than the fumonisins, although no separation between the individual fumonisins is obtained. In addition, polar components that would interfere with the subsequent purifications on silica gel were effectively removed from the aqueous extract.

The most effective separation between the different fumonisins was obtained on silica gel by using two different mobile phases. Both column separations resulted in approximately 70% recovery of FB<sub>1</sub> (Table II). During the first silica gel column separation, using CHCl<sub>3</sub>/CH<sub>3</sub>OH/CH<sub>3</sub>OOH (6:3:1) as the eluant, most of the pigmented material (Steyn et al., 1979) present in culture extracts of *F. moniliforme* MRC 826 was separated from FB<sub>1</sub>, FB<sub>2</sub>, and FB<sub>3</sub>. When this purification step was omitted, some of the pigments were present in the final FB<sub>1</sub> preparation. This purification step did not completely separate FB<sub>1</sub> from FB<sub>2</sub> and FB<sub>3</sub>, but as the aim of this study was directed at the purification of FB<sub>1</sub>, the fractions were combined separately, thus yielding two main fractions. One fraction (fractions 51–90) contained the bulk of the FB<sub>1</sub> as well as about 50% of the FB<sub>2</sub> and far less FB<sub>3</sub>, while the other fraction (fractions 30–50) consisted mainly of FB<sub>3</sub> and some FB<sub>2</sub> (Chart I). The recovery of FB<sub>3</sub> from the first silica gel column was 85% (Table III). The FB<sub>2</sub> was recovered quantitatively from the first silica gel column with 58% being obtained from the fractions containing FB<sub>1</sub> (Table II) and 42% from those containing FB<sub>3</sub> (Table III). The lower recovery for FB<sub>1</sub> (74.5%, Table II) can be explained by the long elution time and its tendency to streak from silica gel.

The advantage of the second silica gel column purification step in the isolation of FB<sub>1</sub> was that FB<sub>1</sub> was completely separated from FB<sub>2</sub> as shown by TLC. However, the material recovered in the combined FB<sub>1</sub> fractions contained much methanol-insoluble material, presumably salts, which only dissolved in aqueous methanol at a pH below 3.5. The subsequent RP C<sub>18</sub> column step was therefore aimed mainly at removing the inorganic material while the gradient elution of FB<sub>1</sub> also removed some yellow pigments that coeluted with FB<sub>1</sub> from the second silica gel column. A high recovery (91%) and relatively low purification (1.5-fold) of FB<sub>1</sub> was achieved in this chromatographic purification step (Table II).

Although the present method was developed mainly to purify FB<sub>1</sub>, it became clear that, with minor modifications in the sequence of the columns and eluants used, FB<sub>2</sub> and FB<sub>3</sub> could also be purified without any major difficulties. Approximately 50% of the FB<sub>2</sub> was purified to over 90% purity by applying the same column purification procedures as for FB<sub>1</sub> (Chart I; Table II). The introduction of another Amberlite XAD-2 column in the purification of the main FB<sub>2</sub>/FB<sub>3</sub> sample (containing the other half of the FB<sub>2</sub>) was to effect the removal of CH<sub>3</sub>OH-insoluble material which coeluted with this fraction from the first silica gel column. As discussed earlier, this material elutes mainly with FB<sub>1</sub> from the second silica gel column. The use of CH<sub>3</sub>CN in place of CH<sub>3</sub>OH during the second Amberlite XAD-2 chromatographic step was necessary because of the lower polarity of FB<sub>2</sub> and FB<sub>3</sub> in comparison to that of FB<sub>1</sub> (Table IV). A very high recovery of FB<sub>2</sub> (94%) and FB<sub>3</sub> (98%) from the Amberlite XAD-2 column was achieved (Table III). FB<sub>2</sub> and FB<sub>3</sub> coeluted from both the second Amberlite XAD-2 column and the subsequent

**Table IV. Chemical and Chromatographic Characteristics of the Fumonisin and Structurally Related Derivatives**

| compound             | $R_f$ value <sup>a</sup> | FB <sub>1</sub>             | FB <sub>2</sub>             |
|----------------------|--------------------------|-----------------------------|-----------------------------|
| FB <sub>1</sub>      | 0.23 (0.15) <sup>b</sup> |                             |                             |
| FB <sub>2</sub>      | 0.30 (0.20)              |                             |                             |
| FB <sub>3</sub>      | 0.30 (0.26)              |                             |                             |
| FB <sub>4</sub>      | 0.37 (0.31)              |                             |                             |
| MMEs(1) <sup>c</sup> | 0.35 (0.27)              | monomethyl ester            |                             |
| MMEs(2) <sup>c</sup> | 0.42 (0.34)              |                             | monomethyl ester            |
| DMEs(1) <sup>c</sup> | 0.48 (0.42)              | dimethyl ester              |                             |
| DMEs(2) <sup>c</sup> | 0.54 (0.46)              |                             | dimethyl ester              |
| FA <sub>1</sub>      | 0.38 (0.71)              | <i>N</i> -acetyl derivative |                             |
| FA <sub>2</sub>      | 0.46 (0.76)              |                             | <i>N</i> -acetyl derivative |

<sup>a</sup> Silica gel TLC using CHCl<sub>3</sub>:CH<sub>3</sub>OH:H<sub>2</sub>O:CH<sub>3</sub>COOH (55:36:8:1) as developing solvent. <sup>b</sup> Values in parentheses represent  $R_f$  values with CHCl<sub>3</sub>:CH<sub>3</sub>OH:CH<sub>3</sub>COOH (6:3:1) as developing solvent. <sup>c</sup> MMEs, monomethyl esters; DMEs, dimethyl esters.

silica gel column but were finally separated on RP C<sub>18</sub>. The latter step is the only chromatographic procedure which effectively separates FB<sub>2</sub> from FB<sub>3</sub>. As indicated in Chart I, FB<sub>4</sub> is also purified during these chromatographic procedures.

Fumonisin B<sub>1</sub>, B<sub>2</sub>, and B<sub>3</sub> are the major naturally produced fumonisins in corn cultures of *F. moniliforme* strain MRC 826. FB<sub>4</sub>, FA<sub>1</sub>, and FA<sub>2</sub> are produced only in minor quantities. A major disadvantage of the main separation between the different fumonisins on silica gel is the fact that several structurally related compounds (MMEs and DMEs) are formed from FB<sub>1</sub>, FB<sub>2</sub>, and probably FB<sub>3</sub> as a result of the presence of CH<sub>3</sub>OH and CH<sub>3</sub>COOH in the mobile phase (Table IV; Chart I). The carboxylic acid group involved in the formation of each ester is not known. The formation of the MMEs complicates the purification of FB<sub>2</sub> and FB<sub>3</sub> especially during their fractionation on silica gel as they eluted in the same region, as indicated by the  $R_f$  values obtained by TLC (Table IV). The same problem is encountered on the C<sub>18</sub> columns where good separation was obtained between FB<sub>1</sub>, FB<sub>2</sub>, and FB<sub>3</sub> but not between FB<sub>3</sub> and the MMEs of FB<sub>1</sub> and FB<sub>2</sub>, respectively. The purification of FB<sub>4</sub>, which appears to be produced only in minor quantities by the fungus, is hampered by the same difficulties. No problems were encountered in the purification of the dimethyl ester derivatives of both FB<sub>1</sub> and FB<sub>2</sub> as they are eluted much earlier from silica gel (higher  $R_f$  values).

FA<sub>1</sub> and FA<sub>2</sub> were obtained after the first silica gel column separation, and NMR spectral analyses indicated that these compounds are the *N*-acetyl derivatives of FB<sub>1</sub> and FB<sub>2</sub>, respectively. These compounds have previously been reported as metabolites produced by *F. moniliforme* in culture (Bezuidenhout et al., 1988). This finding is confirmed in the present study as there is no indication that either FB<sub>1</sub> or FB<sub>2</sub> is converted into these compounds in the presence of CH<sub>3</sub>COOH even when incubated at fairly high temperatures (60 °C).

Although the method described in this paper is not optimal with respect to the quantities of FB<sub>1</sub>, FB<sub>2</sub>, and FB<sub>3</sub> that are purified, it does provide ample amounts of these mycotoxins for biological evaluation in experimental animals. The high concentration of FB<sub>1</sub> present in culture material of this specific fungal isolate facilitates the purification of FB<sub>1</sub> over that of FB<sub>2</sub> and FB<sub>3</sub> as indicated by the much greater ease (lower purification fold; Table II) with which pure FB<sub>1</sub> can be obtained. At present the fumonisin production profiles of other isolates of *F. mo-*

*niliforme* are under investigation in an attempt to select those isolates which will allow optimal purification of each individual fumonisin. Other solvent systems for the purification of the fumonisins on silica gel are also under investigation to prevent the formation of the structurally related compounds, thereby increasing the final yield of the pure fumonisins.

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Received for review April 17, 1991. Accepted July 31, 1991.

Registry No. FA<sub>1</sub>, 117415-48-2; FA<sub>2</sub>, 117415-47-1; FB<sub>1</sub>, 116355-83-0; FB<sub>1</sub> monomethyl ester, 136458-38-3; FB<sub>1</sub> dimethyl ester, 136458-39-4; FB<sub>2</sub>, 116355-84-1; FB<sub>2</sub> monomethyl ester, 136520-22-4; FB<sub>2</sub> dimethyl ester, 136458-40-7; FB<sub>3</sub>, 136379-59-4; FB<sub>4</sub>, 136379-60-7.

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Received for review April 17, 1991. Accepted July 31, 1991.

Registry No. FA<sub>1</sub>, 117415-48-2; FA<sub>2</sub>, 117415-47-1; FB<sub>1</sub>, 116355-83-0; FB<sub>1</sub> monomethyl ester, 136458-38-3; FB<sub>1</sub> dimethyl ester, 136458-39-4; FB<sub>2</sub>, 116355-84-1; FB<sub>2</sub> monomethyl ester, 136520-22-4; FB<sub>2</sub> dimethyl ester, 136458-40-7; FB<sub>3</sub>, 136379-59-4; FB<sub>4</sub>, 136379-60-7.

## Fumonisin—Novel Mycotoxins with Cancer-Promoting Activity Produced by *Fusarium moniliforme*

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Received 2 March 1988/Accepted 22 April 1988

Cultures on corn of *Fusarium moniliforme* MRC 826 are known to cause leukoencephalomalacia in horses and to be toxic and hepatocarcinogenic in rats. Culture material of this *F. moniliforme* isolate has also been shown to exhibit cancer-promoting activity in a short-term cancer initiation-promotion bioassay with diethylnitrosamine-initiated rats and the induction of gamma-glutamyl-transpeptidase-positive (GGT<sup>+</sup>) foci as an endpoint after 4 weeks of promotion. This bioassay was used as a monitoring system to isolate cancer-promoting compounds from cultures of *F. moniliforme* MRC 826. Culture material was successively extracted with ethyl acetate and CH<sub>3</sub>OH-H<sub>2</sub>O (3:1). Most of the cancer-promoting activity was recovered in the CH<sub>3</sub>OH-H<sub>2</sub>O extract and remained in the aqueous phase following partitioning of this extract between CH<sub>3</sub>OH-H<sub>2</sub>O (1:3) and CHCl<sub>3</sub>. The CH<sub>3</sub>OH-H<sub>2</sub>O fraction was chromatographed on an Amberlite XAD-2 column, and the active fraction was eluted with CH<sub>3</sub>OH. This fraction was chromatographed on a silica gel column with CHCl<sub>3</sub>-CH<sub>3</sub>OH-CH<sub>3</sub>COOH (6:3:1) as eluent and further purified on a C<sub>18</sub> reverse-phase column. Two pure compounds were isolated, and these have been chemically characterized and given the trivial names fumonisin B<sub>1</sub> and B<sub>2</sub>. At least 2 g of the major compound fumonisin B<sub>1</sub> was purified from 1 kg of culture material. Fumonisin B<sub>1</sub> in the diet (0.1%) significantly ( $P < 0.001$ ) induced the formation of GGT<sup>+</sup> foci in the livers of initiated as well as noninitiated rats. The cancer-promoting effect of fumonisin B<sub>1</sub> in rats was associated with a toxic effect, as evidenced by a significant ( $P < 0.0005$ ) reduction in weight gain during the 4-week promoting treatment. The principal pathological change in rats treated with fumonisin B<sub>1</sub> was an insidious and progressive toxic hepatitis similar to that induced by toxic culture material of *F. moniliforme* MRC 826.

The toxicological effects of *Fusarium moniliforme* Sheldon in animals have been studied extensively (11). This fungus is known to cause leukoencephalomalacia (LEM) in horses (9) and to be highly toxic to a variety of experimental animals (5, 7, 8) and hepatocarcinogenic in rats (6, 10). Recently, corn samples naturally infected by *F. moniliforme* and implicated in field outbreaks of LEM in the United States have been reported to be hepatocarcinogenic in rats (14). These findings not only suggest that the LEM toxin and the hepatocarcinogen produced by *F. moniliforme* may be the same metabolite, but also imply that this compound(s) occurs naturally in *F. moniliforme*-infected corn. The toxic and/or carcinogenic principle(s) has, however, not been identified to date.

Culture material of *F. moniliforme* MRC 826, isolated from corn intended for human consumption in Transkei, southern Africa, has been shown to cause LEM in horses (7) and to be carcinogenic in rats (6, 10). Attempts to identify the carcinogen(s) produced by this strain have resulted in the isolation (3) and chemical characterization (1) of the potent mutagen fusarin C. However, this compound is apparently not involved in the hepatocarcinogenic activity of the fungus (2, 6). A recent study (2) indicated that culture material of *F. moniliforme* MRC 826, known to be hepatocarcinogenic in rats (6, 10), exhibited cancer-promoting activity in a cancer initiation-promotion model with diethylnitrosamine (DEN)-initiated rats and the induction of gamma-glutamyl-transpeptidase-positive (GGT<sup>+</sup>) foci as an endpoint. Based on these

results, a short-term cancer initiation-promotion bioassay was established to screen different toxic strains of *F. moniliforme* for their cancer-promoting activity (W. C. A. Gelderblom, W. F. O. Marasas, K. Jaskiewicz, S. Combrinck, and D. J. van Schalkwyk, Carcinogenesis, in press). Rats fed diets containing 5% culture material of each strain during the 4-week promotion period revealed a highly significant correlation between the toxicity and cancer-promoting activity of the different strains. This finding strongly suggests that the same metabolite is responsible for the toxic (7, 8) and carcinogenic (6, 10) effects of *F. moniliforme* MRC 826 culture material in rats.

This paper reports on the isolation of cancer-promoting compounds produced by *F. moniliforme* MRC 826 with a short-term cancer initiation-promotion bioassay in rats as a monitoring system. Preliminary data on the toxic effects of one of these pure compounds in rats are also presented.

### MATERIALS AND METHODS

**Chemicals.** DEN was obtained from Sigma Chemical Co., St. Louis, Mo. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and Amberlite XAD-2 was purchased from Merck SA. The reverse-phase packing material (C<sub>18</sub>; Sepralyte, 40 μm) was obtained from Analytichem International, Harbor City, Calif. All the organic solvents were analytical grade.

**Fungus cultures.** Corn cultures of *F. moniliforme* strain MRC 826 were prepared as described previously (8) and incubated in the dark at 25°C for 2 weeks, followed by 2

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TABLE 1. Cancer-promoting activity of *F. moniliforme* MRC 826 culture material and fractions obtained during the solvent partitioning step

| Rat treatment group (n = 5) | Sample   | Culture material (g) | Fraction (g)       | DEN initiation | Mean GGT activity (foci/cm <sup>2</sup> ) ± SD |
|-----------------------------|--|----------------------|--------------------|----------------|--|
| 1                           | Culture material                                       | 200                  |                    | +              | 9.82 ± 4.00                                    |
| 2                           | Culture material                                       | 200                  |                    | -              | N.D. <sup>a</sup>                              |
| 3                           | Rat mash (control)                                     |                      |                    | +              | N.D.   |
| 4                           | Ethyl acetate <sup>b</sup>                             | 1,000                | 99.4               | +              | 0.18 ± 0.30                                    |
| 5                           | CH <sub>3</sub> OH-H <sub>2</sub> O (3:1) <sup>b</sup> | 1,000                | 144.0 <sup>c</sup> | +              | 34.76 ± 13.46                                  |
| 6                           | Residue <sup>b</sup>                                   | 1,000                | 736.0              | +              | 0.56 ± 0.57                                    |

<sup>a</sup> N.D., None detected.

<sup>b</sup> Samples obtained after successive extraction of 1 kg of culture material.

<sup>c</sup> After the first week of promotion, the diet was diluted 1:2 with rat mash.

weeks at 15°C. Cultures were then lyophilized and stored in air-tight containers at 4°C prior to extraction.

**Animals.** Male BD IX rats (ca. 150 g) were caged individually in an animal house with a controlled environment at 25°C and 50% humidity with a 12-h artificial-light cycle. They were fed rat mash prepared from rat cubes (Epol Ltd., Johannesburg) and had free access to drinking water.

**Cancer initiation-promotion bioassay.** Rats were randomized into treatment groups of five each. The standard protocol to demonstrate the cancer-promoting activity of *F. moniliforme* MRC 826 culture material consisted of an initiation treatment (200 mg of DEN per kg, intraperitoneally), followed by the 4-week promoting treatment commencing 1 week later. The latter was effected by incorporating lyophilized culture material of strain MRC 826 at a level of 5% into the diet. Two control groups, one initiated with DEN and fed rat mash and another receiving the promoting treatment without initiation (dimethyl sulfoxide [DMSO] instead; 0.1 ml/100 g of body weight), were included. At least 4 kg of rat mash was prepared per group for the 4-week promotion treatment.

Fractions isolated from culture material were screened for cancer-promoting activity by incorporating them in the rat mash (4 kg) and feeding them to DEN-initiated rats as described above. The amounts incorporated in 4 kg of feed are indicated in Tables 1 and 2. DEN-initiated rats receiving only rat mash were included as a control in each assay during the isolation procedure. All rats were weighed twice weekly and killed after the 4-week promotion treatment, and their livers were subjected to histochemical analyses for GGT.

**Histochemical analyses.** Cryostat sections (5 μm) were prepared from rat livers at -25°C and stained for GGT by the method of Rutenburg et al. (12). At least 2 cm<sup>2</sup> of tissue was scanned, and only foci containing 10 or more cells were counted.

**Extraction and purification. (i) Extraction.** Culture material (1 kg) was successively extracted once with 1 liter of ethyl acetate and three times with 1 liter of CH<sub>3</sub>OH-H<sub>2</sub>O (3:1) by blending and filtering. Both the ethyl acetate and the aqueous methanol extracts as well as the remaining residue were tested for cancer-promoting activity as described above.

**(ii) Solvent partitioning and Amberlite XAD-2 column chromatography.** The CH<sub>3</sub>OH-H<sub>2</sub>O (3:1) extract obtained as described above was evaporated to dryness and partitioned between CH<sub>3</sub>OH-H<sub>2</sub>O (1:3; 200 ml) and CHCl<sub>3</sub> (three portions, 100 ml each). The aqueous phase was divided equally, and one part (ca. 65 g) chromatographed on an Amberlite

TABLE 2. Cancer-promoting activity of fractions obtained by solvent partitioning and Amberlite XAD-2 column fractionation of the CH<sub>3</sub>OH-H<sub>2</sub>O (3:1) extract of *F. moniliforme* MRC 826 culture material

| Rat treatment group (n = 5) | Fraction <sup>a</sup>                                  | Elution vol (liters)  | Fraction wt (g)   | Mean GGT activity (foci/cm <sup>2</sup> ) ± SD |
|-----------------------------|--|-----------------------|-------------------|--|
| 1                           | CHCl <sub>3</sub> extract<br>First XAD-2 fractionation |                       | 12.5              | ND <sup>b</sup>                                |
| 2                           | CH <sub>3</sub> OH-H <sub>2</sub> O (1:3)              | 4.0                   | 55.3              | ND   |
| 3                           | CH <sub>3</sub> OH-H <sub>2</sub> O (1:1)              | 2.0                   | 3.0               | ND   |
| 4                           | CH <sub>3</sub> OH<br>Second XAD-2 fractionation       | 1.0                   | 14.0              | 50.90 ± 3.44                                   |
| 5                           | CH <sub>3</sub> OH-H <sub>2</sub> O (3:1)              | 2.3 (fractions 1-23)  | 8.75 <sup>c</sup> | 9.0 ± 3.24                                     |
| 6                           | CH <sub>3</sub> OH-H <sub>2</sub> O (3:1)              | 2.1 (fractions 24-45) | 0.42 <sup>c</sup> | ND   |
| 7                           | CH <sub>3</sub> OH                                     | 0.4 (fractions 46-49) | 1.32 <sup>c</sup> | ND   |
| 8                           | CH <sub>3</sub> OH                                     | 1.0 (fractions 50-60) | 2.91 <sup>c</sup> | ND   |

<sup>a</sup> XAD-2 fractionations: the first was done after the solvent partitioning step, and one-half (65 g) of the CH<sub>3</sub>OH-H<sub>2</sub>O (1:3) fraction was fractionated; after the second half of the CH<sub>3</sub>OH-H<sub>2</sub>O (1:3) solvent-partitioning fraction was fractionated, the CH<sub>3</sub>OH fraction (15.3 g) was subjected to the second XAD-2 column fractionation.

<sup>b</sup> ND, None detected.

<sup>c</sup> One-half of the fraction was incorporated in the diet during the 4 weeks of promotion.

XAD-2 column (sample-resin, 1:15). The column was successively eluted with H<sub>2</sub>O (1 liter), CH<sub>3</sub>OH-H<sub>2</sub>O (1:3; 4 liters), CH<sub>3</sub>OH-H<sub>2</sub>O (1:1; 2 liters), and finally CH<sub>3</sub>OH (1 liter). The fractions from the XAD-2 column together with the combined CHCl<sub>3</sub> fractions were evaporated onto rat mash and tested for cancer-promoting activity.

After similar fractionation of the remaining CH<sub>3</sub>OH-H<sub>2</sub>O (1:3) solvent partitioning fraction (ca. 65 g) on Amberlite XAD-2, the CH<sub>3</sub>OH eluate was further purified on Amberlite XAD-2. The column was successively eluted with CH<sub>3</sub>OH-H<sub>2</sub>O (3:1; 5 liters) and CH<sub>3</sub>OH (2 liters), collecting 100-ml fractions. Four pooled fractions were prepared (Table 2) and divided into equal portions, and one part was tested for cancer promoting activity.

**(iii) Sephadex LH-20 column fractionation.** The active fraction (4.89 g) eluted from the second Amberlite XAD-2 column with CH<sub>3</sub>OH-H<sub>2</sub>O (3:1) was fractionated on a Sephadex LH-20 column (150 g; 5 by 70 cm) with CH<sub>3</sub>OH-H<sub>2</sub>O (3:1) as the mobile phase, collecting 100-ml fractions. Fractions were analyzed by reverse-phase thin-layer chromatography (RP-TLC) with CH<sub>3</sub>OH-H<sub>2</sub>O (3:1) as the developing solvent, spraying with 0.5% *p*-anisaldehyde solution in CH<sub>3</sub>OH-H<sub>2</sub>SO<sub>4</sub>-CH<sub>3</sub>COOH (85:5:10) and heating at 120°C. Those fractions containing compounds with similar *R<sub>f</sub>* values were combined, and the samples designated A, B, and C (Table 3) were screened for cancer-promoting activity.

**(iv) RP (C<sub>18</sub>) column chromatography.** Sample B (2 g) was further purified on a Sepalyte C<sub>18</sub> RP column (200 g; 2.5 by 60 cm), maintaining the flow rate at 1 ml/min. The column was successively eluted with CH<sub>3</sub>OH-H<sub>2</sub>O (1:1; 200 ml), CH<sub>3</sub>OH-H<sub>2</sub>O (3:1; 200 ml), and CH<sub>3</sub>OH (200 ml). Fractions (50 ml) were collected and analyzed by RP-TLC as described above. One major and two minor compounds eluted with CH<sub>3</sub>OH-H<sub>2</sub>O (3:1), comprising at least 85% of the weight of sample B. The compounds eluting first and last, given the



TABLE 4. Cancer-promoting activity of fumonisin B<sub>1</sub> in rats

| Rat treatment group | No. of rats | DEN initiation | Promotion <sup>a</sup> | Mean GGT activity (foci/cm <sup>2</sup> ) ± SD |
|---------------------|-------------|----------------|------------------------|--|
| 1                   | 5           | +              | -                      | ND <sup>b</sup>                                |
| 2                   | 5           | -              | -                      | ND   |
| 3                   | 4           | -              | +                      | 20.00 ± 8.34 <sup>c</sup>                      |
| 4                   | 4           | +              | +                      | 54.80 ± 10.30 <sup>c</sup>                     |

<sup>a</sup> Fumonisin B<sub>1</sub> at 0.1% in the diet.

<sup>b</sup> ND, None detected.

<sup>c</sup> Means significantly different (*P* < 0.005).

obtained. During the large-scale extraction, approximately 2 g of fumonisin B<sub>1</sub> was purified from 1 kg of the cultured corn, while 10 times less fumonisin B<sub>2</sub> was obtained. The purity of the fumonisin B<sub>1</sub> preparation, as determined by HPLC, was found to be 92%.

**Cancer-promoting activity of fumonisin B<sub>1</sub> in rats.** A dietary level of 0.1% fumonisin B<sub>1</sub> markedly induced the formation of GGT<sup>+</sup> foci in both DEN-initiated and control (DMSO-treated) rats (Table 4). The induction of GGT<sup>+</sup> foci was, however, significantly higher (*P* < 0.0005) in the DEN-initiated (group 4) than the noninitiated (group 3) rats. In both of these groups of rats treated with fumonisin B<sub>1</sub>, a marked reduction in weight gain occurred from the first week of promotion treatment (Fig. 2). At the end of the promotion period, the mean body weights of the rats treated with fumonisin B<sub>1</sub> (groups 3 and 4) were significantly (*P* < 0.0001) lower than those of the nontreated rats (groups 1 and 2).

**Pathological changes in rats caused by fumonisin B<sub>1</sub>.** In the rats that died after 3 days of dosing with fumonisin B<sub>1</sub>, there was toxic hepatitis, characterized by scattered single-cell necrosis accompanied by mild fatty changes, hydropic degeneration, and hyaline droplet degeneration (Fig. 3).

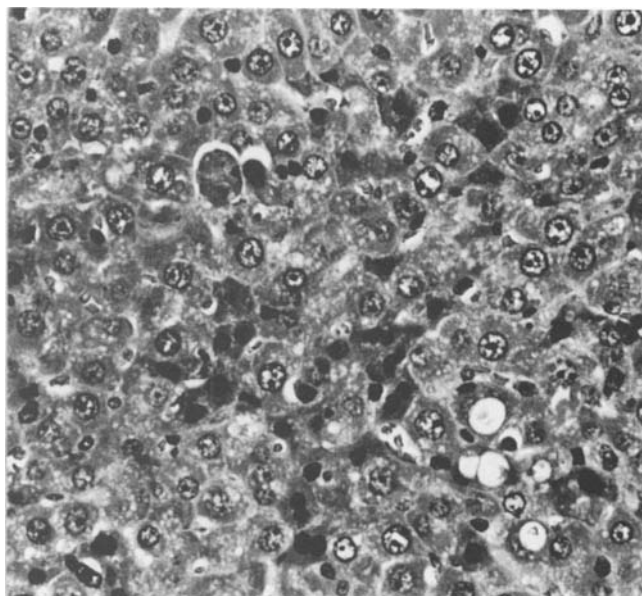


FIG. 3. Histopathological changes in the liver of a rat receiving 0.25 ml of fumonisin B<sub>1</sub> solution (0.95 g in 10 ml of DMSO) per day for 3 days. Note the single-cell necrosis, hyaline droplets, and fatty changes. Magnification, ×25.

Kupffer cells were increased and enlarged. The necrotic cells occurred indiscriminately throughout the lobules, while the degenerative changes were periportal. Hepatocellular nuclei varied in size; few of them were markedly enlarged. Lesions of a lesser degree occurred in some of the other organs. Fatty changes and scant necrosis were present in the proximal convoluted tubules of the kidney. Prominent lymphoid

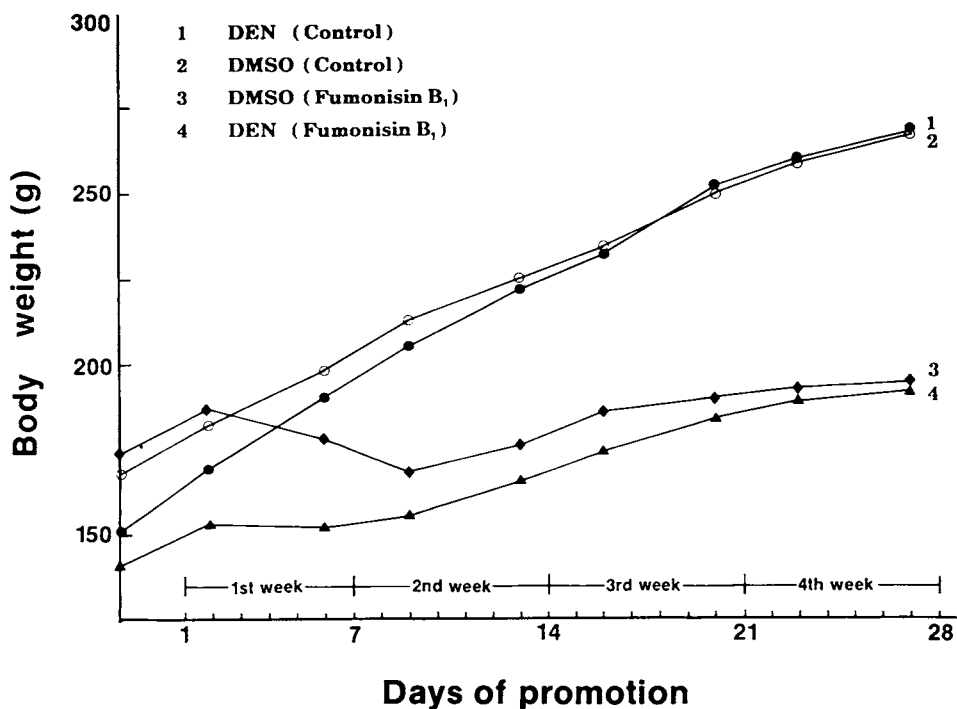


FIG. 2. Mean body weights of rats over the 4-week promotion period. Rats were weighed twice weekly.



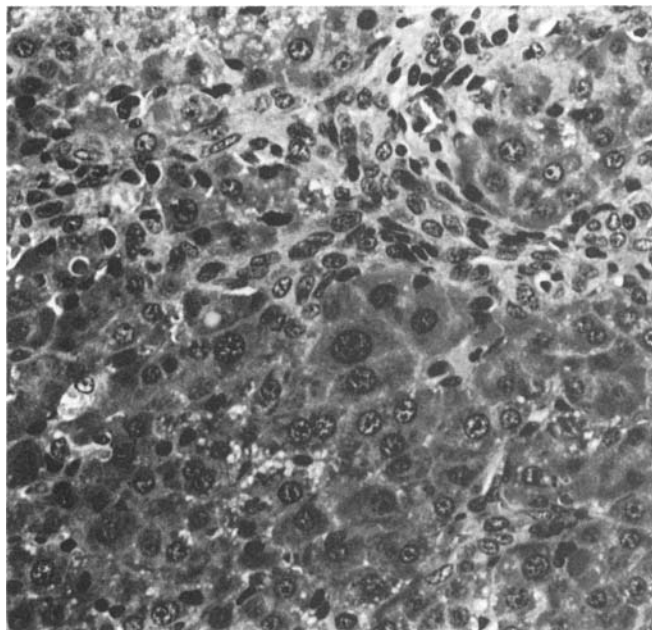


FIG. 4. Histopathological changes in the liver of a rat fed fumonisin B<sub>1</sub> in the diet (0.1%) for 33 days. Note the fibrosis, bile duct hyperplasia, and anisonucleosis at the edge of a hyperplastic nodule. Magnification,  $\times 250$ .

necrosis in the Peyer's patches and scattered focal superficial and midzonal epithelial necrosis occurred in the mucosa of the stomach. Severe, disseminated acute myocardial necrosis and severe pulmonary edema were observed in two of the rats.

In the rats that were killed after 21 and 33 days, chronic toxic hepatitis was present that was further advanced in the latter group.

In the livers of the rats killed after 21 days of dosing with fumonisin B<sub>1</sub>, there was marked hydropic degeneration, single-cell necrosis, and a few hyaline droplets. Early bile duct proliferation and fibrosis radiated out from the portal tract or developed focally within the midzonal area of the hepatic lobules. A few hepatocellular nuclei were markedly enlarged and contained a fine granular chromatin pattern; some had a lobed nucleus.

In the rats that were killed after 33 days on a diet containing 0.1% fumonisin B<sub>1</sub>, the changes were similar but more advanced. The proliferation of bile ducts and fibrosis caused distortion of the lobular structure of the liver that, together with the development of hyperplastic nodules (Fig. 4), gave the liver a distinctly nodular appearance. Many more enlarged nuclei, similar to those observed in the rats killed after 21 days, were seen in this group. The hepatocytes in the hyperplastic nodules had large vesicular nuclei and a voluminous foamy cytoplasm. Numerous mitotic figures, some of which were abnormal, were also present. The lesions in the kidney were similar but less severe than those seen in the rats that died within 3 days. No lesions occurred in any of the other organs.

No comparable lesions were observed in the livers or the kidneys of the rats in the control groups.

#### DISCUSSION

The induction of GGT<sup>+</sup> foci has been used widely as a marker of preneoplastic hepatic lesions during chemical

carcinogenesis (4). In a short-term cancer initiation-promotion bioassay with DEN-initiated rats, hepatocarcinogenic culture material of *F. moniliforme* MRC 826 induced GGT<sup>+</sup> foci within 4 weeks of promoting treatment (Gelderblom et al., in press). This short-term bioassay was used for the isolation of cancer-promoting principles from culture material of *F. moniliforme* MRC 826. A group of related compounds, the fumonisins, were isolated in this way, and some of them have been chemically characterized (Bezuidenhout et al., in press).

The fumonisins were extracted from the culture material in CH<sub>3</sub>OH-H<sub>2</sub>O (3:1) and are polar compounds, since no cancer-promoting activity could be recovered in the ethyl acetate fraction and they preferred an aqueous environment after solvent partitioning with CHCl<sub>3</sub>. Solvent partitioning removed most of the lipophilic compounds that are likely to interfere during the Amberlite XAD-2 chromatographic fractionation. During this fractionation, the fumonisins exhibited a relatively nonpolar character because they could not be eluted by 50% aqueous CH<sub>3</sub>OH (Table 2) but eluted in CH<sub>3</sub>OH-H<sub>2</sub>O (3:1). This resulted in excellent separation, as indicated by the 80% purification obtained during this fractionation step. In contrast, Sephadex LH-20 was far less efficient and resulted in a purification of only 20%. During later bulk purification of the fumonisins, both the second XAD-2 and the Sephadex LH-20 fractionation steps were omitted and replaced by a single chromatographic separation on silica gel. The inclusion of this step resulted in the isolation of pure fumonisins B<sub>1</sub> and B<sub>2</sub> by means of only three chromatographic separations.

The induction of GGT<sup>+</sup> foci by fumonisin B<sub>1</sub> in DEN-initiated as well as noninitiated rats agrees well with the previous finding that culture material of *F. moniliforme* MRC 826 exhibits cancer-promoting activity in the absence of DEN initiation (2). In the present experiment, however, a different batch of lyophilized culture material of this fungus did not induce GGT<sup>+</sup> foci in noninitiated rats. A possible explanation is that the second batch of culture material contained lower levels of fumonisin B<sub>1</sub> and related compounds than the previous one. These results imply that fumonisin B<sub>1</sub> is a complete carcinogen and, together with the other two related compounds, might be responsible for the hepatocarcinogenicity of *F. moniliforme* MRC 826.

The cancer-promoting activity of fumonisin B<sub>1</sub> was also associated with a toxic effect, as evidenced by the significant reduction in weight gain of treated compared with control rats. This again is in agreement with the previous finding that a highly significant correlation exists between the cancer-promoting activity and toxicity of different strains of *F. moniliforme* (Gelderblom et al., in press). Fumonisin B<sub>1</sub> proved to be acutely toxic to rats, and high doses caused death within 3 days. The subacute pathological changes in the livers of rats caused by fumonisin B<sub>1</sub> were also similar to those caused by culture material of *F. moniliforme* MRC 826 and some other strains of this fungus (7, 8). Thus, it appears that fumonisin B<sub>1</sub> is also responsible for the toxicity of *F. moniliforme* MRC 826 in rats.

Experiments are in progress to investigate the carcinogenicity of fumonisin B<sub>1</sub> and related compounds in rats and to determine whether the fumonisins are also responsible for LEM in horses.

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**Table 1.** NMR Data for the Normal and 3-*epi* Series of Fumonisin B<sub>3</sub> and B<sub>4</sub><sup>a</sup>

| atom | FB <sub>3</sub> (3) |                       | 3- <i>epi</i> -FB <sub>3</sub> (5) |                       |
|------|---------------------|-----------------------|------------------------------------|-----------------------|
|      | δ <sub>C</sub>      | δ <sub>H</sub>        | δ <sub>C</sub>                     | δ <sub>H</sub>        |
| 1    | 15.61 Q             | 1.113 d (J 6.7)       | 12.01 Q                            | 1.054 d (J 6.7)       |
| 2    | 51.36 D             | 2.956 qd (J 6.7, 6.8) | 50.63 D                            | 3.125 qd (J 6.8, 3.1) |
| 3    | 71.08 D             | 3.336 m               | 69.65 D                            | 3.568 m               |
| 4    | 32.90 T             |                       | 32.46 T                            |                       |

| atom | FB <sub>4</sub> (4) |                       | 3- <i>epi</i> -FB <sub>4</sub> (6) |                       |
|------|---------------------|-----------------------|------------------------------------|-----------------------|
|      | δ <sub>C</sub>      | δ <sub>H</sub>        | δ <sub>C</sub>                     | δ <sub>H</sub>        |
| 1    | 15.42 Q             | 1.120 d (J 6.6)       | 11.93 Q                            | 1.060 d (J 6.7)       |
| 2    | 51.23 D             | 2.957 qd (J 6.7, 6.7) | 50.56 D                            | 3.120 qd (J 6.8, 3.1) |
| 3    | 70.96 D             | 3.33 m                | 69.57 D                            | 3.58 m                |
| 4    | 32.75 T             |                       | 32.37 T                            |                       |

<sup>a</sup> Solvent DMSO-*d*<sub>6</sub>. NMR analyses were performed on mixtures of stereoisomers of FB<sub>3</sub> and FB<sub>4</sub>.

**Table 2.** <sup>1</sup>H NMR Data for the MTPA Amides (δ, CDCl<sub>3</sub>)

| compound  | atom              | ( <i>S</i> )-MTPA (b) | ( <i>R</i> )-MTPA (a) | Δδ (δ <sub>S</sub> - δ <sub>R</sub> ) |
|-----------|-------------------|-----------------------|-----------------------|---------------------------------------|
| <b>9</b>  | H(1)              | 1.110                 | 1.179                 | -0.069                                |
|           | H(3) <sup>a</sup> | 3.49                  | 3.47                  | +0.02                                 |
| <b>10</b> | H(1)              | 1.025                 | 1.095                 | -0.070                                |
|           | H(3) <sup>a</sup> | 3.60                  | 3.54                  | +0.06                                 |

<sup>a</sup> Chemical shift values obtained from COSY and HETCOR data.

monitored by full-scan MS-MS between *m/z* 330 and *m/z* 730. A collision energy of 32% was used to fragment the molecular ions, and the resultant product ions were monitored as diagnostic indicators for the presence of the toxins.

**NMR Analysis.** NMR spectra were recorded on a Bruker Avance-500DRX spectrometer operating at 500 MHz for <sup>1</sup>H and 125 MHz for <sup>13</sup>C nuclei using standard Bruker pulse sequences. Spectra acquired for DMSO-*d*<sub>6</sub> solutions were referenced to the signals at δ<sub>H</sub> 2.49 and δ<sub>C</sub> 39.50 and for spectra in CDCl<sub>3</sub> to the signals at δ<sub>H</sub> 7.24 and δ<sub>C</sub> 77.00.

**Determination of Fumonisin Analogues by HPLC with Fluorescence Detection.** Determination of fumonisin analogues in corn was conducted by the method of Sydenham et al. (15). Briefly, corn samples were homogenized in methanol–water (3:1), centrifuged, and filtered. An aliquot of the clear extract was applied to a strong anion exchange solid-phase extraction cartridge (Varian, Harbor City, CA) and washed with methanol, and the fumonisins were eluted with 1% acetic acid in methanol. The purified extracts were dried down under nitrogen at 60 °C. Immediately prior to analysis, the residues were redissolved in methanol and an aliquot was derivatized with OPA. The fumonisin analogues were separated and detected by reversed-phase HPLC (Ultracarb 5 ODS column; Phenomenex) with fluorescence detection (Figure 4). The mobile phase was pumped at 1 mL/min and consisted of methanol–sodium dihydrogen phosphate (0.1 M, pH 3.3) (77:23) as generally used for fumonisin determination.

**Preparation of the FB<sub>3</sub> Derivatives.** Fumonisin B<sub>3</sub> Tetramethyl Ester. A solution of FB<sub>3</sub> standard (549 mg) containing a mixture of FB<sub>3</sub> (3) and 3-*epi*-FB<sub>3</sub> (5) was treated with an excess of a diethyl ether solution of diazomethane (prepared from 4.28 g of Diazald). After 10 min, the excess diazomethane was evaporated in a stream of nitrogen and the residue was dried in vacuo to give the tetramethyl esters **7** and **8** (590 mg, 99%) (Figure 2).

**Preparation of the Mosher Amide Derivatives.** Oxalyl chloride (220 mg, 1.74 mmol) was added to a solution of (*R*)-(-)-MTPA (e.e. ≥99%, 82 mg, 0.35 mmol) and dimethyl formamide (DMF) (25.4 mg, 0.35 mmol) in hexane (5 mL) at room temperature. A white precipitate formed immediately. After 60 min, the mixture was passed through a small cotton plug to filter off the formed DMF–Cl. The filtrate was concentrated under reduced pressure to yield the (*S*)-MTPA chloride.

**Table 3.** Fumonisin Levels in Samples of Corn and Commercial Corn Meal

| sample                          | fumonisin levels (μg/kg) <sup>a</sup> |                 |                 |                             |       | % <i>epi</i> /FB <sub>3</sub> |
|---------------------------------|---------------------------------------|-----------------|-----------------|-----------------------------|-------|-------------------------------|
|                                 | FB <sub>1</sub>                       | FB <sub>2</sub> | FB <sub>3</sub> | <i>epi</i> -FB <sub>3</sub> | total |                               |
| commercial corn meal            | 380                                   | 100             | 30              | 2                           | 512   | 6.7                           |
|                                 | 625                                   | 215             | 60              | 5                           | 905   | 8.3                           |
|                                 | 240                                   | 70              | 15              | 2                           | 327   | 13                            |
|                                 | 265                                   | 80              | 25              | 4                           | 374   | 16                            |
| Brazilian corn                  | 1960                                  | 720             | 370             | 45                          | 3095  | 12                            |
|                                 | 4515                                  | 255             | 665             | 95                          | 5530  | 14                            |
|                                 | 3635                                  | 900             | 320             | 60                          | 4915  | 19                            |
|                                 | 2405                                  | 1985            | 400             | 75                          | 4865  | 19                            |
| Transkei home-grown, good corn  | 2350                                  | 540             | 200             | 35                          | 3125  | 18                            |
|                                 | 1370                                  | 465             | 120             | 20                          | 1975  | 17                            |
|                                 | 2980                                  | 780             | 215             | 45                          | 4020  | 21                            |
| Transkei home-grown, moldy corn | 26240                                 | 10070           | 2490            | 200                         | 39000 | 8.1                           |
|                                 | 2665                                  | 830             | 165             | 25                          | 3685  | 15                            |
|                                 | 12480                                 | 6860            | 1740            | 285                         | 21365 | 16                            |
|                                 | 5035                                  | 2050            | 495             | 75                          | 7655  | 15                            |
|                                 | 5365                                  | 2025            | 445             | 30                          | 7865  | 6.7                           |

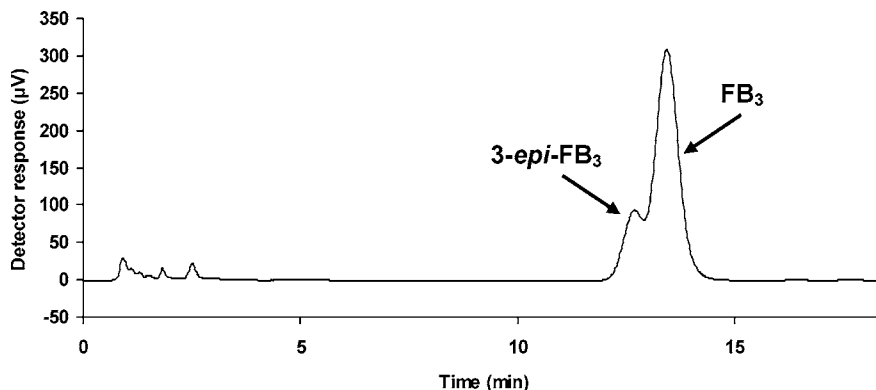
<sup>a</sup> FB<sub>4</sub> was not detected.

A solution of the tetramethyl esters **7** and **8** (265 mg, 0.35 mmol), Et<sub>3</sub>N (352 mg, 3.48 mmol), and DMAP (5 mg) in CH<sub>2</sub>Cl<sub>2</sub> (5 mL) was added to a solution of the (*S*)-MTPA chloride in CH<sub>2</sub>Cl<sub>2</sub> (2 mL) solution. The mixture was stirred for 30 min and then quenched by addition of water (2 mL). The organic solution was washed with 0.5 M HCl, followed by saturated NaHCO<sub>3</sub> solution and water. The CH<sub>2</sub>-Cl<sub>2</sub> solution was dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and evaporated. The product was purified by column chromatography on silica gel with EtOAc–hexane (3:2) as the eluent to give the (*R*)-Mosher amide derivative (180 mg, 59%), an oil, as a mixture of two diastereomers (**9a** and **10a**) (d.r. 82:18); *R<sub>f</sub>* 0.34.

The same protocol was followed but using (*S*)-(+)-MTPA (e.e. ≥99%), to convert a sample of the tetramethyl esters **7** and **8** (265 mg, 0.34 mmol) to a mixture of the two diastereomeric (*S*)-Mosher amide derivatives (**9b** and **10b**) (214 mg, 63%) (d.r. 82:18) (Figure 2).

## RESULTS AND DISCUSSION

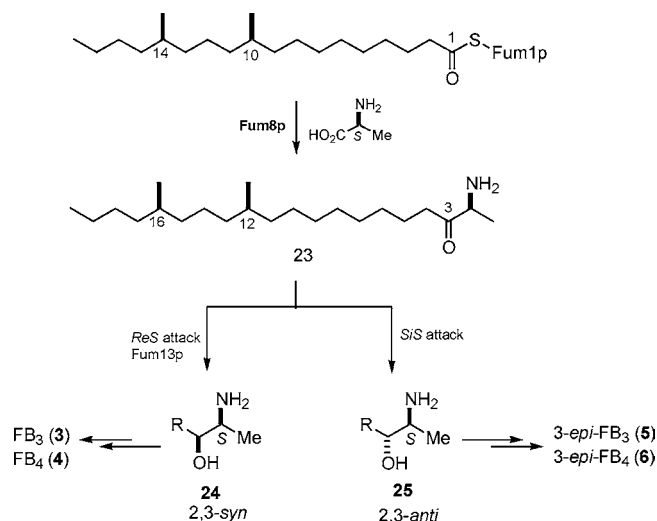
The terminal 2-amino-3-hydroxy motif of the fumonisin C<sub>20</sub> backbone is also present in a number of marine natural products (see Figure 3). In 1989, Gulavita and Scheuer (16) isolated two epimeric aliphatic amino alcohols from a Papua New Guinea sponge, *Xestospongia* sp., and proposed their structures as (2*S*,3*S*,5*E*,7*E*)- and (2*S*,3*R*,5*E*,7*E*)-2-aminotetradeca-5,7-dien-3-ol, *ent*-(**11**) and *ent*-(**12**), respectively. The relative stereochemistry followed from nuclear Overhauser effect studies on the oxazolidinone derivative and the absolute configuration at C(2) by degradation of the diacetyl derivatives to alanine and HPLC analysis of the derivative formed with 1-fluoro-2,4-dinitrophen-5-yl-(2*S*)-alanine amide. Mori and Matsuda (17) reported the total synthesis of both enantiomers of **11** and **12** and assigned the enantiomeric stereochemistry to the natural products isolated by Gulavita and Scheuer (16), since the 2*R* stereoisomers showed the same sign of optical rotation as the natural products. The stereochemistry of the xestoaminols A (**13**), B (**14**), and C (**15**) isolated by Jiménez and Crews (18) from two different sponges of the genus *Xestospongia* was determined by Garrido et al. (19) in their study on the obscuraminols, for example, obscuraminol C (**16**) obtained from the tunicate *Pseudodistoma obscurum*. Sata and Fusetani (20) isolated two new cytotoxic stereoisomeric 2,3-amino alcohols, amaminol A (**17**) and B (**18**), from an unidentified tunicate of the family Polyclinidae. The stereochemical relationship between these two compounds, as in the case of the amino alcohols **11** and **12** isolated by Gulavita and Scheuer (16), is of particular



**Figure 4.** HPLC chromatogram with fluorescence detection of the OPA derivatives of the isolated  $\text{FB}_3$  standard showing the relative areas of the two stereoisomers.

significance to the structure elucidation of the minor metabolites of the fumonisins. A detailed analysis of the  $^1\text{H}$  NMR spectra of **17** and **18** disclosed that the relative stereochemistry of the C(2) and C(3) stereogenic centers differed as indicated by the coupling constant between H(2) and H(3) [ $J_{2,3}$  3.1 Hz for (**17**) vs 7.3 Hz for (**18**)]. The Mosher ester analysis (21–23) indicated that the two compounds had the same 3*S* absolute configuration and were epimeric at C(2). This is in direct contrast to the results obtained by Gulavita and Scheuer (16) on the amino alcohols **11** and **12**, which are C(3) epimers. The absolute configuration of the crucigasterins **19**–**21**, isolated by Jarez et al. (24) from a Mediterranean tunicate *Pseudodistoma crucigaster*, was assigned by chemical degradation to (3*S*,4*R*)-3-hydroxy-4-aminopentanoic acid. The *anti* stereochemistry of the 2-amino-3-hydroxy group of the crucigasterins is reflected by the 3.0 Hz coupling constant for the C(2) and C(3) protons. Analysis of the  $^{13}\text{C}$  NMR data reported by Gulavita and Scheuer (16) and Mori and Matsuda (17) in the work on the amino alcohols **11** and **12** and by Sata and Fusitani (20) on the amaminols **17** and **18** showed characteristic chemical shift values for the methyl group of the *syn*- and *anti*-2,3-amino alcohols. Thus, the methyl group, C(1), in the diacetyl derivative of **11** appeared at  $\delta_{\text{C}}$  18.49 (*syn*) whereas in the diacetyl derivative of **12** it appeared at  $\delta_{\text{C}}$  14.93 (*anti*). The same trend is observed for the amaminols:  $\delta_{\text{C}}$  16.0 for the *syn* compound (**11**) and  $\delta_{\text{C}}$  12.1 for the *anti* compound (**12**). The *anti*-amino alcohol (**22**) (25) showed the methyl groups at  $\delta_{\text{C}}$  12.1 and 11.9, whereas in the *syn*-amino alcohols such as fumonisin B<sub>3</sub> (**3**) and B<sub>4</sub> (**4**) the methyl group appears at  $\delta_{\text{C}}$  15.61 and 15.42. It is thus evident that the  $^{13}\text{C}$  chemical shift values for the methyl group, C(1), define the relative stereochemistry of the 2,3-amino alcohol moiety in these compounds.

Fast atom bombardment and ES mass spectrometry of  $\text{FB}_3$  samples, which exhibited two peaks in the HPLC analysis, showed the  $[\text{M} + \text{H}]^+$  ion at 706 and established the molecular formula  $\text{C}_{34}\text{H}_{59}\text{NO}_{14}$  for each of the two compounds. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra showed in all cases the presence of a minor component 3-*epi*- $\text{FB}_3$  (**5**). The use of two-dimensional correlation spectroscopy (COSY) and heteronuclear correlation (HETCOR) experiments established that the discernible signals of the minor component in the  $^1\text{H}$  and  $^{13}\text{C}$  spectra represent the C(1)–C(4) unit of the backbone. The coupling constant of 6.8 Hz between the C(2) and the C(3) protons of  $\text{FB}_3$  is characteristic of the *syn*-2,3-amino alcohol in these metabolites. The *anti* stereochemistry for the 2,3-amino alcohol unit of the minor metabolite 3-*epi*- $\text{FB}_3$  (**5**) followed from the 3.1 Hz coupling observed for the C(2) and C(3) protons (Table 1). The  $^{13}\text{C}$  chemical shifts for the methyl group in fumonisin B<sub>3</sub> ( $\delta_{\text{C}}$

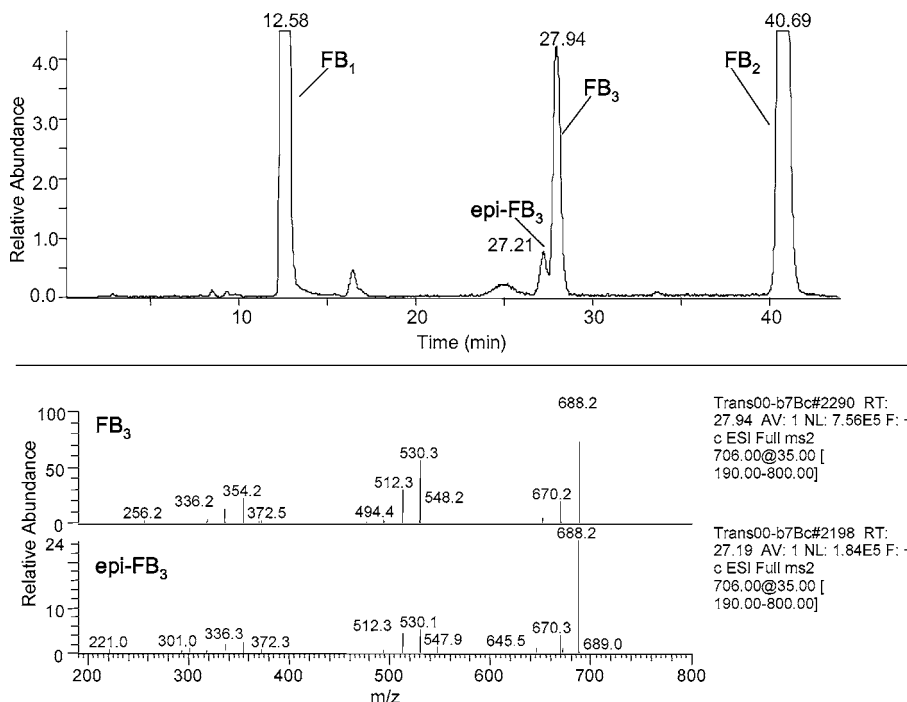


**Figure 5.** Biosynthetic formation of the fumonisins and the 3-*epi* stereoisomers.

15.61Q) and the minor component 3-*epi*- $\text{FB}_3$  (**5**) ( $\delta_{\text{C}}$  12.01Q) confirmed the *anti*-2,3 stereochemistry of the latter.

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of samples of fumonisin B<sub>4</sub> (**4**) isolated over the years sometimes also showed the presence of up to 40% of a minor metabolite 3-*epi*- $\text{FB}_4$  (**6**) with the 2,3-*anti* stereochemistry (Table 1). The *anti* stereochemistry of 3-*epi*- $\text{FB}_4$  (**6**) followed once again from the coupling constant of 3.1 Hz for the C(2) and C(3) protons and the  $^{13}\text{C}$  chemical shift value of 11.93 for the methyl group, C(1) (see Table 1). In contrast, the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of samples of  $\text{FB}_1$  (**1**) and  $\text{FB}_2$  (**2**) prepared by us have always lacked the signals for the corresponding 3-*epi* stereoisomers with the *anti*-2,3 stereochemistry.

The absolute configuration of the C(2) and C(3) stereogenic centers of  $\text{FB}_{1-4}$  has been established as (2*S*,3*S*) (26–32), and consequently, the minor metabolites 3-*epi*- $\text{FB}_3$  (**5**) and 3-*epi*- $\text{FB}_4$  (**6**) must either have the (2*S*,3*R*) or (2*R*,3*S*) configuration. The C(2) absolute configuration for the  $\text{FB}_3$  minor metabolite, 3-*epi*- $\text{FB}_3$  (**5**), was determined using Mosher methodology (21–23). A sample of  $\text{FB}_3$  (**3**) [containing 18% of the minor metabolite 3-*epi*- $\text{FB}_3$  (**5**)] was converted to the tetramethyl ester derivatives **7** and **8** (minor) by treatment with an excess of diazomethane. The amino group was then converted to the (*R*)- and (*S*)-MTPA amides (**7a,b** and **8a,b**) (minor), respectively, by reaction with the (*S*)- and (*R*)-MTPA chlorides [prepared from the (*R*)- and (*S*)-MTPA acid, respectively, following the protocol developed by Ward and Rhee (33)]. The  $\Delta\delta$  ( $\delta_{\text{S}} - \delta_{\text{R}}$ ) values observed for H(1) (−0.069 ppm) and H(3) (+0.02



**Figure 6.** Total ion chromatogram of a Transkeian moldy corn sample and mass spectra of the protonated FB<sub>3</sub> and 3-*epi*-FB<sub>3</sub> analogues.

ppm) of the Mosher amide derivatives (**9**) (see **Table 2**) are in agreement with the 2*S* absolute configuration of the fumonisins. The fact that similar values are observed for the corresponding protons of the Mosher amide derivatives (**10**) implies that the minor metabolite 3-*epi*-FB<sub>3</sub> (**5**) present in samples of FB<sub>3</sub> (**3**) also has the 2*S* absolute configuration and therefore on the basis of its 2,3-*anti* relative stereochemistry, the 3*R* absolute configuration. As a consequence of this result, the assignment of the 2*S*,3*R* absolute configuration to the minor metabolite 3-*epi*-FB<sub>4</sub> (**6**) present in samples of fumonisin B<sub>4</sub> (**4**) is reasonable.

Biosynthetic studies on the fumonisins using [1-<sup>13</sup>C]-, [2-<sup>13</sup>C]-, and [1,2-<sup>13</sup>C<sub>2</sub>]acetate, [methyl-<sup>13</sup>C]-(*2S*)-methionine, and [3-<sup>13</sup>C]-(*2S*)-alanine precursors established that C(3)–C(20) are derived from acetate and C(1), C(2), and the 2-NH<sub>2</sub> group are derived from alanine. The C(12) and C(16) methyl groups are derived from methionine (34–38). An acetate-derived carbonyl group is the source of the C(3) hydroxyl group, whereas the C(5), C(10), C(14), and C(15) hydroxyl groups are likely derived from molecular oxygen (39). The citric acid cycle is believed to be the source of the tricarballic acid moiety (37). Recently, a 15-gene cluster (*FUM1*–*FUM3*, *FUM6*–*FUM8*, *FUM10*, *FUM11*, and *FUM13*–*FUM19*) required for the biosynthesis of the fumonisins in *F. verticilloides* was cloned and characterized (40–42). The data obtained from direct studies of these biosynthetic genes by genetic and biochemical approaches (40–48) provided the basis for a biosynthetic pathway (46–48). The biosynthesis starts with the formation of a dimethylated, saturated C<sub>18</sub> polyketide chain catalyzed by the Fum1p polyketide synthase (see **Figure 5**). The carbon–carbon bond formation between the (*2S*)-alanine and the enzyme-bound C<sub>18</sub> polyketide chain by the pyridoxal-phosphate-dependent aminoacyl transferase enzyme Fum8p, encoded by the *FUM8* gene (42, 43), involves the loss of CO<sub>2</sub> to form the 3-keto intermediate (**23**) with overall retention of the C(2) configuration. Stereospecific reduction of the 3-keto intermediate at the *ReS* face of the carbonyl group by ketoreductase Fum13p results in the formation of the 3*S* hydroxyl group and the *syn*-2,3 amino alcohol motif (**24**) (43, 45, 47). This reduction is analogous to that of 3-ketosphinganine to sphinganine (43) except that the reduction

of the carbonyl group with 3-ketosphinganine reductase must occur at the *Si* face to generate the 3*R* hydroxyl group and the *anti*-2,3 amino alcohol of sphinganine.

The formation of 3-*epi*-FB<sub>3</sub> (**5**) with its 3*R* hydroxyl group and thus the 2,3-*anti* stereochemistry is postulated to occur by reduction at the *SiS* face of the carbonyl group of the 3-keto intermediate (**23**) to give the intermediate (**25**) in the fumonisin biosynthesis by a 3-ketosphinganine type reductase. The presence of such a 3-ketosphinganine type reductase has been proposed to account for the low levels of FB<sub>1–4</sub> production in *FUM13* deletion mutants (43).

Evidence has been presented that the reduction of the 3-keto group is an early step in the biosynthetic pathway, whereas the introduction of the C(5) hydroxyl group by a 2-ketoglutarate-dependent dioxygenase Fum3p, encoded by the *FUM3* gene, is the last step (43, 47, 48). On the basis of this proposed biosynthetic pathway, the presence of 3-*epi*-FB<sub>3</sub> (**5**) and 3-*epi*-FB<sub>4</sub> (**6**) should lead to the formation of the 3-*epi* stereoisomers of FB<sub>1</sub> and FB<sub>2</sub> by the Fum3p enzyme. This is not the case as these 3-*epi* stereoisomers of FB<sub>1</sub> and FB<sub>2</sub> have never been detected by us. This failure of Fum3p to convert 3-*epi*-FB<sub>3</sub> (**5**) and 3-*epi*-FB<sub>4</sub> (**6**) to 3-*epi*-FB<sub>1</sub> and 3-*epi*-FB<sub>2</sub>, respectively, suggests that the metabolites with the 3*R* hydroxyl group, that is, the 2,3-*anti* stereochemistry, are not recognized by Fum3p and thus do not serve as substrates.

The newly described 3-*epi*-FB<sub>3</sub> (**5**) was identified from the FB<sub>3</sub> standard isolated in the PROMEC Unit from various batches of corn patty culture material prepared from *F. verticilloides* MRC 826. In an attempt to avoid the presence of this analogue in the analytical standard, a range of other *F. verticilloides* strains, including MRC 8041 that only produces FB<sub>3</sub>, have been cultured. The 3-*epi*-FB<sub>3</sub> (**5**) has been found to occur in all of these at levels ranging from 21 to 42% of the level of the normal FB<sub>3</sub>. In a limited comparison, two strains grown in liquid medium showed lower levels (around 7–8%) of the *epi* stereoisomer. Of greater concern is the potential natural occurrence of this new fumonisin analogue. A range of corn samples from various sources have recently been analyzed by HPLC with fluorescence detection. 3-*epi*-FB<sub>3</sub> (**5**) was identified by its

retention time as a small peak eluting from the HPLC column immediately prior to normal FB<sub>3</sub>. **Table 3** shows the results of the analytical determination of FB<sub>3</sub> (**3**) and 3-*epi*-FB<sub>3</sub> (**5**) for 16 samples of corn and commercial corn meal. The presence of the stereoisomer was confirmed by LC-MS-MS analysis and the presence of the characteristic fragmentation pattern for fumonisins. **Figure 6** shows the total ion chromatogram for a sample of Transkeian corn, as well as a comparison of the mass spectra for FB<sub>3</sub> (**3**) and for the peak corresponding to 3-*epi*-FB<sub>3</sub> (**5**). The similarity of the spectra and the presence of the fragment ions corresponding to [M + H - H<sub>2</sub>O]<sup>+</sup> (*m/z* 688), [M + H - 2H<sub>2</sub>O]<sup>+</sup> (*m/z* 670), [M + H - TCA]<sup>+</sup> (*m/z* 530), [M + H - H<sub>2</sub>O - TCA]<sup>+</sup> (*m/z* 512), [M + H - 2TCA]<sup>+</sup> (*m/z* 354), and [M + H - H<sub>2</sub>O - 2TCA]<sup>+</sup> (*m/z* 336) provides unequivocal identification of the compounds. The level of 3-*epi*-FB<sub>3</sub> (**5**) in these samples ranged from 6.7 to 21% of the level of FB<sub>3</sub> and contributed only marginally to the total level of fumonisins. On the basis of these data and the fact that it also only occurs at lower levels than FB<sub>3</sub> in culture, it can be concluded that, in general, the influence of 3-*epi*-FB<sub>3</sub> on fumonisin exposure will be small.

The use of an isolated FB<sub>3</sub> analytical standard for chromatographic analysis is problematic due to the presence of the two stereoisomers. Nevertheless, because of their chemical similarity and similar retention times on a reversed-phase HPLC column, the assumption of the same chromatographic response factors for the two isomers would appear to be reasonable and allows accurate analysis to be performed. The response factor calculated for the combination of the two isomers in the standard chromatogram is then applied separately to each isomer peak in the chromatogram of the sample.

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Received for review January 9, 2007. Revised manuscript received March 22, 2007. Accepted March 22, 2007. Financial support from the University of Pretoria and the National Research Foundation, Pretoria, as well as the Wellcome Trust toward the purchase of the LC-MS system is gratefully acknowledged.

JF070061H

## Evidence for the Natural Occurrence of Fumonisin B<sub>1</sub>, a Mycotoxin Produced by *Fusarium moniliforme*, in Corn

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*Fusarium moniliforme*, a common fungal contaminant of corn, was recently shown to produce a group of mycotoxins, the fumonisins, in culture. Moldy home-grown corn collected from an area of the Transkei, southern Africa, was analyzed for the presence of the fumonisin mycotoxins. Fumonisin B<sub>1</sub> (FB<sub>1</sub>) was detected in the sample extract, as independently prepared derivatives, by two high-performance liquid chromatographic procedures. A capillary gas chromatographic-mass spectrometric procedure was used to confirm the identity of the tricarballic acid moiety, present in the esterified hydrolysates of the fumonisins. This is the first conclusive report of the natural occurrence of FB<sub>1</sub> in corn.

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*Fusarium moniliforme* Sheldon, a common fungal contaminant of corn throughout the world (Booth, 1971), has been implicated in animal and human diseases (Marasas et al., 1984b). Various strains of the fungus are known to be highly toxic (Kriek et al., 1981a,b) and carcinogenic (Marasas et al., 1984a; Jaskiewicz et al., 1987) in animals. Since *F. moniliforme* has been associated with human esophageal cancer risk in the Transkei, southern Africa (Marasas, 1982; Marasas et al., 1981, 1988a) and in China (Li et al., 1980; Yang, 1980), recent investigations have focused on the characterization of the carcinogenic compounds produced by this fungus.

The mutagenic activities exhibited by various strains of *F. moniliforme* in the *Salmonella* mutagenicity test resulted in the characterization of the potent mutagen, fusarin C (Wiebe and Bjeldanes, 1981; Gelderblom et al., 1984a; Gaddamidi et al., 1985). However, the lack of carcinogenicity of fusarin C (Gelderblom et al., 1986) makes it unlikely that this mutagen is involved in the carcinogenic effects of the fungus. Recently, several strains of *F. moniliforme* were found to exhibit cancer-promoting activity in a short-term cancer initiation/promotion bioassay in rats using diethylnitrosamine (DEN) as a cancer initiator and the induction of  $\gamma$ -glutamyltranspeptidase positive foci in the liver as end point (Gelderblom et al., 1988b). With use of this bioassay as a monitor for cancer-promoting principles, the fumonisins were iso-

lated (Gelderblom et al., 1988a) and chemically characterized (Bezuidenhout et al., 1988) from culture material of *F. moniliforme* MRC 826, previously shown to be hepatocarcinogenic to rats (Marasas et al., 1984a; Jaskiewicz et al., 1987). In addition to its cancer-promoting ability, fumonisin B<sub>1</sub> (FB<sub>1</sub>; Figure 1), the major compound, also exhibits toxic effects in rats similar to that of the culture material of *F. moniliforme* MRC 826 (Gelderblom et al., 1988a). Recently Marasas et al. (1988b) induced the equine neurotoxic disease leukoencephalomalacia (LEM) in a horse by intravenous injection of FB<sub>1</sub> isolated from strain MRC 826.

A sample of home-grown corn from a high esophageal cancer risk area of the Transkei, southern Africa, has previously been shown to be naturally contaminated with at least four *Fusarium* mycotoxins, i.e. moniliformin, zearalenone, deoxynivalenol, and fusarin C (Thiel et al., 1982; Gelderblom et al., 1984b). This paper details the chemical analysis of this corn sample for the presence of FB<sub>1</sub>.

### EXPERIMENTAL SECTION

**Analytical Standards.** FB<sub>1</sub> was extracted from *F. moniliforme* MRC 826 as previously described (Gelderblom et al., 1988a). The purity of the analytical standard was assessed by thin-layer chromatography (TLC; see TLC analyses). Visual inspection of the plate showed the presence of a minor contam-

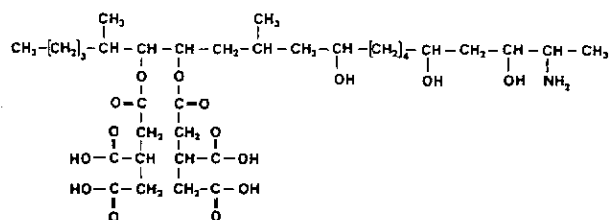


Figure 1. Chemical structure of fumonisin B<sub>1</sub>.

inant at a slightly higher  $R_f$  value to that of the FB<sub>1</sub> spot, in the position of fumonisin B<sub>3</sub> (FB<sub>3</sub>), another fumonisin mycotoxin whose chemical structure has still to be verified. When compared with the FB<sub>1</sub> used by Gelderblom et al. (1988a), the purity of the analytical standard used in this investigation was found to be >92%.

Tricarballic acid (1,2,3-propanetricarboxylic acid, TCA) was purchased from Fluka Chemicals, Buchs, Switzerland. Only one component could be detected by capillary gas chromatography-flame ionization detection (GC-FID) analysis of the esterified derivative of this standard.

**Fungal Culture.** Corn cultures of *F. moniliforme* MRC 826 were incubated for 2 weeks at 25 °C followed by 2 weeks at 15 °C after which they were freeze-dried, ground, and stored at 4 °C prior to analyses (Gelderblom et al., 1984b).

**Corn Samples.** A sample of moldy corn ears of the 1978 crop was obtained from a farm in the Butterworth district, Transkei, during July 1978 (Thiel et al., 1982). Visibly *Fusarium* infected ears were selected and hand-shelled. The kernels (a mixture of *Fusarium*-infected kernels, healthy kernels, and kernels infected with other fungi) were retained as sample M-84 (Thiel et al., 1982; Gelderblom et al., 1984b). Two subsamples, one containing predominantly healthy kernels, and the other predominantly *Fusarium*-infected kernels, were selected from sample M-84 and retained as samples M-84/C and M-84/F, respectively. Hand-selected kernels from first-grade, commercial South African corn together with a sample of commercially available corn meal were used as control samples. Each sample was ground and stored at 4 °C prior to analyses.

**Chemical Analyses.** 1. **Extraction.** Samples of corn (25 g, 5 g for culture samples) were extracted with CH<sub>3</sub>OH-H<sub>2</sub>O (3:1, 50 mL), by blending for 5 min and filtering. A 25-mL aliquot of the filtrate was evaporated to dryness at 50 °C, dissolved in CH<sub>3</sub>OH-H<sub>2</sub>O (1:3, 25 mL), and partitioned with CHCl<sub>3</sub> (2 × 50 mL). The aqueous phase was evaporated to dryness and resuspended in CH<sub>3</sub>OH-H<sub>2</sub>O (1:3, 10 mL, 50 mL for culture samples). Aliquots of these extracts (2 mL for HPLC

and 1 mL for GC) were further purified by application to a Sep-Pak C<sub>18</sub> cartridge, washing with CH<sub>3</sub>OH-H<sub>2</sub>O (1:3, 10 mL), and eluting the toxin with CH<sub>3</sub>OH-H<sub>2</sub>O (3:1, 10 mL). The eluate was evaporated to dryness.

2. **TLC Analysis.** Aliquots of the purified sample extracts (in CH<sub>3</sub>OH-H<sub>2</sub>O, 1:3) were spotted on Silica Gel 60 TLC plates (Merck). The plates were developed in CHCl<sub>3</sub>-CH<sub>3</sub>OH-CH<sub>3</sub>COOH (6:3:1), dried, sprayed with a solution of 0.5% *p*-anisaldehyde in CH<sub>3</sub>OH-CH<sub>3</sub>COOH-H<sub>2</sub>SO<sub>4</sub> (85:10:5, v/v), heated for 5 min at 110 °C, and visually inspected. FB<sub>1</sub> showed as a brown-purple spot with an  $R_f$  ~0.25.

3. **Maleyl Derivatization and High-Performance Liquid Chromatographic (HPLC) Analyses.** The purified extracts were maleylated and analyzed by HPLC according to the method of Siler and Gilchrist (1982), with minor modifications. Briefly, the purified extract residues were dissolved in 0.05 M Na<sub>2</sub>CO<sub>3</sub> (pH 9.2, 5 mL) and treated with an excess of 1 M maleic anhydride solution in dioxane (3 × 10 μL), while the pH was adjusted to 9 with 0.1 M NaOH, following each addition of maleic anhydride. Following a reaction time of 10 min, the mixture was adjusted to between pH 6 and 7 with use of 0.1 M HCl. The derivatized extracts were then evaporated to dryness at 50 °C and redissolved in CH<sub>3</sub>OH-H<sub>2</sub>O (1:1, 2 mL). HPLC separations were performed on a C<sub>18</sub> reversed-phase column using a Waters Model 510 pump, and the UV absorption of the eluate was monitored with a Waters 481 variable-wavelength detector. The detailed chromatographic conditions are summarized in Table I. Data were collected with a Waters 745 data module, and quantitative determination of FB<sub>1</sub> was by comparison of the peak areas in the samples to that of the peak area of a similarly derivatized FB<sub>1</sub> standard. Sample extracts spiked with FB<sub>1</sub> were similarly chromatographed.

4. **Extraction, Fluorescamine Derivatization, and HPLC Analysis.** Samples of corn (25 g) were extracted with CH<sub>3</sub>OH-H<sub>2</sub>O (3:1, 50 mL) by shaking for 30 min. The extracts were filtered, and a 5-mL aliquot of the filtrate was evaporated to dryness at 50 °C. The residue was partially redissolved in CH<sub>3</sub>OH-H<sub>2</sub>O (7:3, 1 mL) and fully solvated following the addition of CH<sub>3</sub>OH (1 mL). The solution was transferred to the top of a prepared chromatographic column (10-mm i.d.) containing activated silica gel (2 g, 70-230 mesh; Merck) suspended between two layers of anhydrous Na<sub>2</sub>SO<sub>4</sub> (1 g), in CH<sub>3</sub>OH. The column was washed with CH<sub>3</sub>OH (15 mL), the toxins were eluted with 0.1% CH<sub>3</sub>COOH-CH<sub>3</sub>OH (20 mL), and the eluate was evaporated to dryness. The purified residues were redissolved in 0.05 M NaHCO<sub>3</sub> (pH 8.6, 1 mL), and an aliquot (25 μL) was diluted to 500 μL in a plastic microfuge tube (1.8-mL capacity) with the same NaHCO<sub>3</sub> solution. While vortex mixing, fluorescam-

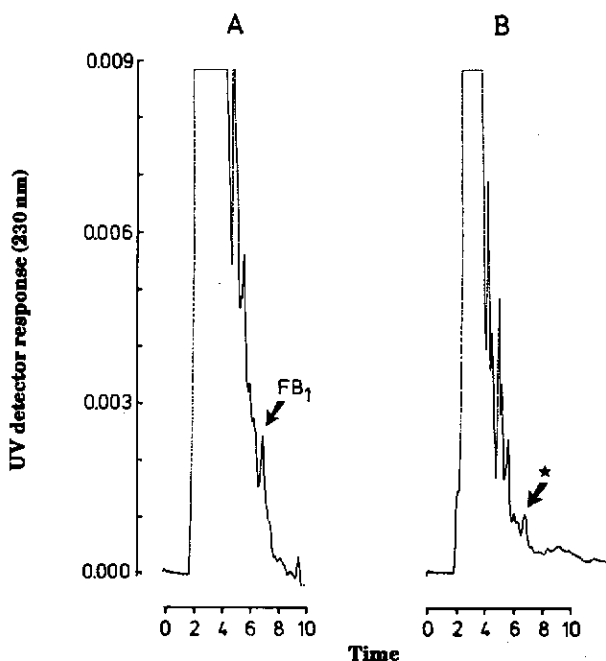
Table I. Chromatographic Conditions for the HPLC and GC Analyses of Fumonisin B<sub>1</sub> (FB<sub>1</sub>) and Tricarballic Acid (TCA) Derivatives

| parameter                  | maleyl deriv of FB <sub>1</sub>  | fluorescamine deriv of FB <sub>1</sub>                     | esterified TCA                                 |   |
|----------------------------|--|--|--|---|
|                            | HPLC/UV  | HPLC/fluorescence  | GC-FID   | GC-MS (EI)                                |
| chromatographic procedure  |  |  |  |   |
| column                     | Waters Novapak C <sub>18</sub> (4 μm), 150 mm × 4.6 mm (i.d.)            | Phenomenex Ultracarb ODS 30 (7 μm), 250 mm × 4.6 mm (i.d.) | DB-5 (0.25-μm film), 30 m × 0.32 mm (i.d.)     | DB-5 (0.25-μm film) 60 m × 0.32 mm (i.d.) |
| mobile phase               | 0.05 M KH <sub>2</sub> PO <sub>4</sub> -CH <sub>3</sub> OH (3:7, pH 3.5) | 0.1 M acetate buffer (pH 4.0)-CH <sub>3</sub> CN (1:1)     | helium   | helium                                    |
| flow rate                  | 1 mL/min   | 1 mL/min   | 35 cm/s  | 30 cm/s                                   |
| injection vol, μL          | 5 or 10  | 20   | 1  | 1   |
| splitless (on time), min   |  |  | 0.5  | 0.5                                       |
| injector temp, °C          |  |  | 140  | 140                                       |
| detector temp, °C          |  |  | 280  | 280                                       |
| temp profile               |  |  |  |   |
| init temp, °C              |  |  | 50 or 3 min                                    | 70 for 1 min                              |
| final temp, °C             |  |  | 280  | 280                                       |
| rate, °C min <sup>-1</sup> |  |  | 15   | 15  |
| detector fuel gases        |  |  | air, 300 mL/min;<br>H <sub>2</sub> , 30 mL/min |   |
| detector wavelengths, nm   | 230  | 390 (excitation),<br>475 (emission)                        |  |   |
| detector sensitivity       | 0.010 AUFS   | 20-nm slit width   | 2 <sup>7</sup>                                 |   |
| mass range, m/z            |  |  |  | 50-350                                    |

**Table II.** Levels ( $\mu\text{g/g}$ ) of Fumonisin B<sub>1</sub> (FB<sub>1</sub>) and Tricarballic Acid (TCA) Assayed as Their Maleyl and Butyl Ester Derivatives, Respectively

| sample <sup>a</sup>        | FB <sub>1</sub> measd as maleyl deriv <sup>b</sup> | TCA calcd as due to FB <sub>1</sub> (maleyl deriv results) | TCA measd as Bu ester deriv <sup>c</sup> |
|----------------------------|--|--|--|
| hand-selected control corn | <10 <sup>d</sup>                                   | <4.9   | <0.5                                     |
| commercial corn meal       | <10  | <4.9   | 13                                       |
| M-84/C                     | <10  | <4.9   | 21                                       |
| M-84                       | 44   | 22   | 101                                      |
| M-84/F                     | 83   | 41   | 164                                      |
| MRC 826                    | 9280   | 4530   | 6400                                     |

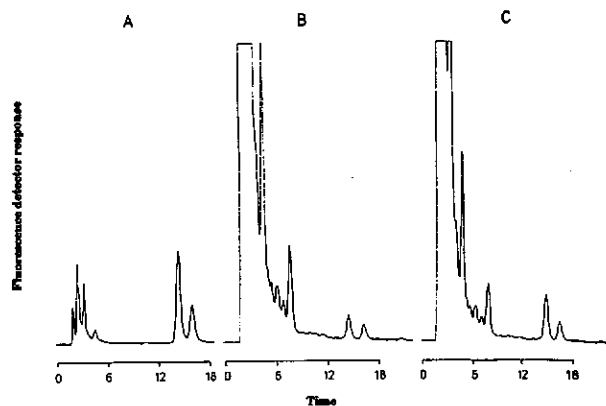
<sup>a</sup> Key: M-84/C = healthy corn kernels; M-84 = moldy corn kernels; M-84/F = *Fusarium*-infected corn kernels; MRC 826 = culture material of *F. moniliforme* MRC 826. <sup>b</sup> Determined by HPLC/UV; detection limit approximately 10  $\mu\text{g/g}$ . <sup>c</sup> Determined by GC-FID; detection limit 0.5  $\mu\text{g/g}$ . <sup>d</sup> Subsequent analysis indicated that this was a contaminant peak eluting in the position of FB<sub>1</sub>.



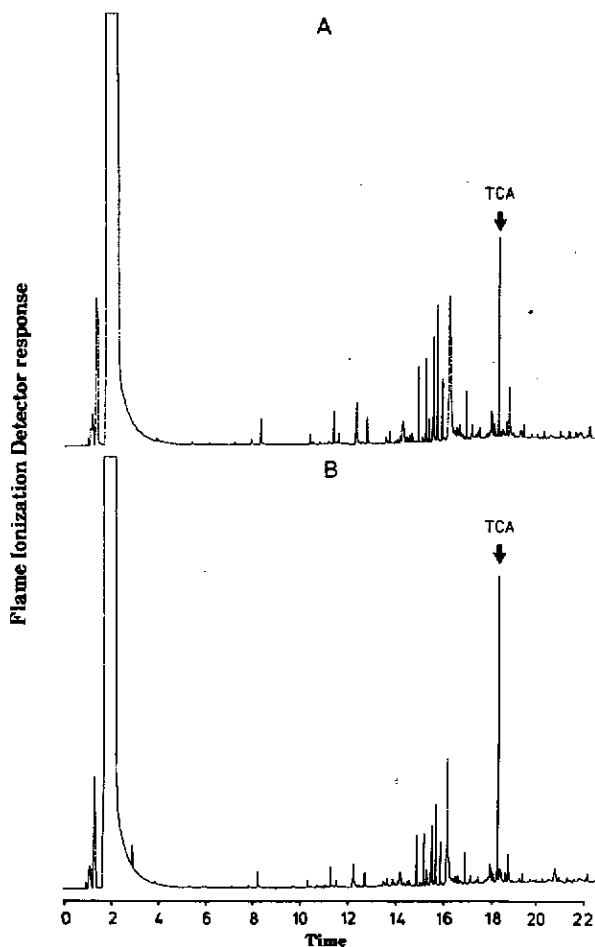
**Figure 2.** HPLC chromatogram of (A) the maleyl derivative of sample M-84 and (B) a similarly prepared extract of hand-selected corn kernels showing the presence of a contaminant peak (\*), which elutes in the chromatographic position of fumonisin B<sub>1</sub> (FB<sub>1</sub>).

ine (50  $\mu\text{L}$ , 2 mg/100  $\mu\text{L}$  of acetone; Aldrich) was added to the tube. Mixing was continued for 1 min, and the mixture was then centrifuged for 1 min at 9000g. Aliquots (20  $\mu\text{L}$ ) of the derivatized extracts were separated by reversed-phase HPLC (Table I) using a Waters 510 pump coupled to a Perkin-Elmer 650S fluorescence detector.

**5. Hydrolysis, Derivatization, and Gas Chromatographic Analyses.** Purified sample extracts were hydrolyzed (under N<sub>2</sub>) with 6 M HCl (2 mL) at 95 °C for 3 h. Each sample was then cooled and an aliquot of each subjected to esterification and acylation according to the method of Labadarios et al. (1984). Briefly, an aliquot (500  $\mu\text{L}$ ) of each hydrolysate was transferred to an appropriate tube, and the excess acid was removed by freeze-drying under reduced pressure. Esterification of the residue was performed under nitrogen with isobutyl alcohol containing 3 M HCl (250  $\mu\text{L}$ ) and heated at 100 °C for 45 min. The acidified isobutyl alcohol was removed by freeze-drying under reduced pressure and the residue acylated with heptafluorobutyric anhydride (100  $\mu\text{L}$ ) by heating for 10 min (under nitrogen) at 150 °C. The samples were then cooled in ice, freeze-



**Figure 3.** HPLC chromatogram of (A) the fluorescamine derivative of 65 ng of fumonisin B<sub>1</sub> (FB<sub>1</sub>), (B) a similarly prepared derivative of sample M-84/F, and (C) the same sample extract spiked with derivatized FB<sub>1</sub>.



**Figure 4.** Capillary GC chromatogram of (A) a hydrolyzed-esterified extract of M-84/C showing a peak eluting at 18.3 min and (B) the same sample extract spiked with esterified tricarballic acid (TCA).

dried, and dissolved in ethyl acetate (100  $\mu\text{L}$ ). Capillary gas chromatographic separations of 1- $\mu\text{L}$  aliquots of each derivatized hydrolysate were performed on an apolar capillary column connected to a Carlo Erba Mega 5300 gas chromatograph fitted with a flame ionization detector and a Waters 745 data module. The prevailing chromatographic conditions are outlined in Table I. Each sample was analyzed in triplicate, and samples spiked with FB<sub>1</sub> were similarly treated. Quantitative determinations were done by comparison of the peak areas of

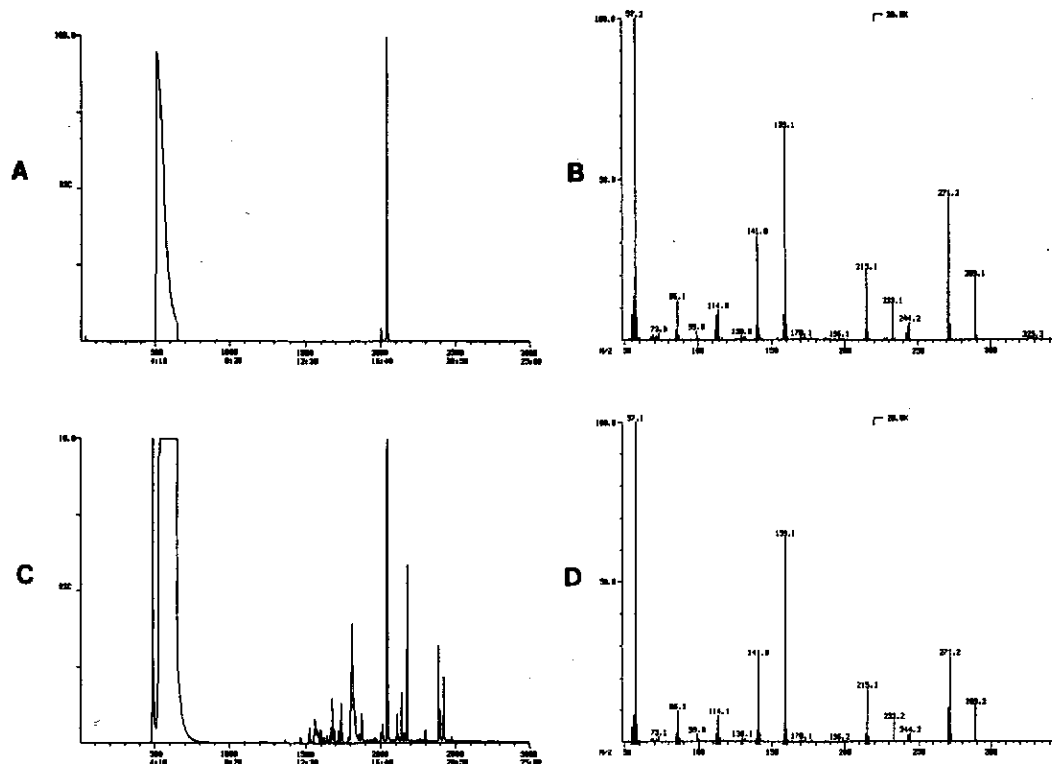


Figure 5. (A) Total ion chromatogram (TIC) of esterified tricarballic acid (TCA) showing a major peak eluting at 17.1 min, (B) the mass spectrum (MS) of esterified TCA, (C) TIC of a hydrolyzed-esterified extract of M-84/F also showing a major peak at 17.1 min, and (D) MS of the peak eluting at 17.1 min shown in (C).

the derivatized TCA moiety, against a calibration curve of similarly derivatized TCA standards (found to be linear over the range 25–150 ng). Capillary gas chromatographic-mass spectrometric data of the TCA butyl ester peak present in the samples were obtained on a Finnigan MAT 4500 gas chromatograph-mass spectrometer under the conditions specified in Table I.

## RESULTS AND DISCUSSION

Extracts of a fungal culture (*F. moniliforme* MRC 826), two samples of control corn, and the three Transkeian corn subsamples were prepared and analyzed by HPLC for the presence of FB<sub>1</sub>, as its maleyl derivative. The results generated are given in Table II. The chromatogram obtained from a 5- $\mu$ L aliquot of a corn sample extract (M-84), as its corresponding maleyl derivative, is shown in Figure 2A. Base-line separation of the FB<sub>1</sub> peak was not possible due to the presence of substrate matrix interferences.

Figure 2B shows the chromatogram obtained from a 10- $\mu$ L aliquot of an extract of hand-selected control corn kernels as the maleyl derivative. While the degree of matrix interference is much lower than that observed in Figure 2A, a small peak was observed at the chromatographic position of FB<sub>1</sub> (corresponding to <10  $\mu$ g/g of FB<sub>1</sub>; Table II). A similar peak was also observed in the sample of commercially available corn meal as well as in extracts of three other control corn samples (data on the latter samples are not included). The presence of this peak in several control corn samples indicated the possibility that it was an interfering compound found intrinsically in corn.

Therefore, a number of matrix-related factors clearly demonstrate the limitations of the maleyl derivative procedure for the determination of FB<sub>1</sub> in naturally contaminated corn samples. Using the maleyl derivative procedure, the average corrected recovery (in triplicate) of

FB<sub>1</sub> from hand-selected control corn spiked at 100  $\mu$ g/g was found to be 66.3% with a standard deviation of 1.4%.

Further evidence for the presence of FB<sub>1</sub> was obtained by the extraction, fluorescent labeling, and chromatographic separation of extracts of the Transkeian corn samples. Figure 3A shows the chromatogram of a fluorescamine-derivatized FB<sub>1</sub> standard, where two well-resolved peaks may be seen at the retention times of 14.3 and 16.5 min, respectively. Figure 3B shows the chromatogram obtained from a similarly derivatized extract of sample M-84/F, and Figure 3C, the same extract spiked with derivatized FB<sub>1</sub>. HPLC separation of a preformed fluorescamine-primary amine complex often results in dual peaks for primary amines, as was the case for FB<sub>1</sub> (Figure 3A). These peaks result from the formation of the acid alcohol and the lactone derivatives of the fluorescent complex both exhibiting identical fluorescent characteristics (Perrett, 1985; Rosenthal, 1985). Due to the necessity for optimization, this fluorescamine derivatization method could not be used quantitatively, and hence, its application to the naturally contaminated corn subsamples merely yielded supportive evidence for the presence of FB<sub>1</sub>.

Only a single peak could be detected by GC-FID in a hydrolyzed, esterified, and acylated FB<sub>1</sub> standard, under the conditions outlined in Table I (GC-FID data). Esterification of an aliquot of the FB<sub>1</sub> hydrolysate (without the subsequent acylation step) resulted in the detection of the same chromatographic peak. This peak was also observed in an esterified derivative of an authentic TCA standard. These observations suggested that the FB<sub>1</sub> had been successfully hydrolyzed but that in the process only the esterified TCA moiety could be detected and not the anticipated aminopentol moiety.

By the hydrolysis/esterification procedure, the levels of the TCA moiety present in each sample were deter-

mined and the results are given in Table II. Figure 4A shows the GC-FID chromatogram obtained from a hydrolyzed-esterified extract of M-84/C, while in Figure 4B the same extract spiked with similarly esterified FB<sub>1</sub> standard is illustrated. A well-resolved peak corresponding to the isobutyl ester of TCA can be observed at a retention time of 18.3 min (Figure 4A,B).

In order to verify the identity of the TCA moiety present in the Transkeian corn samples, hydrolyzed-esterified extracts of each sample were analyzed by GC-MS (chromatographic conditions are given in Table I). Figure 5A details the total ion chromatogram (TIC) of the esterified TCA standard, with a single peak eluting at a retention time of 17.1 min, the mass spectrum (MS) of which is shown in Figure 5B. Figure 5C displays the TIC of the hydrolyzed-esterified extract of M-84/F, where a well-defined peak can also be seen at 17.1 min. The MS of this peak is given in Figure 5D. The excellent agreement between the two spectra (Figures 5B,D) verified the presence of TCA in the hydrolyzed-esterified extract of the Transkeian corn sample.

The TCA contribution due to the FB<sub>1</sub> (determined as its maleyl derivative), present in each sample, was calculated, and the results are given in Table II. The levels of TCA determined experimentally (by GC-FID) were invariably higher in each sample than the corresponding TCA content contributed by the FB<sub>1</sub>. In the case of the hand-selected control sample, the peak observed in the chromatographic position of FB<sub>1</sub>, as its maleyl derivative (Figure 2B) was undoubtedly a contaminant peak since no corresponding TCA was detected in the sample. Since a similar sized peak was also observed in the maleylated extracts of the commercial corn meal and M-84/C samples, the levels of TCA determined experimentally in these samples could not be directly attributed to the presence of FB<sub>1</sub>.

The fact that the TCA levels in five to six samples were higher than could be explained by the presence of FB<sub>1</sub> was not unexpected, as it has already been shown that TCA-containing compounds other than FB<sub>1</sub> (such as fumonisin B<sub>2</sub>; FB<sub>2</sub>) are produced by *F. moniliforme* (Gelderblom et al., 1988a). In the case of the MRC 826 culture material, the FB<sub>1</sub> accounted for >70% of the total TCA detected experimentally in the hydrolyzed-esterified extract, which agrees with the observation that FB<sub>1</sub> is the major fumonisin produced by this fungus in culture (Gelderblom et al., 1988a). In samples M-84 and M-84/F, FB<sub>1</sub> contributed less to the total TCA than in the culture material, implying that more of the other fumonisins were present in these samples. Attempts to confirm the presence of FB<sub>1</sub> and other related fumonisins (FB<sub>2</sub> and FB<sub>3</sub>) in extracts of the Transkeian corn samples by TLC were inconclusive, since the detection limit of the method (500 µg/g for each toxin) was far higher than the levels present in the samples.

None of the chromatographic methods presented should be considered as fully developed analytical procedures for the determination of FB<sub>1</sub> in corn. However, the application of three chromatographic procedures to a naturally contaminated sample provided conclusive evidence for the presence of FB<sub>1</sub> in corn. This evidence suggests that humans in an area of the Transkei, southern Africa, may well be exposed to the cancer-promoting *F. moniliforme* mycotoxins, the fumonisins.

Subsequent to the acceptance of this publication, Voss et al. (1989) reported the cooccurrence of FB<sub>1</sub> and FB<sub>2</sub> in two corn samples associated with outbreaks of equine LEM in the United States.

#### ABBREVIATIONS USED

DEN, diethylnitrosamine; FB<sub>1</sub>, fumonisin B<sub>1</sub>; FB<sub>2</sub>, fumonisin B<sub>2</sub>; FB<sub>3</sub>, fumonisin B<sub>3</sub>; GC-FID, gas chromatography-flame ionization detection; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; LEM, leukoencephalomalacia; MS, mass spectrum; TCA, tricarballic acid; TIC, total ion chromatogram; UV, ultraviolet.

#### ACKNOWLEDGMENT

We express our gratitude to the following: S. Stockenström and L. van der Westhuizen for technical assistance throughout the course of this study; I. M. Moodie for interest, advice, and critical review of the manuscript; G. Grierson for the generation of mass spectral data; D. Botes, C. Meyer, and B. Burger for useful discussions and suggestions.

Registry No. FB<sub>1</sub>, 116355-83-0.

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Received for review March 30, 1989. Accepted August 14, 1989.

## Survey of Fumonisin Production by *Fusarium* Species

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Received 5 November 1990/Accepted 15 January 1991

**Fumonisin B<sub>1</sub> (FB<sub>1</sub>) and B<sub>2</sub> (FB<sub>2</sub>), two structurally related mycotoxins with cancer-promoting activity, were recently isolated from corn cultures of *Fusarium moniliforme* MRC 826. These toxins have been reported to be produced also by isolates of *F. proliferatum*. Contamination of foods and feeds by *F. moniliforme* has been associated with human esophageal cancer risk, and FB<sub>1</sub> has been shown to be the causative agent of the neurotoxic disease leukoencephalomalacia in horses. Because of the toxicological importance of the fumonisins, the potential to produce FB<sub>1</sub> and FB<sub>2</sub> was determined in a study of 40 toxic *Fusarium* isolates representing 27 taxa in 9 of the 12 sections of *Fusarium*, as well as two recently described species not yet classified into sections. With the exception of one isolate of *F. nygamai*, fumonisin production was restricted to isolates of *F. moniliforme* and *F. proliferatum*, in the section *Lisola*. The *F. nygamai* isolate produced 605 µg of FB<sub>1</sub> g<sup>-1</sup> and 530 µg of FB<sub>2</sub> g<sup>-1</sup>, and the identity of the toxins was confirmed by capillary gas chromatography-mass spectrometry. This is the first report of the production of the fumonisins by *F. nygamai*.**

*Fusarium moniliforme* contamination of feeds has been associated with several diseases in animals, including leukoencephalomalacia (LEM) in horses (12), and the incidence of *F. moniliforme* infection of home-grown corn has been shown to be correlated with the high incidence of human esophageal cancer in Transkei, southern Africa (9, 10, 16), and in China (8, 27).

Fumonisin B<sub>1</sub> (FB<sub>1</sub>) and B<sub>2</sub> (FB<sub>2</sub>), structurally related mycotoxins with cancer-promoting activity, were recently isolated from corn cultures of *F. moniliforme* Sheldon strain MRC 826 (3), and their structures were elucidated (1). FB<sub>1</sub> has since been shown to induce LEM in horses after either intravenous (11) or per os (6) administration and to induce pulmonary edema in swine after intravenous administration (5).

Both FB<sub>1</sub> and FB<sub>2</sub> have been found to occur naturally in corn and in feeds associated with field outbreaks of LEM in horses (20, 21, 23, 25, 26). Higher concentrations of both toxins have been detected in corn samples from areas with a high esophageal cancer rate in Transkei than in samples from low-rate areas (22).

At present there is no experimental proof that the fumonisins, which are rat liver cancer promoters, are causative agents of esophageal cancer in humans. However, the toxicological importance of the fumonisins emphasizes the need to establish the extent of human and animal exposure to these toxins. One approach is to screen different fungal isolates that commonly occur on foods and feeds for their potential to produce the fumonisins. A recent report (19) documented the production of FB<sub>1</sub> and FB<sub>2</sub> by cultures of both *F. moniliforme* and *F. proliferatum* (Matsushima) Nirenberg isolated from feeds associated with LEM in horses and pulmonary edema in swine in the United States.

In the present study 40 toxic *Fusarium* isolates representing 27 taxa in 9 of the 12 sections of *Fusarium* as classified by Nelson et al. (17), as well as two recently described species not yet classified into sections, were screened for their potential to produce FB<sub>1</sub> and FB<sub>2</sub>.

### MATERIALS AND METHODS

***Fusarium* isolates.** All *Fusarium* isolates used in this investigation were obtained from the culture collection of the Research Institute for Nutritional Diseases, Medical Research Council, Tygerberg, South Africa. Cultures on corn of all the isolates tested were known to be highly toxic to ducklings, causing four of four deaths when fed to 1-day-old Pekin ducklings for 14 days, as previously described (15). The origin of each isolate, the substrate from which it was isolated, and the *Fusarium* taxon to which it belongs are given in Table 1. The *Fusarium* taxa are also arranged in sections as described by Nelson et al. (17), except for the two recently described species *F. nygamai* Burgess & Trimboli (2) and *F. napiforme* Marasas, Nelson & Rabie (13), which have not yet been classified into sections.

**Culture techniques.** Lyophilized conidia of the different *Fusarium* isolates were suspended in sterile water and used to inoculate moistened yellow corn kernels (400 g of kernels and 400 ml of water) in 2-liter glass fruit jars previously autoclaved at 121°C for 1 h on each of 2 consecutive days. Cultures were incubated in the dark at 25°C for 21 days, dried overnight at 45°C, ground to a fine meal in a laboratory mill, and stored at 0°C until analyzed.

**Reference standards.** FB<sub>1</sub> and FB<sub>2</sub> standards were isolated and purified from cultures of *F. moniliforme* MRC 826 as previously described (3). The identity and purity of each standard were assessed by thin-layer chromatography, high-performance liquid chromatography, and nuclear magnetic resonance spectroscopy.

**Determination of fumonisins.** FB<sub>1</sub> and FB<sub>2</sub> were determined by a recently developed high-performance liquid chromatography method (20). Briefly, fumonisins were extracted from a sample of the culture material with methanol-water (3:1, vol/vol). The extract was purified on a strong anion-exchange cartridge, and an aliquot was derivatized with *o*-phthaldialdehyde. The derivatized fumonisins were separated on a reversed-phase column, monitored by fluorescence detection, and quantified by comparison of peak areas with those obtained with reference standards of FB<sub>1</sub> and FB<sub>2</sub>.

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TABLE 1. Fumonisin production by *Fusarium* species

| Section, MRC <sup>a</sup> no., and species | Origin        | Substrate | Fumonisin concn $\mu\text{g g}^{-1}$ |                 |
|--|---------------|-----------|--------------------------------------|-----------------|
|  |               |           | FB <sub>1</sub>                      | FB <sub>2</sub> |
| <b>Spicarioides</b>                        |               |           |                                      |                 |
| 2801 <i>F. decemcellulare</i>              | South Africa  | Avocado   | ND <sup>b</sup>                      | ND              |
| <b>Sporotrichiella</b>                     |               |           |                                      |                 |
| 43 <i>F. sporotrichioides</i>              | France        | Corn      | ND                                   | ND              |
| 3424 <i>F. poae</i>                        | South Africa  | Barley    | ND                                   | ND              |
| 1577 <i>F. tricinctum</i>                  | United States | Ivy       | ND                                   | ND              |
| <b>Roseum</b>                              |               |           |                                      |                 |
| 3302 <i>F. avenaceum</i>                   | South Africa  | Medicago  | ND                                   | ND              |
| <b>Arthrosporiella</b>                     |               |           |                                      |                 |
| 2905 <i>F. semitectum</i>                  | South Africa  | Corn      | ND                                   | ND              |
| 4815 <i>F. camptoceras</i>                 | Costa Rica    | Cocoa     | ND                                   | ND              |
| 3520 <i>F. camptoceras</i> <sup>c</sup>    | South Africa  | Soil      | ND                                   | ND              |
| <b>Gibbosum</b>                            |               |           |                                      |                 |
| 2330 <i>F. equiseti</i>                    | United States | Cereal    | ND                                   | ND              |
| 4463 <i>F. equiseti</i>                    | Transkei      | Soil      | ND                                   | ND              |
| 3311 <i>F. acuminatum</i>                  | South Africa  | Medicago  | ND                                   | ND              |
| 4028 <i>F. acuminatum</i> <sup>d</sup>     | South Africa  | Soil      | ND                                   | ND              |
| 3687 <i>F. scirpi</i>                      | Australia     | Soil      | ND                                   | ND              |
| 3528 <i>F. longipes</i> <sup>e</sup>       | South Africa  | Soil      | ND                                   | ND              |
| 4458 <i>F. longipes</i>                    | New Guinea    | Soil      | ND                                   | ND              |
| <b>Discolor</b>                            |               |           |                                      |                 |
| 3307 <i>F. sambucinum</i>                  | South Africa  | Medicago  | ND                                   | ND              |
| 4666 <i>F. sambucinum</i>                  | South Africa  | Potato    | ND                                   | ND              |
| 1115 <i>F. graminearum</i> Gr.2            | Transkei      | Corn      | ND                                   | ND              |
| 4917 <i>F. graminearum</i> Gr.1            | South Africa  | Barley    | ND                                   | ND              |
| 5052 <i>F. graminearum</i> Gr.2            | South Africa  | Wheat     | ND                                   | ND              |
| 3636 <i>F. reticulatum</i>                 | South Africa  | Medicago  | ND                                   | ND              |
| 1386 <i>F. compactum</i>                   | South Africa  | Groundnut | ND                                   | ND              |
| <b>Lateritium</b>                          |               |           |                                      |                 |
| 1925 <i>F. lateritium</i>                  | Zimbabwe      | Coffee    | ND                                   | ND              |
| <b>Liseola</b>                             |               |           |                                      |                 |
| 826 <i>F. moniliforme</i>                  | Transkei      | Corn      | 7,100                                | 3,000           |
| 1065 <i>F. moniliforme</i>                 | Transkei      | Corn      | 85                                   | 10              |
| 4315 <i>F. moniliforme</i>                 | Transkei      | Corn      | 2,645                                | 325             |
| 4317 <i>F. moniliforme</i>                 | Transkei      | Corn      | 205                                  | 40              |
| 4318 <i>F. moniliforme</i>                 | Transkei      | Corn      | 105                                  | ND              |
| 4319 <i>F. moniliforme</i>                 | Transkei      | Corn      | 180                                  | 40              |
| 4321 <i>F. moniliforme</i>                 | Transkei      | Corn      | 1,330                                | 140             |
| 2059 <i>F. proliferatum</i>                | South Africa  | Sorghum   | 20                                   | 160             |
| 2301 <i>F. proliferatum</i>                | United States | Corn      | 870                                  | 450             |
| 2302 <i>F. proliferatum</i>                | United States | Corn      | 290                                  | 65              |
| 2383 <i>F. proliferatum</i>                | Sierra Leone  | Corn      | 660                                  | 200             |
| 1077 <i>F. subglutinans</i>                | Transkei      | Corn      | ND                                   | ND              |
| 3823 <i>F. anthropilum</i>                 | South Africa  | Oats      | ND                                   | ND              |
| <b>Elegans</b>                             |               |           |                                      |                 |
| 1492 <i>F. oxysporum</i>                   | South Africa  | Groundnut | ND                                   | ND              |
| <b>Newly described species</b>             |               |           |                                      |                 |
| 4003 <i>F. nygamai</i>                     | South Africa  | Soil      | 605                                  | 530             |
| 4150 <i>F. nygamai</i> <sup>f</sup>        | Namibia       | Millet    | ND                                   | ND              |
| 4134 <i>F. napiforme</i>                   | Namibia       | Sorghum   | ND                                   | ND              |

<sup>a</sup> MRC, Medical Research Council.<sup>b</sup> ND, Not detected ( $<1 \mu\text{g g}^{-1}$ ).<sup>c</sup> Lamprecht et al. (7).<sup>d</sup> Rabie et al. (18).<sup>e</sup> Van Wyk et al. (24).<sup>f</sup> Marasas et al. (14).

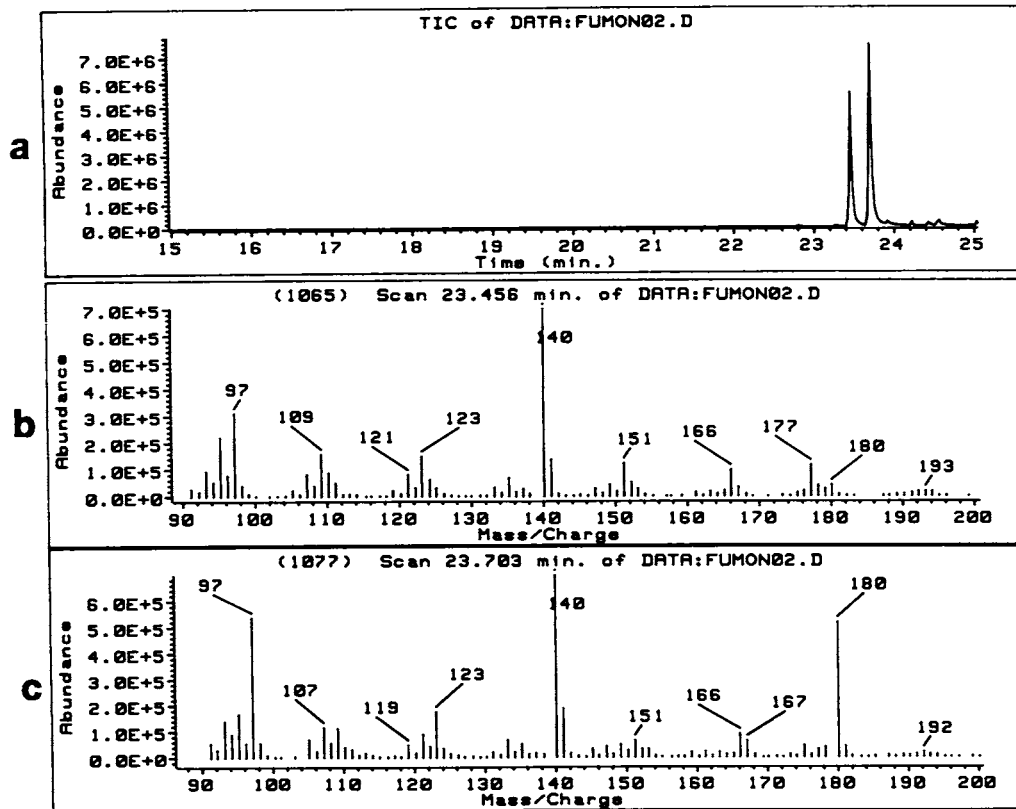


FIG. 1. (a) Total ion chromatogram (TIC) of the acylated hydrolysates of authentic fumonisin standards. (b) Partial mass spectrum of the amino-tetraol moiety of  $FB_2$ . (c) Similar mass spectrum of the amino-pentol moiety of  $FB_1$ .

**Confirmation of the presence of  $FB_1$  and  $FB_2$ .** Corn culture extracts were prepared and purified on strong anion-exchange cartridges and then hydrolyzed with 2 M potassium hydroxide (25). The hydrolysates were acidified with 1 M hydrochloric acid and purified further on short Amberlite XAD-4 columns (25). Aliquots of the purified hydrolysates were either derivatized with *o*-phthalaldehyde and analyzed by high-performance liquid chromatography or derivatized with trifluoroacetylimidazole and analyzed by capillary gas chromatography-mass spectrometry. The amino-pentol moiety of  $FB_1$  and the amino-tetraol moiety of  $FB_2$  (present in the hydrolysates) were determined by both chromatographic techniques. The retention times and mass spectra observed in the culture extracts were compared with those obtained for the amino-pentol and amino-tetraol present in similarly hydrolyzed fumonisin standards.

## RESULTS AND DISCUSSION

Results of fumonisin analyses of corn cultures of 40 toxic *Fusarium* isolates are summarized in Table 1. With the exception of one isolate of *F. nygamai*, fumonisin production was restricted to section Liseola. All the isolates tested of two of the *Fusarium* spp. in section Liseola, *F. moniliforme* and *F. proliferatum*, produced both  $FB_1$  and  $FB_2$  except for *F. moniliforme* strain MRC 4318, which produced only  $FB_1$ . One isolate of each of two other species in this section, *F. subglutinans* (Wollenweber & Reinking) Nelson, Toussoun & Marasas, and *F. anthropilum* (A. Braun) Wollenweber, did not produce any chemically detectable  $FB_1$  or  $FB_2$ .

Six of the seven *F. moniliforme* isolates from corn from a high-risk area of human esophageal cancer in the Transkei (4) produced both  $FB_1$  and  $FB_2$ , whereas the seventh isolate (MRC 4318) produced only  $FB_1$ . The highest producer of both  $FB_1$  ( $7,100 \mu\text{g g}^{-1}$ ) and  $FB_2$  ( $3,000 \mu\text{g g}^{-1}$ ) was *F. moniliforme* MRC 826, the strain from which the fumonisins were originally isolated and characterized (1, 3). Cultures of MRC 826 also had the highest cancer promotion activity compared with those of 10 other isolates of *F. moniliforme* from Transkeian corn, in a short-term cancer initiation-promotion bioassay in rat liver (4). The two other highest producers of fumonisins (MRC 4315 and MRC 4321, Table 1) similarly exhibited high cancer promotion activity in the cancer initiation-promotion bioassay (4). Of the three lowest producers of fumonisins (MRC 4317, MRC 4318, and MRC 4319; Table 1), two (MRC 4317 and MRC 4318) exhibited no cancer promotion activity, whereas the third (MRC 4319) registered the second-highest activity (4). With the exception of *F. moniliforme* MRC 4319, the fumonisin production by all *F. moniliforme* strains correlated well with the cancer promotion activity of the cultures.

The observation that all four isolates of *F. proliferatum* tested produced both  $FB_1$  and  $FB_2$  (Table 1) confirms a previous report (19) on fumonisin production by two isolates of *F. proliferatum* from feeds associated with field outbreaks of equine LEM and porcine pulmonary edema, respectively. The concentrations reported by Ross et al. (19) were in excess of  $1,600 \mu\text{g g}^{-1}$  for  $FB_1$  and  $150 \mu\text{g g}^{-1}$  for  $FB_2$ . In the present study, four *F. proliferatum* isolates, either from sorghum in South Africa or from corn in the United States

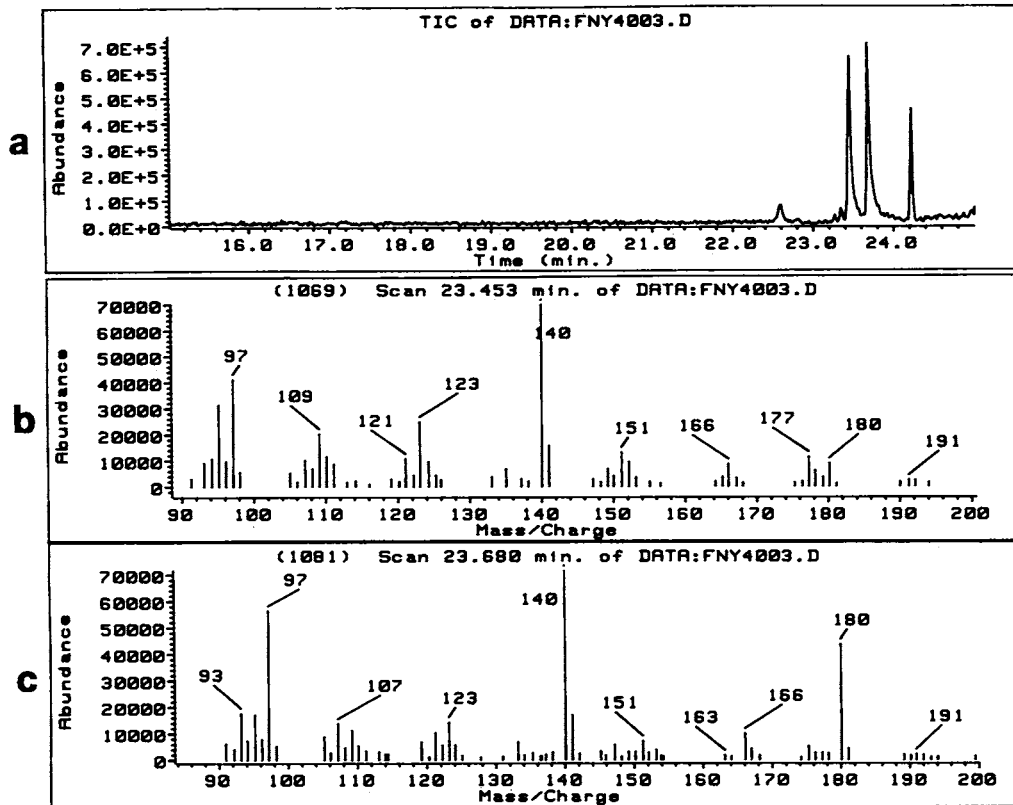


FIG. 2. (a) Total ion chromatogram (TIC) of the acylated hydrolysate of an extract of *F. nygamai* MRC 4003. (b) Partial mass spectrum of the peak eluting at 23.45 min. (c) Similar mass spectrum of the peak eluting at 23.68 min.

and Sierra Leone, produced  $FB_1$  and  $FB_2$  in cultures on corn, at levels ranging from 20 to 660  $\mu\text{g g}^{-1}$  and 65 to 450  $\mu\text{g g}^{-1}$ , respectively. One South African isolate of *F. proliferatum* (MRC 2059) from sorghum differed from all other fumonisin-producing strains thus far analyzed, in that it produced more  $FB_2$  (160  $\mu\text{g g}^{-1}$ ) than  $FB_1$  (20  $\mu\text{g g}^{-1}$ ).

In addition to the two fumonisin-producing *Fusarium* species in section *Liseola*, i.e., *F. moniliforme* and *F. proliferatum*, only one other toxic *Fusarium* isolate produced chemically detectable levels of  $FB_1$  and  $FB_2$ ; this was *F. nygamai* MRC 4003 (Table 1). The identity of the fumonisins produced by this isolate was confirmed by the hydrolysis of a sample extract followed by *o*-phthalaldehyde derivatization and high-performance liquid chromatographic analysis as well as by acylation and analysis by capillary gas chromatography-mass spectrometry.

Figure 1a shows the total ion chromatogram of the hydrolyzed, acylated products of authentic fumonisin standards. The two chromatographic peaks eluting at 23.45 and 23.70 min correspond to the amino-tetraol and -pentol moieties of  $FB_2$  and  $FB_1$ , respectively. Figures 1b and c show the partial mass spectra (90 to 200  $m/z$ ) of the peaks.

Figure 2a illustrates the total ion chromatogram of an extract of *F. nygamai* MRC 4003, and Fig. 2b and c show the mass spectra of the two major peaks observed in Fig. 2a. The excellent agreement between the two sets of retention times and mass spectra confirmed the production of both  $FB_1$  and  $FB_2$  by *F. nygamai* MRC 4003. The presence of the amino-pentol moiety of  $FB_1$  and the amino-tetraol moiety of  $FB_2$  in the hydrolyzed extracts of a culture of *F. nygamai* MRC

4003 was also confirmed by HPLC analysis of the *o*-phthalaldehyde-derivatized hydrolysate.

The recently described species *F. nygamai* (2) is related to but excluded from the section *Liseola* by the production of chlamydo-spores but has not yet been assigned to a section. *F. nygamai* MRC 4003 was isolated from soil in the Kruger National Park, South Africa, and is one of the authentic isolates cited (as M-2368) in the original description (2). Cultures of *F. nygamai* MRC 4003 on corn produced 605  $\mu\text{g g}^{-1}$  of  $FB_1$  and 530  $\mu\text{g g}^{-1}$  of  $FB_2$  (Table 1). This is the first report of fumonisin production by *F. nygamai*.

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## CHAPTER III

### TOXICOLOGICAL EFFECTS AND CARCINOGENESIS STUDIES OF FUMONISIN B<sub>1</sub>

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## TOXICOLOGICAL EFFECTS AND CARCINOGENESIS STUDIES OF FUMONISIN B<sub>1</sub>

### 3.1 *Induction of leukoencephalomalacia (LEM)*

A major toxic syndrome associated with culture material of *F. verticillioides* is the induction of equine LEM that was first induced by naturally fungal contaminated maize in 1902 (Butler, 1902). The actual involvement of *F. verticillioides* was only

verified during the 1970's by Wilson & Maronpot (1971) and Marasas *et al.* (1976) with pure cultures of the fungus in maize. The first evidence that FB<sub>1</sub> is the causative principle for LEM resulted from a study following the intravenous application of 0.125 mg FB<sub>1</sub>/kg bw/day for 7 days (Marasas *et al.*, 1988). The principal lesions were severe oedema of the brain and early bilaterally symmetrical, focal necrosis of the medulla oblongata. In a subsequent experiment the characteristic lesion of liquefactive necrosis of the white matter in the brain was induced in two horses. The dosages utilized were 1,25 to 4 mg/kg in one horse and 1 to 4 mg/kg in the other horse yielding an effective total dose of 29.7 and 42.1 mg FB<sub>1</sub>, respectively. Chemical pathological changes associated with liver function were carefully monitored as high doses could result in hepatosis without inducing the clinical signs of LEM. The apparent dose ranges compared well to the levels ranging between 0.6 to 2.1 mg FB<sub>1</sub>/kg bw/day associated with a natural outbreak of LEM in horses fed maize screenings (Wilson *et al.*, 1990). It was suggested that maize contaminated up to a level of 10 mg FB per kg could pose an increased risk for horses to develop LEM (Viljoen and Marasas, 2002). This level is slightly lower than that proposed by the Joint Evaluating Committee on Food Additives (JECFA) which defines a minimum dose between 15-22 mg FB/kg of the diet (WHO, 2001).

### 3.2 *Carcinogenesis studies with purified fumonisin B<sub>1</sub>*

#### 3.2.1 Long-term studies in male BD IX rats

Following the characterisation of the fumonisin B mycotoxins from culture material of *F. verticillioides* MRC 826 a long-term toxicological study was

conducted in male BD IX rats at a dietary level of 50 mg FB<sub>1</sub>/kg (Gelderblom *et al.*, 1991). The experimental diet used was very similar to that used by Jaskiewicz *et al.* (1987). The kinetics of the toxic and/or carcinogenic changes was followed during the course of the experiment by killing subgroups of rats at 6, 12 and 16 months with the remaining rats terminated after 16 to 26 months depending on their health status. The control diet contained low levels of FB<sub>1</sub> (0.5 mg/kg) that occurs naturally in the maize while no trace of aflatoxin was detected. Changes associated with the development of cirrhosis and cholangiofibrosis (adenofibrosis) were already present after 6 months, while at 12 months the livers of the rats were distorted with a nodular appearance, described as a chronic toxic hepatitis progressing to cirrhosis. From 18 months onwards some of the hepatocyte nodules manifested dysplasia and nuclear atypia characteristic of preneoplastic changes, a few of which were transformed into hepatocellular carcinoma. Of the 15 rats that were killed between 18 and 26 months, 10 developed hepatocellular carcinoma of which two metastasised to the lung and heart and one to the kidneys. Another prominent lesion that was consistently present from 6 months onwards was cholangiofibrosis, which was considered in some rats to develop into cholangiocarcinoma towards the end of the experiment. The culture material of strain MRC 826, used in the two previous experiments, also induced both types of hepatic lesions. Two lesions that were induced by the culture material, i.e. mild basal cell hyperplasia in the oesophagus and cardiac changes were not reproduced by FB<sub>1</sub>. Histopathological changes in the kidney were not commonly observed, although FB<sub>1</sub> tends to cause some chronic lesions towards the end of the experiment. No specific lesions were induced in the kidneys of the BD IX rats treated with the culture material of strain MRC 826.

In order to study dose-response effects with respect to FB<sub>1</sub>-induced hepatocarcinogenesis in BD IX rats, a follow-up study using low dietary levels of fumonisin B<sub>1</sub> (1, 10 and 25 mg FB<sub>1</sub>/kg diet) was performed using the same diet (Gelderblom *et al.*, 1996a). Only mild toxic effects were observed in the liver, and apart from the induction of hepatocyte foci and nodules staining

positively for the placental form of glutathione-S-transferase (PGST<sup>+</sup>) up to 10 mg FB<sub>1</sub>/kg diet, no evidence of hepatocellular carcinoma was noticed.

Several observations regarding the long-term studies using either the maize culture material or purified FB<sub>1</sub> have to be considered:

- FB<sub>1</sub> is responsible for the hepatocarcinogenic effects of *F. verticillioides* and the liver is the major target organ in male BD IX rats.
- FB<sub>1</sub>-induced hepatocarcinogenesis was associated with a chronic toxic hepatitis, eventually leading to cirrhosis.
- All the hepatotoxic effects induced by the culture material of strain MRC 826 in rats were reproduced by FB<sub>1</sub>.

### 3.2.2 Long-term studies in male Fischer rats

More recently, a study in male Fischer rats showed that FB<sub>1</sub> causes dysplastic nodules and bile duct lesions in the liver (Lemmer *et al.*, 2004). Male Fisher rats were fed FB<sub>1</sub> at different dosage levels over a period of 5 months using the AIN 76 diet. The dosage levels used were 250 mg FB<sub>1</sub>/kg diet for 5 weeks followed by 100 mg FB<sub>1</sub>/kg diet for the remainder of the experiment. The FB<sub>1</sub> dietary treatment was discontinued after 5 months at which time liver biopsies were collected from a subpopulation of the rats. The liver biopsies were continued on individual rats for a further 6 months and the rats terminated after 12 months. In the rats terminated after 12 months, hepatic lesions were described and shown to be very similar to those reported in male BD IX rats receiving either culture material (Marasas *et al.*, 1984) and/or pure FB<sub>1</sub> (Gelderblom *et al.*, 1991) and in male Fisher rats receiving naturally contaminated maize (Wilson *et al.*, 1985). Therefore, irrespective of the diets used, very similar hepatotoxic effects were induced in the liver of male BD IX and Fisher 344 rats. FB<sub>1</sub> induced a variety of preneoplastic and neoplastic hepatic lesions, including GSTP positive lesions, dysplastic liver nodules, cholangiofibrotic lesions, intraductal cholangiocarcinomas, and one unequivocal trabecular/-pseudoglandular hepatocellular carcinoma (Lemmer *et al.*, 2004). Proliferating cells with the morphology of oval cells were found inside/adjacent to PGST<sup>+</sup> lesions, dysplastic nodules, and cholangiofibrotic lesions, suggesting that oval cells may be involved in FB<sub>1</sub>-induced hepato-



and cholangiocarcinogenesis in the liver. Furthermore, immunohistochemical analysis demonstrated that proliferating oval cells and bile ductules, hepatic nodules, cholangiofibrotic lesions, and cystic lesions expressed the OV-6 antigen, indicating that all of these cells may have a common cell of origin. These provocative findings suggested that oval cells (putative liver progenitor cells) may be involved in the generation of the dysplastic nodules and hepatocellular carcinomas seen in FB<sub>1</sub>-fed male Fischer rats. It is conceivable that both the hepatocellular and bile duct tumours induced by FB<sub>1</sub> are derived from a common ('stem') cell of origin. The short-term dietary exposure (250 mg FB<sub>1</sub>/kg for 5 weeks) also developed adenomas and cholangiofibrosis in the liver after one year indicating the irreversible nature of these neoplastic lesions.

### *3.3 Dietary modulation and synergistic interactions of FB<sub>1</sub>-induced hepato-carcinogenesis*

#### 3.3.1 Role of different dietary constituents

A critical evaluation of the different diets used in studies, investigating the carcinogenic properties of maize culture material of *F. verticillioides* and FB<sub>1</sub>, has recently been published (Gelderblom *et al.*, 2004). Studies using naturally contaminated maize, maize cultures of the fungus and purified FB<sub>1</sub> in male Fischer 344 or male BD IX rats yield remarkably similar lesions in the liver with specific dose response effects. When utilizing a maize only diet (Wilson *et al.*, 1985) and the semi purified diet (Marasas *et al.*, 1984; Jaskiewicz *et al.*, 1987), the choline and methione content seemed to be adequate considering the minimum requirements for rats (Keenan *et al.*, 2000). In the corn-only diet, vitamins B12 and D were absent while vitamin E and folate were very low. The latter seems not to play a role as intestinal bacteria serve as a source although the disruption of the folate receptor by FB<sub>1</sub> is likely to alter folate metabolism in the cell (Stevens *et al.*, 1997). The low vitamin E levels in the corn-only diet could also be important to increase the susceptibility of FB<sub>1</sub>-induced hepatotoxic effects. Low calcium and phosphate present as phytate, result in a low calcium-to-phosphorus molar ratio (<1:3) that could lead to nephrocalcinosis that was observed when rats were fed a high dosage of FB<sub>1</sub>

(Gelderblom *et al.*, 2001). Another important dietary constituent is the protein content with the corn (9.4%) and semi-purified (10.2%) diets falling well within the minimal requirements for rats. A high protein diet was reported to effect nephropathy as indicated by the NIH 31 diet used in the long-term FB<sub>1</sub> toxicology study in male Fischer rats that contained approximately twice the required protein content with 80 to 90 % of rats developed the syndrome (Howard *et al.*, 2001). It is not known at present whether the high protein diet played a role in the development of kidney carcinoma induced by FB<sub>1</sub> in the long-term study. This aspect will be further debated in section 3.4.

### 3.3.2 Role of dietary iron

Dietary iron overload has been identified as a risk factor for the development of hepatocellular carcinoma although the underlying mechanisms are poorly understood (Mandishona *et al.*, 1998). Several commonalities exist in the hepatotoxic effects induced by iron and the fumonisins, suggesting a possible synergistic interaction regarding the development of liver cancer. Both stimulated the induction of membrane lipid peroxidation resulting in oxidative cellular damage including DNA leading to the development of liver tumors, either directly or indirectly. However, induction of liver cancer was associated with the development of chronic persistent cirrhosis with FB<sub>1</sub> while it was absent in the iron model (Asare *et al.*, 2006). Excessive iron overload (1 to 2% of carbolylic iron) significantly potentiated lipid peroxidation induced by FB<sub>1</sub> in the liver over a period of 5 weeks, which was not associated with an increased hepatotoxic effect (Lemmer *et al.*, 1999). FB<sub>1</sub>, however, shifted the iron distribution from the hepatocytes to the Kupffer cells, presumably due to phagocytosis of the necrotic iron-loaded hepatocytes. Dietary iron significantly reduced the number and size of hepatocyte PGST<sup>+</sup> foci and nodules, which was related to the augmentation of the hepatic regenerative response to the FB<sub>1</sub>-induced hepatotoxic effect. The decreased relative liver weight induced by FB<sub>1</sub> was normalised by iron, suggesting that the associated inhibition of hepatocyte proliferation was countered. In this regard the mito-stimulatory effect of iron in the liver has been reported (Stal *et al.*, 1995) and therefore could counter the FB<sub>1</sub>-induced selection process related to cancer promotion.

### 3.3.3 Role of tea antioxidants

The possible role of lipid peroxidation in the hepatocarcinogenic effects of FB<sub>1</sub> initiated a study on the possible role of antioxidants on cancer promotion in rat liver utilizing diethylnitrosamine (DEN) as cancer initiator (Marnewick *et al.*, 2009). Different herbal infusions, unfermented and fermented rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*) herbal teas, and green and black teas (*Camellia sinensis*) were investigated. Unfermented rooibos and honeybush enhanced the hepatotoxic effects of FB<sub>1</sub> when considering certain clinical chemical parameters associated with liver and kidney damage, suggesting specific FB<sub>1</sub>/iron/polyphenolic interactions related to pro-oxidant effects. Green tea significantly ( $P < 0.05$ ) enhanced the FB<sub>1</sub>-induced reduction of the oxygen radical absorbance capacity, while fermented herbal teas and unfermented honeybush tea significantly ( $P < 0.05$ ) decreased FB<sub>1</sub>-induced lipid peroxidation in the liver. The latter was ascribed to the known reduced antioxidant potencies of these teas in different *in vitro* antioxidant assays. The teas exhibited varying effects on FB<sub>1</sub>-induced changes in the activities of catalase, glutathione peroxidase (GPx) glutathione reductase (GR) as well as the glutathione (GSH) status. All the teas reduced the relative amount of the larger foci suggesting an interference with the FB<sub>1</sub>-selection process of initiated cells. Unfermented rooibos and honeybush teas significantly ( $P < 0.05$ ) to marginally ( $P < 0.1$ ) reduced the total number of foci ( $> 10\mu\text{m}$ ), respectively, suggesting an increased removal of initiated cells presumably due to apoptosis. Fermentation appeared to reduce the protective effect of the herbal teas. Differences in the major polyphenolic components and certain FB<sub>1</sub>/polyphenolic/tissue interactions may explain the varying effects of the different teas on the oxidative parameters, hepatotoxic effects and cancer promotion by FB<sub>1</sub>.

### 3.3.4 Role of aflatoxin B<sub>1</sub>

AFB<sub>1</sub> is an important carcinogenic mycotoxin reported to co-occur with FB<sub>1</sub> on dietary staples of humans (Ono *et al.*, 2001). Both AFB<sub>1</sub> and FB<sub>1</sub> was shown to be weak cancer initiators when utilising the resistant hepatocyte liver model, which was related to their inhibitory effect on cell proliferation and the induction of apoptosis likely to remove initiated cells from the liver

(Gelderblom et al, 2002). A sequential model was therefore selected, as the simultaneous application of the two carcinogens could have countered the cancer initiating potency in the liver as described above. The separate treatment of rats with the mycotoxins only resulted in mild hepatotoxic effects while the sequential treatment resulted in cirrhotic livers with numerous PGST<sup>+</sup> regenerative and dysplastic nodules, indicating a strong synergistic interaction. The sustained toxic effects of AFB<sub>1</sub> rendered the liver more susceptible to the initiating potency of FB<sub>1</sub>, similar to other hepatotoxicants such as CCl<sub>4</sub> known to effect regenerative cell proliferation (Gelderblom *et al.*, 2001). The major effect of FB<sub>1</sub> in the sequential model, however, could be related to the cancer promoting properties which exceeded the properties of the 2-AAF/PH promoting stimulus. A more significant interaction was noticed between AFB<sub>1</sub> vs FB<sub>1</sub> as compared to FB<sub>1</sub> vs 2-AAF, when considering the size of the preneoplastic lesions, which were ascribed to the promotion of the different subsets of initiated hepatocytes by FB<sub>1</sub>. As an apparent threshold for cancer initiation and promotion by FB<sub>1</sub> exists, the synergistic interaction of the two mycotoxins may alter the potential to develop early preneoplastic lesions and hence their role in the development of certain phases in cancer development.

### 3.4 Perspectives

FB<sub>1</sub> was shown to be responsible for most of the toxicological effects of *F. verticillioides* culture material in experimental and domestic animals. Two studies in horses showed the causative role of FB<sub>1</sub> in the development of LEM while intravenous dosing in pigs provided evidence for the involvement of pulmonary edema (Harrison *et al.*, 1990). A chronic hepatotoxic effect in rats was associated with the development of two types of carcinomas, i.e. hepatocellular and cholangiocarcinoma in male BD IX rats when fed over a period of two years. Subchronic levels significantly reduced the induction of hepatocellular and cholangiocarcinoma similar to that obtained with the fungal culture material. A discontinued feeding study in male Fischer 344 rats showed that a short-term exposure of 250 mg FB<sub>1</sub>/kg for five weeks and a subsequent 100 mg FB<sub>1</sub>/kg diet for 5 months resulted in the induction of hepatoadenomas and cholangiofibromas in these rats after a period on 1

year. Rats treated only with the five-week FB<sub>1</sub> dietary regimen also developed similar lesions, providing evidence of the irreversible nature of the neoplastic lesion initiated in the liver. In one rat this lesion developed into a hepatocellular carcinoma. The incidence of cholangiofibromas was increased when FB<sub>1</sub> was used in combination with a cancer promoting 2-AAF dietary treatment (Lemmer *et al.*, 2004). A possible role of oval cells in the development of adenomatous lesions has been suggested via the activation of a facultative stem cell compartment that could be the source of both the hepatocellular and cholangiocellular tumors induced by FB<sub>1</sub>. Both types of tumours were also induced in male Fischer rats by a naturally infected maize sample associated with an outbreak of LEM containing an apparent level of 50 mg FB/kg, indicating that the fumonisins are responsible for the hepatotoxicity in both rat species. The lack of hepatotoxic and carcinogenic effects of a chronic exposure of male Fischer rats to FB<sub>1</sub> up to a level of 150mg/kg diet is somewhat surprising. Instead chronic kidney toxicity was reported with the development of kidney tumours in the mid and high dose groups. Of interest was the development of rare anaplastic variants in 50% and 80% of the animals that developed tumors (Howard *et al.*, 2001; Hard *et al.*, 2001).

The nephrocarcinogenicity properties of FB<sub>1</sub> could be related to specific dietary interactions such as a high protein diet selectively sensitising the kidneys due the chronic progressive nephropathy (CPN). Recently it was reported that many renal tubule proliferative lesions developed in kidneys of especially male Fischer and Sprague-Dawley rats when chronically fed a high protein diet culminating in advanced CPN in older rats (Hard *et al.*, 2005). One of these proliferative lesions includes atypical tubule hyperplasia (ATH) that develops into adenomas and is therefore regarded as a precursor lesion for kidney carcinogenesis. When considering the toxic and cancer promoting properties of the fumonisins, specific FB<sub>1</sub>/high protein diet interactions are likely to prevail whereby ATH could be promoted into adenomas and carcinomas. The induction of ATH in rats fed a high protein diet is also age related which is of interest as no neoplastic lesions were noticed in the kidneys up to six months in any of the FB<sub>1</sub>-treated rats, while hyperplastic tubule, ATH, adenomas and carcinomas were noticed after two years (NTP,

2001). This would suggest that renal adenomas and carcinomas develop in the presence of advanced CPN, which complicate attempts to mechanistically define the role of FB<sub>1</sub>. The presence of tubule hyperplasia, ATH and adenomas was also noticed in the females fed the two highest dose levels, although to a far lower extent (NTP, 2001; Hard *et al.*, 2001). This is in agreement with the notion that male rats are more sensitive to CPN, which coincided with the higher tumor response when compared to females. However, it should be noted that lower FB<sub>1</sub> dose levels were used in the females (Howard *et al.*, 2001). The marginal dose response effect regarding the induction of renal tumors by FB<sub>1</sub> further suggests a possible interaction with advanced CPN.

The interactions between CPN and carcinogens are complex and should be treated with caution in order to exclude any confounding effect in relation to FB<sub>1</sub>-induced nephrocarcinogenesis. The use of male Fischer rats receiving a high protein diet in chronic nephrotoxicity and nephrocarcinogenicity models was questioned due to the formation of ATH and adenomas (Hard *et al.*, 2004). The NTP study, therefore, is likely to provide additional information on FB/dietary interactions and further elaborate on the cancer promoting properties of the fumonisins utilising different experimental protocols. This must be considered against the background that most of the long-term rat studies conducted thusfar provide evidence that the fumonisins are liver carcinogens (Sections 1.1 and 3.2). Of interest is that the NIH 31 diet (20.1% protein) was recently replaced by the NIH 2000 diet containing 14.5% protein to be used in long term toxicity testing (Rao *et al.*, 1996). A similar argument could also be used when considering the hepatocarcinogenicity of FB<sub>1</sub> in B6C3F<sub>1</sub> female mice (Howard *et al.*, 2001a). These mice are known to spontaneously develop adenoma and carcinomas over a period of two years (Haseman *et al.*, 1994) and the dose dependent increase in the number of neoplastic lesions can be ascribed to the cancer promoting properties of FB<sub>1</sub>.

The liver cancer model utilising male BD IX rats, therefore, provides a far better model to be utilised in determining risk assessment parameters, as more information is available regarding the underlying mechanisms of cancer

induction. In a long-term study in BD IX rats, early neoplastic lesions were already present after 6 months (Gelderblom *et al.*, 1991) while the cancer initiating and promoting properties have been well defined (See details in Chapter IV) which provide, beyond any doubt, that the fumonisins are hepatocarcinogenic. Except for the myocardial endocardial fibrosis and oesophageal basal cell hyperplasia obtained, all the lesions induced by the fungal culture material of *F. verticillioides* in male BD IX rats were reproduced by FB<sub>1</sub>. The association of cardiac lesions with the consumption of culture material of *F. verticillioides* is, however, still unresolved but could be related to the high dose of the culture material that was used. The original study by Kriek *et al.* (1981a) with culture material of *F. verticillioides* reported on acute and proliferative endocardial lesions whereas culture material of a moniliformin-producing strain *F. subglutinans* caused myocardial degeneration and fibrosis (Kriek *et al.*, 1977). These differences were confirmed by Marasas *et al.* (1984) who reported endothelial hyperplasia of the ventricular endocardium in male BD IX rats fed culture material of *F. verticillioides* MRC 826 that did not produce moniliformin (Kriek *et al.*, 1981b). In contrast Jaskiewicz *et al.* (1987) reported a high incidence of myocardial disseminated fibrosis in male BD IX rats using the same culture material of MRC 826 while the incidence of endocardial fibrosis was far lower. Myocardial fibrosis was associated with moniliformin whilst endocardial damage was linked to an unknown compound produced by *F. verticillioides*. The only difference between the two studies was the use of freeze-dried material instead of oven-dried culture material used by Marasas *et al.* (1984).

Although the culture material of MRC 826 is known to affect different organs in different animal species (Kriek *et al.* 1981b), the kidney was not an important target organ in male BD IX rats fed culture material of *F. verticillioides* MRC 826 (Kriek *et al.*, 1981a, b; Marasas *et al.*, 1984; Jaskiewicz *et al.*, 1987). The long-term feeding study using pure FB<sub>1</sub> up to a level of 1.6 FB<sub>1</sub>/kg/day showed no carcinogenic effects in the kidney of male BD IX rats (Gelderblom *et al.*, 1991). The protein level in the semi-purified diet used in all these studies was 10.2% (Gelderblom *et al.*, 2004). Dose response effects were noticed in the liver during a chronic feeding study using low dietary levels (1,

10 and 25 mg FB<sub>1</sub>/kg) representing an intake of 0.03, 0.3 and 0.8 mg FB<sub>1</sub>/kg/day over a period of 2 years. Lesions in rats receiving the highest dose (0.8 mg FB<sub>1</sub>/kg/day) were typical of those described previously in rats receiving an apparent FB<sub>1</sub> dose of 1.6 ug/kg bw/day while only mild changes were noticed at an intake of 0.3 mg FB<sub>1</sub>/kg bw/day (Gelderblom *et al.*, 2001). Typical ground glass foci were induced at the lowest dose of 0.03 mg FB<sub>1</sub>/kg bw/day indicating that cancer initiation was also effected at this dose level. In the kidneys typical toxic lesions were noticed in the tubular epithelium, mainly the proximal convoluted tubules at an intake of 0.3 mg FB<sub>1</sub>/kg bw/day.

In a study with purified FB<sub>1</sub>, fed to male Sprague Dawley rats no hepatotoxic changes were observed in the liver of the rats fed a 50 mg FB<sub>1</sub>/kg diet representing an estimated intake of 4.7 mg FB<sub>1</sub>/kg bw/day over a period of 4 weeks (Voss *et al.*, 1993, 1995a). Lesions characteristic of those induced by fungal culture material were obtained in the liver at a higher dosage (13.6 mg FB<sub>1</sub>/kg bw/day) diet. In the male rats, shown to be more sensitive than female, changes were observed at a dose of 14 mg FB<sub>1</sub>/kg bw/day. Studies in male Fisher rats at different locations using different diets indicated that very similar toxicological effects were induced in the liver either by incorporating FB<sub>1</sub> in the diet or by gavage dosing (Gelderblom *et al.*, 1994, 1996, 2001a). Mild changes were observed in the liver at 3.5 mg FB<sub>1</sub>/kg bw/day dietary level for 21 days, which is in agreement with the long-term study in BD IX rats (Gelderblom *et al.*, 1996b; 2001b). In contrast, no hepatotoxic effects were reported in the liver at a dietary intake of up to 5.7 mg FB<sub>1</sub>/kg bw/day over 90 days while kidney lesions were noticed at an intake of 0.62 mg FB<sub>1</sub>/kg bw/day with a NOEL of 0.21 mg FB<sub>1</sub>/kg bw/day (Voss *et al.*, 1995b). The latter study is in accordance with the findings of the NTP study where male Fischer rats developed renal adenoma and carcinoma while no toxic lesions were obtained in liver at an exposure level of 6.6 mg FB<sub>1</sub>/kg bw/day over a period of two years (Howard *et al.*, 2001). A NOEL for kidney tumors in male Fischer rats was defined to be between an intake of 0.7 and 2.2 mg FB<sub>1</sub>/kg bw/day. A NOEL for nephrotoxicity was set at 0.2 mg FB<sub>1</sub>/kg bw/day which was obtained during the 90 day and two year chronic feeding NTP studies (Vos *et al.*, 1995; Howard *et al.*, 2001). Based on the notion that nephrotoxicity is a prerequisite



for renal carcinogenesis the intake level was considered by Joint FAO/WHO Expert Committee on Food Additives (JECFA) to obtain the Provisional Tolerable Daily Intake (PMTDI) for fumonisins in humans. However, a dose of 0.7 mg FB<sub>1</sub>/kg bw/day for 2 years induced kidney lesions without the induction of ATH, a precursor lesion of the renal tumors which raised some doubts about the cell death/compensatory regenerative model for FB<sub>1</sub>-induced nephrocarcinogenesis proposed by Dragan *et al.*, (2001). This is further complicated by the induction of proliferative lesion in the kidney due to the development of CPN.

The possible role of different dietary constituents on the FB<sub>1</sub>-induced toxicological effects has been addressed recently (Gelderblom *et al.*, 2004). However, very few studies regarding the role of diet and FB<sub>1</sub> carcinogenicity have been conducted. The interaction between FB<sub>1</sub> and dietary iron has recently been addressed indicating that a protective effect was obtained against cancer promotion, presumably due to the mitogenic properties of iron. In a subsequent long-term study the role of low levels of dietary iron was re-evaluated in a discontinued FB<sub>1</sub> feeding study (Werner, 2002). A dual role of iron on FB<sub>1</sub>-induced hepatocarcinogenesis was proposed in that (i) iron overload enhanced the induction of hepatocyte nodules in the presence of FB<sub>1</sub> while (ii) after removal of FB<sub>1</sub>, the continued iron supplementation impaired the progression of the hepatic nodules. The exact mechanism involved is not known but the differential modulation of FB<sub>1</sub> and iron on cell proliferation and oxidative damage could be of importance in altering the growth of preneoplastic lesions. The interaction between antioxidants and FB<sub>1</sub>-induced hepato- and nephrotoxicity has been investigated by monitoring the protective effect of royal jelly, obtained from worker honey bees, in Sprague-Dawley rats (El-Nekeety *et al.*, 2007). Co-treatment of the rat with the royal jelly significantly reduced the toxic effects of FB<sub>1</sub> in the liver and kidneys in a dose-dependent manner. The protection was associated with a significant increase in the FB<sub>1</sub>-induced reduction in glutathione peroxidase and superoxide dismutase while it also counteracted the increase in lipid peroxidation, presumably related to the free oxygen radical scavenging and antioxidant properties of the royal jelly. Garlic and cabbage extracts have

been shown to protect against FB-induced developmental toxicity in Sprague-Dawley rats (Abdel-Wahhab *et al.*, 2004). The extracts significantly reduced the number of skeletal malformations, presumably due to the presence of diallyl sulphide known to protect against oxidative damage (Horie *et al.*, 1989). These studies provide further evidence that different dietary constituents may interfere with fumonisin-induced toxic effects which could be used in developing chemopreventative initiatives.

The synergistic interactions between FB<sub>1</sub> and other toxins and/or different dietary components, although complex, are of importance when considering the toxic and carcinogenic properties. The combined hepatotoxic effects of AFB<sub>1</sub> and FB<sub>1</sub> in growing barrows also exceed that of the individual mycotoxins (Harvey *et al.*, 1995). A similar finding was recorded using growing barrows regarding the combined effects of FB<sub>1</sub> and deoxynivalenol while the hematological and immunological parameters showed increased responses as a result of the combined treatment (Harvey *et al.*, 1996). Co-administration of FB<sub>1</sub> increased the mortality caused by AFB<sub>1</sub> in rats in an additive manner (Mckean *et al.*, 2006). In New Zealand white rabbits co-administration of AFB<sub>1</sub> and FB<sub>1</sub> showed synergistic liver and kidney toxicological effects when considering the clinical chemical data and histology analyses (Orsi *et al.*, 2007). FB<sub>1</sub> also significantly enhanced the development of tumours in AFB<sub>1</sub> treated rainbow trout (Carlson *et al.*, 2001). Several studies utilising different cell culture systems reported on the synergistic and/or additive effects of FB<sub>1</sub> in combination with different toxins. A synergistic cytotoxic effect was noticed between FB<sub>1</sub> and ochratoxin A when monitoring cytotoxicity in rat brain glioma cells, human intestinal Caco cells and green monkey kidney Vero cells (Creppy *et al.*, 2004). Additive effects regarding the increase in TBARS and decrease in GSH levels by FB<sub>1</sub>, ochratoxin A, and beauvericin were reported in porcine kidney epithelial cells (Klarić *et al.*, 2006). However, depending on the time of exposure and dose utilised synergistic and/or antagonistic effects were obtained. In human lymphoblastoid cells (Jurkat T) FB<sub>1</sub> increased cell proliferation while it enhanced the inhibitory effects of alpha-zearalenol (Luongo *et al.*, 2006). In hepatoma cells (HepG2), FB<sub>1</sub> exhibited an antagonistic effect against AFB<sub>1</sub>-

induced cytotoxic effects while an additive effect was noticed in human bronchial epithelial cells (Mckean *et al.*, 2006). These interactive models should to impact on setting realistic risk assessment parameters, not only for the fumonisins, but also other naturally occurring mycotoxins.

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## Toxicity and carcinogenicity of the *Fusarium moniliforme* metabolite, fumonisin B<sub>1</sub>, in rats

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A semi-purified corn-based diet containing 50 mg/kg of pure (not < 90%) fumonisin B<sub>1</sub> (FB<sub>1</sub>), isolated from culture material of *Fusarium moniliforme* strain MRC 826, was fed to a group of 25 rats over a period of 26 months. A control group of 25 rats received the same diet without FB<sub>1</sub>. Five rats from each group were killed at 6, 12, 20 and 26 months. The liver was the main target organ in the FB<sub>1</sub>-treated rats and the hepatic pathological changes were identical to those previously reported in rats fed culture material of *F.moniliforme* MRC 826. All FB<sub>1</sub>-treated rats that died or were killed from 18 months onwards suffered from a micro- and macronodular cirrhosis and had large expansile nodules of cholangiofibrosis at the hilus of the liver. Ten out of 15 FB<sub>1</sub>-treated rats (66%) that were killed and/or died between 18 and 26 months developed primary hepatocellular carcinoma. Metastases to the heart, lungs or kidneys were present in four of the rats with hepatocellular carcinoma. No neoplastic changes were observed in any of the control rats. Chronic interstitial nephritis was present in the kidneys of FB<sub>1</sub>-treated rats killed after 26 months. No lesions were observed in the esophagus, heart or forestomach of FB<sub>1</sub>-treated rats and this is contrary to previous findings when culture material of the fungus was fed to rats. It is concluded that FB<sub>1</sub> is responsible for the hepatocarcinogenic and the hepatotoxic but not all the other toxic effects of culture material of *F.moniliforme* MRC 826 in rats.

### Introduction

The carcinogenic and toxicological effects of *Fusarium moniliforme* Sheldon in animals have received world-wide attention as the fungus is a common contaminant of corn, a major dietary staple of humans and animals (1-7). Attempts to identify the toxins and carcinogens produced by *F.moniliforme* resulted in the characterization of moniliformin (8) fusariocin (9) and fusarin C (10). These toxins are, however, not responsible for the toxic and carcinogenic action of *F.moniliforme* in animals. Recent studies on the cancer promoting activity in rat liver exhibited by different strains of *F.moniliforme* (11) resulted in the isolation (12) and characterization (13) of the liver cancer promoters, the fumonisin B (FB\*) mycotoxins. Subsequent studies have indicated that FB<sub>1</sub>, the major fumonisin produced in culture (12), causes leukoencephalomalacia (LEM) in horses and is hepatotoxic in rats and horses (12,14,15).

Short-term feeding studies with FB<sub>1</sub> in rats showed that, apart from the hepatotoxic effects, gamma-glutamyltranspeptidase

positive (GGT<sup>+</sup>) hyperplastic foci are induced in the absence of any other known carcinogen in the diet (12,16) indicating that it also acts as a cancer initiator. It was concluded that since FB<sub>1</sub> can affect both the cancer initiating and promotional phases in liver cancer development, it is probably responsible for the hepatocarcinogenic effect of *F.moniliforme* culture material in rats (5,6). At present the only information available on the carcinogenic activity of FB<sub>1</sub> is indirect and based on short-term studies with FB<sub>1</sub> (12,16) and on long-term feeding trials using culture material of *F.moniliforme* strain MRC 826 (5,6). This specific strain has been shown to produce high levels of FB<sub>1</sub> (17). In addition, a sample of corn infected with *F.moniliforme* and implicated in a natural outbreak of LEM in horses in the USA, induced precancerous hepatic lesions during a long-term feeding trial in rats (18). This finding, together with the fact that the fumonisins occur naturally in corn as evidenced by the field outbreaks of LEM, and the detection of FB<sub>1</sub> in corn samples associated with LEM (19,20) as well as in home-grown corn intended for human consumption (21,22), emphasize the importance of obtaining more information about the toxic and carcinogenic potential of the fumonisins.

In this study the toxicological effects of FB<sub>1</sub> in rats, were evaluated.

### Materials and methods

#### Chemicals

FB<sub>1</sub> was purified from culture material of *F.moniliforme* MRC 826 as described previously (12). The purity of the mycotoxin (not <90%) was verified by HPLC according to the method of Alberts *et al.* (17) using a pure analytical standard. The stock sample of FB<sub>1</sub> was kept as a dry powder at 4°C until used.

#### Animals

Fifty inbred male BD IX rats initially weighing 70-80 g were caged individually in a controlled environment at 23°C and 50% humidity with a 12 h artificial light cycle. The rats were randomly divided into two groups of 25 rats each. The FB<sub>1</sub>-treated and control groups were housed in separate stands. Food and water were available *ad libitum*. Rats were observed daily for the presence of clinical signs and weighed weekly.

#### Treatments and diets

All the rats received a modified (5) cereal-based diet (23). The diet consisted of 75% sifted white corn meal while minimal levels of protein, analytical grade minerals, and vitamins were added as a supplement to ensure adequate growth and maintenance of the rats. The diet was fed in a milled form and analyzed regularly for the presence of the carcinogen aflatoxin B<sub>1</sub> (24) as well as for FB<sub>1</sub> (17).

The FB<sub>1</sub>-containing diet (50 mg FB<sub>1</sub>/kg diet) was prepared as follows: FB<sub>1</sub> (300 mg) stock sample dissolved in methanol (50 ml) was evaporated onto a subsample (200 g) of the diet whereafter it was dried in a fume hood at room temperature for 12 h. Subsequently the subsample was thoroughly mixed into the diet (6 kg) to obtain the desired concentration of FB<sub>1</sub>. The control diet was treated in a similar way using only an equal volume of methanol. Each diet was prepared in 6 kg quantities at a time and stored at 4°C until used.

#### Experimental

Five rats from each group were killed at 6, 12 and 20 months respectively, to assess the progression of lesions. Where rats in the FB<sub>1</sub>-treated group died during the course of the experiment, an equal number of rats from the control group were killed. The remainder of the surviving rats (five of each group) were killed at the termination of the experiment after 26 months. In all cases rats were anesthetized by the i.p. administration of a sodium pentobarbitone solution (6% m/v). Blood was collected from the abdominal aorta for clinical chemical analyses

\*Abbreviations: FB<sub>1</sub>, fumonisin B<sub>1</sub>; LEM, leukoencephalomalacia; GGT<sup>+</sup>, gamma-glutamyltranspeptidase positive; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

and the organs were screened macroscopically for any abnormalities. The liver of each rat was weighed whereafter specimens were collected. Freshly cut slices of the liver were preserved in acetone at 4°C for GGT histochemical analysis (25). The remainder of the liver tissue and specimens of all the other organs were collected and preserved in 10% buffered formalin for routine histopathological examination. Serum samples, obtained from the blood from each rat killed at 20 and 26 months respectively, were analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), GGT, bilirubin, total protein, globulin, albumin, cholesterol, urea and creatinin by Technicon autoanalyses.

## Results

Control and experimental rats became obese during the final stages of the experiment (20 months and onwards). The weight gain of the control group was significantly ( $P < 0.01$ ) more than that of the FB<sub>1</sub>-group from 12 months onward (Table I). Apart from the rats that were killed intentionally, five rats of the FB<sub>1</sub>-treated group died, mainly due to pneumonia, between 18 and 24 months [at 18, 19, 23 (two rats) and 24 months respectively]. The survival rate of the rats receiving the control diet was 96%. The FB<sub>1</sub> content of the control diet was in the order of 0.5 mg FB<sub>1</sub>/kg while no aflatoxin B<sub>1</sub> could be detected.

Pathological changes occurred consistently in the liver of the FB<sub>1</sub>-treated rats (Table I). The terminology used in this paper for a number of lesions induced by FB<sub>1</sub> in the liver may differ from that used in previous papers on the toxicological effects of cultures of *F.moniliforme* MRC 826 in rats (5,6). Bile duct hyperplasia (5) is used synonymously with bile duct proliferation (6), while cholangiofibrosis which is a synonym of adenofibrosis (5,6,26), has become preferable. Most of the nodules in the cirrhotic livers in this study were the result of regeneration and are thus referred to as regenerative nodules (26), and not as hyperplastic or neoplastic nodules (5,6,12,26). These regenerative nodules are thus also representative of the process referred to as nodular regenerative hyperplasia or regenerative nodular hyperplasia in the medical literature. The other terminology used is consistent with that of Stewart *et al.* (26).

All the rats in the FB<sub>1</sub>-treated group that died or were killed from 18 months onward suffered from a micro- and macronodular cirrhosis and had large expansile nodules of cholangiofibrosis at the hilus of the liver. The changes terminating in cirrhosis and cholangiofibrosis were already present in the liver of rats killed 6 months after the initiation of the experiment and included scattered areas of fibrosis, bile duct hyperplasia and lobular distortion. The liver of the rats killed from 12 months onwards were distorted and had a nodular appearance and a variegated color due to the presence of fatty changes, necrosis, hemorrhage and an irregular blood supply. The severity of the lesions and the distortion increased progressively towards the end of the experiment. Histologically the changes were consistent with those of a chronic toxic hepatitis progressing to cirrhosis but also included the development of GGT<sup>+</sup> hyperplastic foci, hepatocellular carcinoma and cholangiocarcinoma.

The liver of all the FB<sub>1</sub>-treated rats in the terminal stages of the experiment contained a multitude of regenerative nodules and also manifested the architectural distortion and extensive fibrosis characteristic of cirrhosis. The regenerative nodules varied from very small to 3.0 cm in diameter and replaced the normal parenchyma throughout the liver of all the FB<sub>1</sub>-treated rats. Individual nodules were markedly larger in the later stages of the experiment than those seen at 6 and 12 months respectively. Histologically most of the nodules consisted of mature, well-structured, single-layered hepatic cords. In these nodules fatty changes, hyaline droplet degeneration, and necrosis which varied in extent from nodule to nodule and also between animals, occurred. Some of the nodules contained areas in which the cytoplasm of hepatocytes had a ground glass appearance seen as a homogeneous, pale, opaque, eosinophilic and expanded cytoplasm. These foci of cells (= GGT<sup>+</sup> hyperplastic foci), in contrast to the bulk of those in the regenerative nodules, stained positive for GGT.

Some of the regenerative nodules also manifested dysplasia, nuclear atypia and the cellular characteristics of preneoplastic

Table I. Main pathological changes in the liver of rats fed a diet containing 50 mg/kg FB<sub>1</sub>

| Duration (months)  | Body wt gain (g) <sup>a</sup> | Liver wt (% of body wt) <sup>a</sup> | Pathological changes <sup>b</sup> |     |        |                   |
|--------------------|-------------------------------|--------------------------------------|-----------------------------------|-----|--------|-------------------|
|                    |                               |                                      | RN                                | CF  | Cirrh. | HCC. <sup>c</sup> |
| 6                  |                               |                                      |                                   |     |        |                   |
| Treated            | 330.2 ± 14.5a                 | ND <sup>d</sup>                      | 5/5                               | 4/5 | 0/5    | 0/5               |
| Control            | 381.6 ± 25.4a                 | ND                                   | 0/5                               | 0/5 | 0/5    | 0/5               |
| 12                 |                               |                                      |                                   |     |        |                   |
| Treated            | 353.0 ± 18.4a                 | ND                                   | 5/5                               | 5/5 | 0/5    | 0/5               |
| Control            | 434.0 ± 60.6b                 | ND                                   | 0/5                               | 0/5 | 0/5    | 0/5               |
| 20                 |                               |                                      |                                   |     |        |                   |
| Treated            | 404.2 ± 24.5a                 | 4.2 ± 0.3a                           | 5/5                               | 5/5 | 5/5    | 3/5               |
| Control            | 482.2 ± 53.7b                 | 2.6 ± 0.3B                           | 0/5                               | 0/5 | 0/5    | 0/5               |
| 26                 |                               |                                      |                                   |     |        |                   |
| Treated            | 454.8 ± 88.8a                 | 8.6 ± 3.4a                           | 5/5                               | 5/5 | 5/5    | 4/5               |
| Control            | 618.4 ± 56.8B                 | 2.3 ± 0.2B                           | 0/5                               | 0/5 | 0/5    | 0/5               |
| 18–25 <sup>e</sup> |                               |                                      |                                   |     |        |                   |
| Treated (died)     | ND                            | ND                                   | 5/5                               | 5/5 | 5/5    | 3/5               |
| Control (killed)   | ND                            | ND                                   | 0/5                               | 0/5 | 0/5    | 0/5               |

<sup>a</sup>Means in a column followed by the same letter do not differ significantly ( $P > 0.05$ ). If the letters differ but the cases do not, then  $P < 0.01$ ; if the letters and cases differ, then  $P < 0.001$ .

<sup>b</sup>RN, regenerative nodules; CF, cholangiofibrosis; Cirrh., cirrhosis and HCC, hepatocellular carcinoma.

<sup>c</sup>Lung, heart and/or kidney metastases—4/15 of the FB<sub>1</sub>-treated rats that were killed or died between 18–26 months.

<sup>d</sup>ND — not determined.

<sup>e</sup>Survival rate: controls, 96%; treated, 80%.

changes, and a few transformed into hepatocellular carcinoma (Figure 1). It was uncommon to find more than a single hepatocellular carcinoma in the liver of any of the rats. Hepatocellular carcinomas developed in six of the ten rats killed between 18 and 24 months. In two of these the neoplasms metastasized to the heart and lungs, and in one rat to one of the kidneys. Of the five rats killed at 26 months, four developed hepatocellular carcinoma of which one metastasized to the kidney in one of the animals. In total, 10 out of the 15 rats (66%) that were killed and/or died between 18 and 26 months, developed hepatocellular carcinoma. Apart from a few foci of atypical and/or dysplastic cells in the liver of two rats killed at 26 months, no preneoplastic or neoplastic lesions were observed in the control rats during the course of the experiment.

The histological differentiation of the hepatocellular carcinomas varied between the different individuals. The poorly differentiated carcinomas manifested marked pleomorphism, both nuclear and cytoplasmic, a high mitotic rate and numerous foci of necrosis. In these, the neoplastic cells were fusiform and they invariably metastasized. In all the animals the neoplastic cells were arranged in a trabecular pattern and were subdivided by thin connective tissue strands. In one animal the neoplasm infiltrated the blood vessel walls and lumps of neoplastic cells projected into the lumen of the hepatic veins. In all cases the cells of the metastatic foci closely resembled those of the primary neoplasm in the liver. Some metastatic neoplasms, however, were less well differentiated than the primary neoplasm in the liver.

Cholangiofibrosis was consistently present from 6 months onwards. In the initial stages this lesion was characterized by the presence of well-differentiated tubular structures embedded in large amounts of concentrically arranged fibrous connective tissue. From 18 months onward cholangiofibrosis consistently manifested only as irregular, duct-like structures or cell cords lined by an epithelium consisting of intermingled tall columnar cells and numerous goblet cells (Figure 2). This epithelial lining was well differentiated but appeared to be multi-layered in certain areas. Individual cells became necrotic and mitotic figures were rare. The large quantities of mucus produced by this epithelium contained numerous exfoliated epithelial cells and led to the distention and eventual rupture of what appeared to be blindly ending tubules. In the ruptured tubules, mucus also accumulated between the epithelial lining and its base which consisted of thin strands of connective tissue. Focal aggregates of lymphocytes were common within the connective tissue septa. These lesions

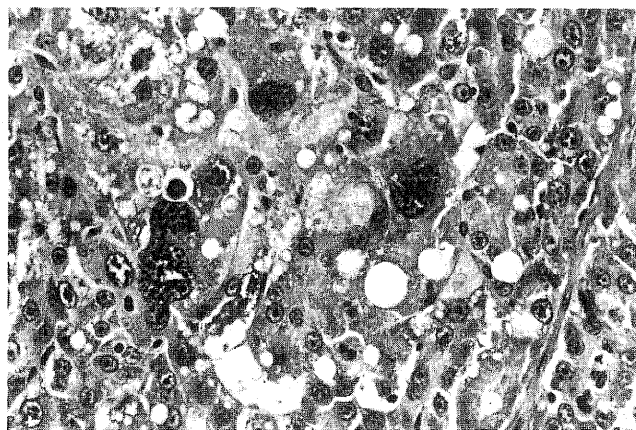


Fig. 1. Hepatocellular carcinoma manifesting marked nuclear and cytoplasmic pleomorphism in a rat chronically exposed to FB<sub>1</sub>. (H & E,  $\times 400$ ).

were expansive, non-infiltrative and did not metastasize in any of the rats, even in those where the mass reached a very large diameter (up to 3 cm). These latter lesions are considered to have progressed to cholangiocarcinoma.

Lesions consistently occurred in the kidneys and lungs of both the control and FB<sub>1</sub>-treated groups. The lungs of the majority of the rats that were either killed or died of their own accord from 18 months onwards, manifested a mild to moderately severe, incidental interstitial pneumonia and lymphocytic bronchitis. There was no difference in the severity of the latter lesions between the two groups. The severity and nature of the lesions in the kidneys, which included a focal to diffuse interstitial lymphocytic nephritis and mild membranoproliferative glomerulonephritis, did not differ visibly between the FB<sub>1</sub>-treated and control groups up to 20 months. Chronic and outspoken lesions occurred in the kidneys of FB<sub>1</sub>-treated rats killed at 26 months. Macroscopically the kidneys were atrophic, pale, irregular in outline and contained numerous cortical and medullary retention cysts, some of which were  $\sim 0.5$  cm in diameter. Histologically the changes were non-specific and included fibrosis, a scattered lymphocytic interstitial infiltrate, retention cysts, hyaline casts, hydropic and hyaline droplet degeneration and scant necrosis of the proximal tubular epithelium. The various glomerular changes included thickening of the glomerular loops, atrophy of the glomerular tuft, expansion of the urinary space and thickening, due to fibrosis, of Bowman's capsule.

Marked elevations of the serum levels of AST, GGT, ALP, creatinine and bilirubin (conjugated and unconjugated) were observed in the FB<sub>1</sub>-treated rats killed at 20 and 26 months respectively (data not shown). There was no difference in the total protein content while the albumin:globulin ratio was significantly reduced ( $P < 0.0005$ ) in the FB<sub>1</sub>-treated group at 26 months due to an increased serum globulin level. Although the serum cholesterol levels were significantly higher ( $P < 0.0005$ ) in the FB<sub>1</sub>-treated group than the controls at 20 months, the difference between the groups killed at 26 months was not statistically significant.

## Discussion

The major toxicological and carcinogenic effects induced by culture material of *F. moniliforme* MRC 826 in the liver of rats were reproduced in the present long-term feeding experiment

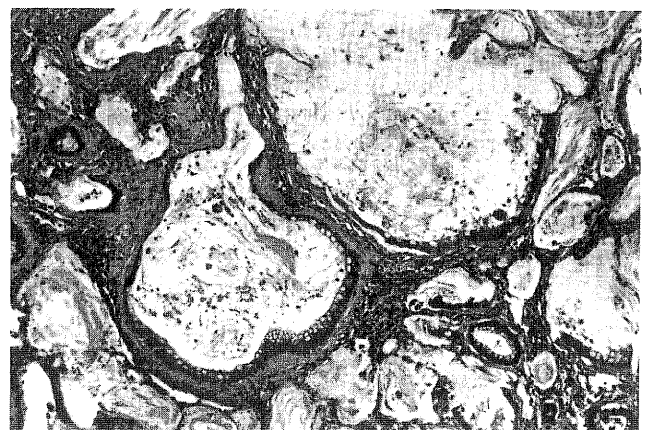


Fig. 2. Cholangiofibrosis in a rat chronically exposed to FB<sub>1</sub> showing dilated tubules distended by large amounts of mucus and cellular debris. (H & E,  $\times 100$ ).

using pure FB<sub>1</sub> at a dietary level of 50 mg/kg. This dietary level of FB<sub>1</sub> is not unrealistically high as naturally contaminated corn samples have been found to contain concentrations up to 27 mg FB<sub>1</sub>/kg (19) and 46.9 mg FB<sub>1</sub>/kg (22).

In the present experiment hepatocellular carcinomas developed in livers affected by a chronic toxic hepatitis which culminated in cirrhosis. Whether regeneration in cirrhosis can lead *de novo* to neoplasia is still not clear but it has been reported that altered cell populations present in cirrhotic livers in humans are associated with the development of malignant lesions (27). In the present study several animals displayed regenerative nodules containing groups of dysplastic cells with multiple, polyploid nuclei and which were regarded as nodules in which a final transformation into neoplasia had not yet occurred.

Apart from hepatocellular carcinoma, FB<sub>1</sub> also induced cholangiofibrosis consistently and, towards the end of the experiment, cholangiocarcinoma in the liver of the treated rats. Identical lesions were seen in rats fed culture material of *F.moniliforme* MRC 826 (5,6) in which cholangiofibrosis was considered to have progressed to neoplasia referred to either as ductular carcinoma (6) or as cholangiocarcinoma (5). It does appear that the nature of cholangiofibrosis, which is specific for rats, remains controversial, but that on a morphological basis, there is no reason why in its end-stage it should not be classified as a neoplasm; the stage at which it progresses to a neoplasm, however, remains unclear. Cholangiofibrosis is, in fact, considered to be a precursor lesion for cholangiocarcinoma in rats exposed to certain carcinogens (28,29) although it may persist without progressing to carcinoma (30).

The growth rate of the control rats fed the cereal-based diet was similar (5) to that normally obtained when using a cereal-based commercial diet (rat cubes; Epol Ltd, Johannesburg, SA). The diet fed was marginally deficient in vitamins such as thiamine, riboflavin, vitamin E and also contained low levels of vitamin B<sub>12</sub>, folate, biotin, methionine and choline when compared to a diet such as AIN-76<sup>TM</sup> (31). Diets deficient in lipotropes such as choline and methionine have been shown to induce liver cancer in rats (32) while those low in lipotropes render the liver more susceptible to the carcinogenic action of carcinogens (33). Thus the diet used in the present study may have had an enhancing effect on the action of FB<sub>1</sub> in the liver. Subsequent studies will therefore be conducted to study the modulating role of various dietary components such as vitamins and lipotropes on the carcinogenic activity of the fumonisins in rats.

Deficiencies in vitamins A and E and in the lipotropes vitamin B<sub>12</sub> and folate, have also been implicated in the etiology of human esophageal cancer in the Transkei, southern Africa (34). However, in the present experiment, using a cereal-based diet that has been shown to enhance the induction of esophageal carcinogenesis by methylbenzyl nitrosamine in rats (23), no effect of FB<sub>1</sub> on the esophagus of the rats was noticed. This indicates that FB<sub>1</sub> is probably not responsible for the basal cell hyperplasia induced by culture material of the fungus in rats (5,6). Several other factors such as snuff, tobacco, home-brewed beer and zinc deficiency have been reported to induce basal cell hyperplasia in dietary experiments in rats (35,36), suggesting that the relevance of this relatively non-specific lesion in the development of esophageal cancer in both humans and animals, should be interpreted with care. Based on the present results and those of previous studies it appears that rats are not a good model in which to study the possible etiological significance of FB<sub>1</sub> in esophageal carcinoma.

The clinical chemistry results from 20 months and onwards supported the histopathological observations that the liver is the main target organ affected by FB<sub>1</sub>. This finding is in agreement with that reported previously in which extracts of the fungus, containing both FB<sub>1</sub> and FB<sub>2</sub>, significantly increased the levels of the serum enzymes ALT, AST, ALP and the total bilirubin level in rats (4). In the present study the levels of only AST, GST and bilirubin (total, conjugated and unconjugated) were significantly higher in the FB<sub>1</sub>-treated than in the control rats, whereas the increases in ALT and ALP were not statistically significant.

The kidneys of the FB<sub>1</sub>-treated rats were also markedly affected towards the end of the experiment, more so than was observed in rats fed culture material of *F.moniliforme* (2). Other lesions induced by culture material of *F.moniliforme* MRC 826, including endothelial hyperplasia and/or intraventricular thrombosis (6), myocardial disseminated fibrosis and endocardial fibrosis (5), and acanthosis, dysplasia and papillomas in the squamous forestomach (5), were not observed in the rats fed the FB<sub>1</sub>-containing diet. The present study indicates that FB<sub>1</sub> is responsible for the hepatocarcinogenic and the hepatotoxic but not all the other toxic effects of *F.moniliforme* MRC 826 in rats. Thus FB<sub>1</sub> may also be responsible for the hepatotoxicity of cultures of *F.moniliforme* in other animal species (1,2,4,6).

Extrapolation of the available data on the toxicity and carcinogenicity of the fumonisins and/or cultures of *F.moniliforme* in experimental animals to humans must take cognisance of the fact that the liver appears to be the primary target organ in a variety of animal species. Although the fungus as well as the fumonisins have been epidemiologically associated with the prevalence of human esophageal cancer (22,37), the role of specific fungal contamination of dietary staples and the prevalence of liver cancer in southern Africa as a whole need to be considered in future studies. The contamination of dietary staples with the fumonisin mycotoxins (22) in areas with a high prevalence of human esophageal cancer and to a lesser extent of liver cancer (38,39) in Transkei, poses a major challenge to investigate the possible role of these naturally occurring food-borne carcinogens in the multifactorial nature of the etiology of both these diseases.

### Acknowledgements

The authors wish to thank Pieter W.de Lange, Colin W.Woodroof, Marita E.Cawood, Sonja Stockenström and Marlène Schlechter for competent technical assistance.

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Received on January 2, 1991; revised on April 18, 1991; accepted on April 22, 1991

## Toxicological effects in rats chronically fed low dietary levels of fumonisin B<sub>1</sub>

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Received 25 September 2000; received in revised form 9 November 2000; accepted 6 December 2000

### Abstract

The toxicity of low dietary levels of fumonisin B<sub>1</sub> (FB<sub>1</sub>), i.e. 1, 10 and 25 mg FB<sub>1</sub>/kg diet, were monitored in rats over a period of 24 months. No effects on the body weight gain and feed intake profiles were noticed, while the relative liver weight was significantly ( $P < 0.05$ ) reduced in the FB<sub>1</sub>-treated rats. Mild toxic effects, including single cell necrosis (apoptosis), proliferation of bile duct epithelial cells (DEC), and early signs of fibrosis, bile duct hyperplasia and in one case, adenofibrosis, were noticed in the liver of the rats fed the highest (25 mg/FB<sub>1</sub>/kg diet) dietary level. A significant ( $P < 0.05$ ) increase in the level of oxidative damage was also noticed in the liver of the rats of high dosage dietary group. The toxic effects were less severe in the 10 mg FB<sub>1</sub>/kg dietary group, whilst only a few ground glass foci were observed in the 1 mg FB<sub>1</sub>/kg dietary group. Hepatocyte nodules, staining positively for glutathione-S-transferase (placental form, PGST), were observed macroscopically in the 25 mg FB<sub>1</sub>/kg treated group and to a lesser extent in the 10 mg FB<sub>1</sub>/kg treated rats. The most prominent toxic lesions by FB<sub>1</sub> (10 and 25 mg FB<sub>1</sub>/kg dietary groups) in the kidneys were restricted to the tubular epithelium manifesting as granular cast, necrosis, apoptosis, calcification and the presence of regenerative foci in the proximal convoluted tubules. The existence of a cytotoxic/proliferative threshold with respect to cancer induction by FB<sub>1</sub> in rat liver became apparent, with a dietary level of < 10-mg FB<sub>1</sub>/kg diet as a no effect threshold for the induction of hepatocyte nodules. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Toxicity of fumonisin B<sub>1</sub> in rats

### 1. Introduction

Chronic feeding studies in male BD IX rats over 26 months showed that 66% of the rats developed liver cancer at a dietary level of 50 mg fumonisin B<sub>1</sub> (FB<sub>1</sub>)/kg diet (Gelderblom et al.,

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1991). The cancer developed from severely cirrhotic livers, while cholangiofibrosis also appeared to be a common lesion in the majority of the livers of the rats. Very few hepatotoxic effects were noticed in the liver of male and female Fischer rats, even upto a dietary level of 150 mg FB<sub>1</sub>/kg fed over a period of 2 years (NTP TR 496, 1999). These long-term, as well as short-term feeding studies in rats (Voss et al., 1993, 1995; Gelderblom et al., 1996a) indicated that the liver and kidneys are the major target organs affected by FB<sub>1</sub>.

Toxicological and carcinogenic studies to assess the biological effects of FB<sub>1</sub> in vivo in rats (Gelderblom et al., 1992) and in vitro utilising primary hepatocytes (Gelderblom et al., 1992; Norred et al., 1992; Gelderblom et al., 1993) failed to detect any genotoxic effects. In spite of these findings, it was shown that FB<sub>1</sub> closely mimics the effects of the genotoxic hepatocarcinogens with respect to their ability to induce cancer initiation and promotion in the liver (Gelderblom et al., 1992, 1994, 1996b). Studies utilising a short-term, cancer initiation/promotion model in the rat liver, indicated that, with FB<sub>1</sub>, a concurrent hepatotoxic effect is required for the induction of cancer initiation (Gelderblom et al., 1994). Cancer promotion, however, can be induced at dietary levels of FB<sub>1</sub> that were less hepatotoxic (Gelderblom et al., 1996b). It is not known whether cancer will develop in the liver of rats in the absence of chronic hepatotoxic effects.

The present study further investigates the toxicological effects of low dietary levels of FB<sub>1</sub> in male BD IX rats as part of an ongoing process to establish risk assessment parameters of fumonisins for humans.

## 2. Materials and methods

### 2.1. FB<sub>1</sub> standard

FB<sub>1</sub> was purified as described elsewhere (Ca-wood et al., 1991) to a purity of between 92 and 95% as verified by HPLC (Shephard et al., 1990). Contaminants consisted of the mono-methyl esters (1–2%) of FB<sub>1</sub> and H<sub>2</sub>O (1–2%). The mono-

methyl esters also exhibited similar biological activity with respect to cancer initiation in rat liver (Gelderblom et al., 1993). The stock sample of the mycotoxin was kept as a dried powder in an airtight container at 4°C.

### 2.2. Animals and diets

Inbred BD IX rats, after having reached a body weight of approximately 100 g, were randomly divided into three treatment groups of 20 rats each. They were caged individually in a controlled environment facility with a 12-h artificial light cycle, an environmental temperature varying from 23 to 25°C and humidity of 50%.

The rats were fed a semi-synthetic diet (Van Rensburg et al., 1985) earlier used in long-term carcinogenesis studies in rats (Gelderblom et al., 1991). This corn based diet consisted of sifted corn meal (750 g/kg diet) and a vitamin and mineral supplement compiled to be marginally deficient in vitamins and lipotropes (Gelderblom et al., 1991). Aflatoxin (Thiel et al., 1986) and fumonisin (Shephard et al., 1990) levels in the corn meal supplement were analysed on a regular basis. Depending on the matrices analysed, the detection limits of these methods were 0.1–0.5 and 10–50 µg/kg for aflatoxin B<sub>1</sub> and FB<sub>1</sub>, respectively.

The experimental diets were prepared by dissolving the calculated amount of FB<sub>1</sub> into methanol (ca 50 ml) and mixing it into a sub-sample (200 g) of the diet that was then allowed to dry overnight in a extraction cabinet. Only methanol was added to the control diet. The sub-samples were mixed into the corresponding stock diets (6-kg) and stored under N<sub>2</sub> at 4°C for periods of not longer than 4 weeks.

### 2.3. Treatments

Water and food were available ad libitum. The different groups of the experimental rats were fed diets containing, respectively 1, 10 and 25 mg FB<sub>1</sub>/kg. All the rats were weighed once weekly and feed intake was measured daily over a period of 14 days after 2, 8 and 14 months, respectively. The 14 month FB<sub>1</sub> intake of the rats fed the

control, 1, 10 and 25 mg FB<sub>1</sub>/kg diets were used as the average FB<sub>1</sub> intake to calculate the cumulative FB<sub>1</sub> intakes over the 24 month period. From 18 months after commencement of the experiment, rats in poor condition were euthanised for necropsy purposes. All surviving rats were killed after 24 months by intraperitoneally administering an overdose of sodium pentobarbital and subjected to a necropsy.

#### 2.4. Clinical pathological analyses

Serum samples were prepared from blood collected from the abdominal aorta of rats after euthanasia. These samples were analysed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamine aminotransferase (GLT), alkaline phosphatase (ALP), and total cholesterol by Technicon autoanalyses. Low- and high-density lipoproteins (LDL and HDL) and triacylglycerol (TAG) were determined by methods described earlier (Fincham et al., 1992).

#### 2.5. Necropsy

Routine necropsies were performed on all the rats. At necropsy, the body and liver weight of the rats were determined and the organs observed macroscopically for the presence of any abnormalities. Specimens collected from the major lobes of the liver were preserved in 10% buffered formalin for histopathology and histochemical staining to determine the presence of GSTP and gamma glutamyltranspeptidase (GGT) foci. The remainder of the liver was frozen at  $-80^{\circ}\text{C}$  in saline, while the rest of the organs were preserved in 10% buffered formalin for histological examination.

#### 2.6. Histopathological and histochemical analyses

GSTP staining (Tatematsu et al., 1988) was performed using the avidin–biotin–peroxidase complex (ABC) and affinity-purified biotin-labelled goat anti-rabbit IgG serum (Vector Laboratories, Burlingame, CA). The paraffin wax-embedded sections of the acetone-preserved material were routinely processed through petroleum benzene and a graded alcohol series

before being stained with the reagents in the ABC kit. Rabbit GSTP-antiserum (kindly donated by Dr E Farber, Department of Pathology, University of Toronto) was used at a dilution of 1:800. The sections were counter-stained with Carazzi's haematoxylin to provide blue-stained nuclei, while the GSTP<sup>+</sup> cells were characterised by the presence of a reddish-brown pigment. Negative and positive controls for the stain were also included to test for the specificity of anti-GSTP antibody binding. The procedures outlined by Ogawa et al. (1980) were followed for the GGT staining. The presence of GGT<sup>+</sup> and GSTP<sup>+</sup> altered lesions were detected by light microscopy (10–20X magnification). The GSTP<sup>+</sup> foci were further categorised into lesions containing < 5 cells; 5–20 cells and > 20 stained cells per focus. Tissue sections of all the preserved organs were routinely processed for histopathological evaluation and stained with haematoxylin and eosin.

#### 2.7. Determination of thiobarbituric acid reactive material (TBARS)

The liver homogenate (1 ml) (0.1–2.0 mg protein/ml phosphate buffer containing 0.1 M EDTA) was mixed with 2 ml of a cold 10% trichloroacetic acid (TCA) solution containing 0.01% butylated hydroxytoluene (BHT). Following centrifugation (3000 rpm), 2 ml of the supernatant was added to 2 ml of a 0.67% TBA solution in a test tube and immersed in boiling water for 10 min. The mixture was then allowed to cool and the absorbance measured at 532 nm (Esterbauer and Cheeseman, 1990). Lipid peroxidation was expressed as nmole malondialdehyde (MDA) equivalents per mg protein using a molar extinction coefficient of  $1.56 \times 10^5$  M/cm at 532 nm for MDA (Buege and Aust, 1978). Determination of MDA as described above is known not to be specific as various substances such as sucrose, metal ions and whole tissue homogenates may also react with TBA or influence the assay procedure. The term thiobarbituric acid-reacting substances (TBARS) are, therefore, used to describe the reaction product in the assay (Esterbauer and Cheeseman, 1990). Non-specific lipid peroxidation was prevented by the incorporation of EDTA in



the buffers and BHT in the reaction solutions for the TBARS assay.

### 2.8. Statistical analyses

Data were subjected to analysis of variance (ANOVA) using the Statistical Analysis System (SAS Institute Inc., Cary, NC). Differences between mean were determined by the Tukey Studentised Range method. The differences between the feed intake profiles at months 2, 8 and 14 (Table 1) were analysed by the parametric paired-difference *t*-test. The Kruskal-Wallis test was used to determine whether the median of the lesions in the different organs, the liver and kidneys differed significantly between the four treatment groups.

## 3. Results

### 3.1. Feed intake and body weight

Fumonisin analyses of commercial corn meal

samples that were used in the diet revealed mean levels (FB<sub>1</sub> + FB<sub>2</sub>) of approximately 0.22 mg/kg (ranging between 0 and 0.6 mg FB/kg). Based on these data the control diet contained levels of approximately 0.16 mg FB/kg. No aflatoxin contamination was detected. The survival rate of the animals, prior to termination that started between 18 and 24 months after commencement was in the order of 80%. Rats terminated before the end of the experiment, (prior to 24 months) suffered from mild to severe interstitial pneumonia.

There were no significant differences between the body weights of the FB<sub>1</sub>-treated rats and the control group at 2, 8 and 14 months (Table 1). The total body weight gain for the duration (24 months) of the experiment (Table 4) also did not differ significantly ( $P > 0.05$ ) between the different treatment groups. However, the relative liver weight (g/100 g body weight) of the rats fed the FB<sub>1</sub>-containing diets was significantly lower (either  $P < 0.01$  or  $P < 0.05$ ) than the control group at 24 months. The feed intake (g feed/100 g body weight) profiles were significantly ( $P <$

Table 1  
Body weight, intake of feed and FB<sub>1</sub>, and the cumulative amount of FB<sub>1</sub> ingested at 2, 8 and 14 months, respectively, after commencement of the experiment<sup>a</sup>

| Treatment (mg FB <sub>1</sub> /kg diet) | Mean body weight (BW) (g) | Feed intake (g/100 g BW per day) | FB <sub>1</sub> intake (mg/100 g BW per day) | Cumulative (mg FB <sub>1</sub> /100 g BW) <sup>b</sup> |
|---|---------------------------|----------------------------------|--|--|
| <i>2 months</i>                         |                           |                                  |  |  |
| Ctrl ( <i>n</i> = 20)                   | 217 ± 26                  | 5.26 ± 0.88a                     | 0.0008 <sup>c</sup>                          | 0.05 ± 0.02  |
| 1 ( <i>n</i> = 20)                      | 223 ± 23                  | 4.98 ± 0.43a                     | 0.005 ± 0.001                                | 0.2 ± 0.1  |
| 10 ( <i>n</i> = 20)                     | 218 ± 22                  | 5.03 ± 0.84a                     | 0.06 ± 0.01                                  | 2.8 ± 0.6  |
| 25 ( <i>n</i> = 20)                     | 209 ± 18                  | 5.48 ± 0.70a                     | 0.14 ± 0.02                                  | 7.8 ± 1.1  |
| <i>8 months</i>                         |                           |                                  |  |  |
| Ctrl ( <i>n</i> = 20)                   | 335 ± 38a                 | 4.16 ± 0.67B                     | 0.0007 <sup>c</sup>                          | 0.17 ± 0.04  |
| 1 ( <i>n</i> = 18)                      | 340 ± 47a                 | 4.23 ± 0.45B                     | 0.005 ± 0.001                                | 1.1 ± 0.3  |
| 10 ( <i>n</i> = 20)                     | 345 ± 38a                 | 4.02 ± 0.82B                     | 0.04 ± 0.01                                  | 10.1 ± 2.6   |
| 25 ( <i>n</i> = 19)                     | 347 ± 28a                 | 4.11 ± 0.51B                     | 0.10 ± 0.01                                  | 26.0 ± 5.2   |
| <i>14 months</i>                        |                           |                                  |  |  |
| Ctrl ( <i>n</i> = 18)                   | 419 ± 33a                 | 3.01 ± 0.46C                     | 0.0005 <sup>c</sup>                          | 0.21 ± 0.05  |
| 1 ( <i>n</i> = 16)                      | 437 ± 63a                 | 3.08 ± 0.47C                     | 0.003 ± 0.001                                | 1.4 ± 0.05   |
| 10 ( <i>n</i> = 17)                     | 413 ± 35a                 | 3.39 ± 0.37C                     | 0.03 ± 0.01                                  | 13.7 ± 0.5   |
| 25 ( <i>n</i> = 18)                     | 387 ± 55                  | 3.47 ± 0.43D                     | 0.08 ± 0.01                                  | 36.5 ± 4.6   |

<sup>a</sup> Values are expressed as the mean ± S.D. of the various values obtained from the rats remaining in each group at each time period. The means followed by the same letter do not differ significantly. If the letters and cases differ, then  $P < 0.01$ ; if the letter differs then  $P < 0.05$ .

<sup>b</sup> Values represent the cumulative FB<sub>1</sub> intake from the start of the experiment.

<sup>c</sup> Values calculated from the FB<sub>1</sub> content of commercial maize meal used in compiling the diet (see Section 2). BW; body weight.

Table 2  
Clinical pathological data of rats euthanised 24 months after commencement of the experiment<sup>a</sup>

| Treatment (mg FB <sub>1</sub> /kg diet) | LDL cholesterol (mmol/l) | Total cholesterol (mmol/l) | Plasma TAG (mmol/l) | HDL Cholesterol (mmol/l) | % HDL           |
|---|--------------------------|----------------------------|---------------------|--------------------------|-----------------|
| <i>Plasma lipid profiles:</i>           |                          |                            |                     |                          |                 |
| Control                                 | 1.03a (0.07)             | 1.84a (0.40)               | 3.37a (1.46)        | 0.73a (0.16)             | 42.18a (4.89)   |
| 1                                       | 1.04a (0.08)             | 1.76ab (0.34)              | 3.94a (1.91)        | 0.63ab (0.22)            | 40.35a (6.64)   |
| 10                                      | 1.02a (0.05)             | 1.45b (0.38)               | 4.26a (2.13)        | 0.48b (0.20)             | 35.48a (7.52)   |
| 25                                      | 1.12a (0.22)             | 1.93a (0.28)               | 3.52a (0.92)        | 0.76a (0.15)             | 39.34a (4.06)   |
| <i>Serum parameters:</i>                |                          |                            |                     |                          |                 |
|   | Creatinine (µmol/l)      | AST (U/l)                  | ALT (U/l)           | GLT (U/l)                | ALP (U/l)       |
| Control                                 | 43.67a (5.92)            | 103.13a (21.68)            | 29.86a (3.76)       | 3.56a (1.24)             | 119.63a (18.45) |
| 1                                       | 48.36ab (5.70)           | 120.44a (16.18)            | 31.00a (17.00)      | 4.09a (0.94)             | 125.50a (26.73) |
| 10                                      | 52.56b (6.95)            | 168.36B (23.42)            | 43.36a (14.82)      | 3.63a (0.92)             | 120.75a (18.4)  |
| 25                                      | 54.13b (8.44)            | 189.00B (14.80)            | 42.80a (12.25)      | 3.25a (1.28)             | 120.63a (29.11) |

<sup>a</sup> Values are expressed as the mean ± S.D. (in parenthesis) of nine or 10 animal per group. Means followed by the same letter do not differ statistically ( $P > 0.05$ ). If the letters differ, then  $P < 0.05$  and when letters and case differ, then  $P < 0.01$ .

0.001) reduced in the older rats during the time course of the experiment (Table 1). The cumulative FB<sub>1</sub> intake of the different groups was calculated at 2, 8 and 14 months using the daily FB<sub>1</sub> intakes monitored at that specific period. The 14-month FB<sub>1</sub> intake profiles of the rats fed the control, 1, 10 and 25 mg FB<sub>1</sub>/kg diets were used as the average FB<sub>1</sub> intake to calculate cumulative FB<sub>1</sub> intakes over the 24 month period. The feed wastage during the 14-day dietary intake experiments was  $0.36 \pm 0.21\%$ .

### 3.2. Clinical pathological analyses

After 24 months the total cholesterol and the HDL cholesterol were significantly ( $P < 0.05$ ) lower in the rats fed the 1 and 10 mg FB<sub>1</sub>/kg diets than in the controls and the high dose group (Table 2). There were no significant difference ( $P > 0.05$ ) between the control and the 25 mg FB<sub>1</sub>/kg diet groups. FB<sub>1</sub> treatment did not alter the levels of TAG, LDL and the % HDL. Serum GLT and ALP were not altered, while AST was significantly ( $P < 0.01$ ) and ALT markedly (but not significantly) increased in a dose response manner in the 10 and 25 mg/kg FB<sub>1</sub>-treated

groups. Creatinine levels were significantly elevated above the controls in the rats fed 10 and 25 mg FB<sub>1</sub>/kg diets ( $P < 0.05$ ) and markedly (but not significantly,  $P < 0.1$ ) in the 1 mg FB<sub>1</sub>/kg treatment.

### 3.3. Enzyme altered foci and/or nodules

GGT<sup>+</sup> foci, with irregular outlines, were not readily detected and only a few liver sections from the highest dose of FB<sub>1</sub> with an average (S.D.) of  $0.611 \pm 0.442$  foci/cm<sup>2</sup>. The foci contained more than 10 cells with a diameter ranging from 435 to 630 µm (data not shown). No foci were detected in either the controls or the rats receiving 1 and 10 mg FB<sub>1</sub>/kg diet. The number of GSTP<sup>+</sup> positive lesions (< 5 cells), minifoci (5–20 cells) and foci and nodules (> 20 cells) did not differ significantly between the control, 1 and 10 mg FB<sub>1</sub>/kg diet groups although more rats contained foci in their livers in the 10 mg FB<sub>1</sub>/kg group. The number of mini foci (5–20 cells/focus) and foci (> 20 cells/focus) were significantly ( $P < 0.01$ ) higher in the group fed the 25 mg FB<sub>1</sub>/kg diet (Fig. 1). All the rats (14/14) treated with the 25 mg FB<sub>1</sub>/kg diets exhibited foci in their livers while

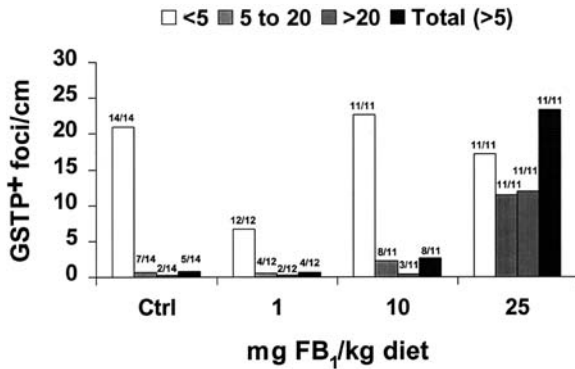


Fig. 1. The distribution of GSTP<sup>+</sup> single cells, mini foci and foci in liver tissue sections of rats treated with three different dietary levels (1, 10 and 25 mg FB<sub>1</sub>/kg) over a period of 24 months. Values are the mean with the number of rats with lesions in the liver given in parenthesis.

only 5/14, 4/12, 8/11 rats had altered hepatocyte foci in the control 1, 10 mg FB<sub>1</sub>/kg treated groups, respectively.

### 3.4. TBARS generation

The FB<sub>1</sub>-treated rats showed no significantly increase in the production of TBARS at dietary levels of 1 and 10 mg dietary levels. A significant ( $P < 0.05$ ) increase was noticed in the liver of the rats fed the 25 mg FB<sub>1</sub>/kg dietary level (Fig. 2).

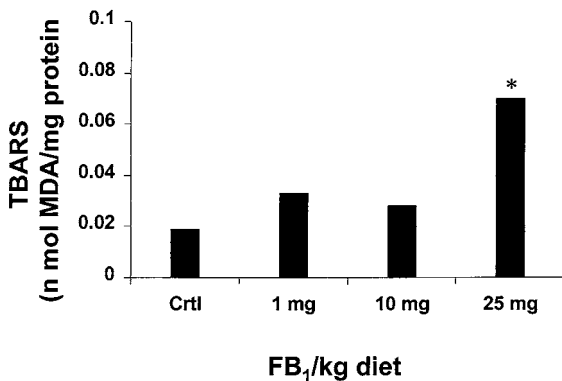


Fig. 2. Dose-response of TBARS generation in vivo in rat liver homogenates as a function of dietary FB<sub>1</sub> levels. Data are the mean of triplicate determinations. \* $P < 0.05$ .

### 3.5. Gross and histopathological changes

The number of lesions and the extent of the changes observed at necropsy and histopathologically differed according to the dosage of FB<sub>1</sub> consumed in the diet over the duration of the experiment. The lesions detected in the individual rats reflected changes of three basic categories — those associated with exposure to FB<sub>1</sub> in the diet, those associated with secondary acute bacterial infections (septicaemia and abscessation), and age-associated changes (Table 3). Except for the marginally significant ( $P < 0.1$ ) increase in the lesions in the liver, no differences in the frequency of lesions could be observed in other organs that can be ascribed to the FB<sub>1</sub>-dietary treatment. Those seen in the other organs were either age-associated changes or the consequences of acute bacterial infections. The prevalence of organ changes caused by secondary bacterial infections (abscesses in organs such as the lungs, prostate, and spleen, as well as peritonitis and meningitis) was noticeably higher in the rats fed the high dosage (25 mg/kg diet) of FB<sub>1</sub>. No difference in the prevalence of these infections was detected between the lower dosage groups and the control animals. Interstitial pneumonia occurred in a large proportion of the rats of all groups and resulted in the development of chronic bronchitis that in certain cases progressed to bronchiectasia. These pneumonic changes also seemed to enhance the development of secondary bacterial infections. Age-associated lesions that occurred in rats of the various treatment groups and in the control rats included prostatic hyperplasia and prostatitis, calcification and atrophy of the testes, and the occurrence of various neoplasms. The neoplasms seen were a gastric carcinoma and squamous cell carcinoma of undetermined location in the 25 mg FB<sub>1</sub>/kg dietary group, lymphoma, small intestinal carcinoma and a sub-cutaneous fibrosarcoma in the 10 mg FB<sub>1</sub>/kg dietary group, and a subcutaneous fibrosarcoma in the control group. No neoplasms were detected in the 1 mg FB<sub>1</sub>/kg dietary group.

The lesions seen in the liver and kidneys of the animals affected reflected the consequence of exposure of these animals to dietary FB<sub>1</sub> (Table 4).

Table 3

Frequency of lesion in the organs of rats in the treatment (FB<sub>1</sub>) and control groups<sup>a</sup>

| Organ       | 25 mg FB <sub>1</sub> /kg diet (n = 17) | 10 mg FB <sub>1</sub> /kg diet (n = 18) | 1 mg FB <sub>1</sub> /kg diet (n = 14) | Control diet (n = 16) |
|-------------|---|---|--|-----------------------|
| Kidney      | 11                                      | 10                                      | 10                                     | 10                    |
| Liver       | 16 <sup>(*)</sup>                       | 12                                      | 10                                     | 9                     |
| Lung        | 15                                      | 11                                      | 11                                     | 11                    |
| Prostate    | 8                                       | 4                                       | 4                                      | 4                     |
| Testis      | 11                                      | 2                                       | 8                                      | 8                     |
| Thyroid     | 1                                       | 2                                       | –                                      | –                     |
| Lymph node  | –                                       | 1                                       | –                                      | –                     |
| Muscle      | 3                                       | 1                                       | 1                                      | –                     |
| Spleen      | 1                                       | –                                       | –                                      | –                     |
| Bladder     | 1                                       | –                                       | 1                                      | –                     |
| Brain       | 1                                       | –                                       | 1                                      | –                     |
| Heart       | 2                                       | –                                       | –                                      | –                     |
| Pancreas    | 2                                       | –                                       | –                                      | –                     |
| Peritoneum  | 1                                       | –                                       | 1                                      | –                     |
| Stomach     | 2                                       | –                                       | –                                      | –                     |
| Intestine   | –                                       | 1                                       | –                                      | –                     |
| Septicaemia | 7*                                      | 3                                       | 2                                      | 1                     |
| Neoplasms   | 2                                       | 3                                       | –                                      | 1                     |

<sup>a</sup> Values differ significantly from the control treatment; (\* $P < 0.05$ ; (<sup>\*</sup>)  $P < 0.1$ ).

### 3.6. Liver

The lesions that could be ascribed to fumonisin treatment were present in all the FB<sub>1</sub> treatment groups. Minor changes were detected in some of the rats treated with the 10 mg FB<sub>1</sub>/kg diet, while only minimal changes were noticed in the 1 mg FB<sub>1</sub>/kg when compared with the control groups. In the rats that received 25 mg FB<sub>1</sub>/kg diet, apoptosis, proliferation of duct epithelial cells (DEC) and mild fibrosis, that in some cases caused bridging between the portal tracts, were detected. These changes resulted in a slight distortion in the architecture of the liver in some rats. One rat in this group showed a large focal area of adenofibrosis. Of the rats killed at 24 months in the groups fed 10 and 25 mg FB<sub>1</sub>/kg, respectively, 12% (2/17) and 55% (10/18) had hepatocyte nodules that were macroscopically visible in their liver. The number of nodules varied between 1–2 and 1–5 nodules per liver in the rats fed the 10 or 25 mg FB<sub>1</sub>/kg diets, respectively. The nodules in the high-dose group were slightly larger (1–6 mm), than those (1–3 mm) in the rats fed 10 mg FB<sub>1</sub>/kg. All the nodules exhibited mild to severe fatty changes and had a ground glass to

basophilic appearance imparted by the staining reaction of the hepatocytes in the nodules. Other pathological changes in the liver included mild peri-acinar fatty changes, mild to prominent anisonucleosis, and a few apoptotic bodies that were scattered throughout the hepatic lobules.

### 3.7. Kidneys

The most prominent lesions were present in the rats that received the 25 mg FB<sub>1</sub>/kg diet (Table 4). The lesions seen in glomeruli were consistently present in all the experimental groups and in the control group. The severity of these glomerular lesions was apparently enhanced by the presence of increased levels of FB<sub>1</sub> in the diet. The prevalence of hyaline casts and cortical cysts was also associated with the presence of the glomerular lesions and did not appear to be correlated with the dosage level of FB<sub>1</sub>. The lesions that appear to have been caused by FB<sub>1</sub> were restricted to the tubular epithelium and included the presence of granular casts, necrosis, apoptosis, calcification, and the presence of regenerative foci (that may also be interpreted as hyperplastic foci) of tubular epithelium in the proximal convoluted tubules.

Some of the changes associated with septicaemia (such as degenerative changes in the tubular epithelium, acute tubular necrosis and the presence of granular casts) would not differ from those caused by the toxicological effects of FB<sub>1</sub>. The presence of these changes may influence interpretation of the significance of some of the more acute lesions present in the kidneys.

#### 4. Discussion

The consequences of the intake of low dietary levels of FB<sub>1</sub> in the present study reflected the ability of the compound to induce discernible toxic effects at dosages of, as low as, 0.03 mg/kg body weight per day (1 mg FB<sub>1</sub>/kg diet) in the diet over a period of 18–24 months. No hepato-

Table 4

Frequency of histopathology lesions observed in the liver and kidneys of rats in the FB<sub>1</sub> treatment and control groups<sup>a</sup>

| Parameter                          | Treatments (mg FB <sub>1</sub> /kg diet) |              |             |              |
|------------------------------------|--|--------------|-------------|--------------|
|                                    | 25                                       | 10           | 1           | Control      |
| Survival rate                      | 17/20                                    | 18/20        | 14/20       | 16/20        |
| Body weight gain                   | 390.5 (54.0)                             | 399.2 (39.5) | 407 (48.1)  | 387.4 (41.4) |
| Liver weight (%)                   | 2.3 (0.3)*                               | 2.2 (0.3)**  | 2.2 (0.1)** | 2.5 (0.2)    |
| <i>Liver histology</i>             |  |              |             |              |
| Anisokaryosis                      | 13*                                      | 8*           | 2           | –            |
| Ground glass foci                  | 7*                                       | 3(*)         | 4*          | –            |
| Cell swelling                      | 2  | 1            | 2           | 1            |
| Fatty changes                      | 9  | 4            | 6           | 10           |
| Hepatocyte nodules                 | 9*                                       | 0            | 0           | 0            |
| Basophilic foci                    |  |              |             |              |
| Bile duct hyperplasia              | 3  | –            | –           | –            |
| Apoptosis                          | 2  | 3            | 2           | 1            |
| Portal fibrosis                    | 5*                                       | –            | –           | –            |
| OVC hyperplasia                    | 2  | –            | –           | –            |
| Lobular distortion                 | 5*                                       | –            | –           | –            |
| Bridging fibrosis                  | 1  | –            | –           | –            |
| Adenofibrosis                      | 1  | –            | –           | –            |
| <i>Kidney histology</i>            |  |              |             |              |
| Granular casts                     | 7  | –            | –           | –            |
| Hyaline casts                      | 6  | 5            | 3           | 3            |
| Cortical cysts                     | 6  | 2            | 5           | 1            |
| Periglomerular infiltrate          | 10                                       | 8            | 9           | 8            |
| Capsular cell proliferation        | 4  | 4            | 5           | 5            |
| Hyaline droplets in glomerulus     | 3  | 1            | –           | 1            |
| Peri-glomerular scarring           | 5  | 1            | 3           | –            |
| <i>PCTs</i>                        |  |              |             |              |
| Fatty changes                      | 5*                                       | –            | –           | –            |
| Apoptosis                          | 2*                                       | –            | –           | 1            |
| Cell swelling                      | 3(*)                                     | 2(*)         | –           | –            |
| <i>DCTs</i>                        |  |              |             |              |
| Necrosis of epithelium             | 4*                                       | 3(*)         | –           | –            |
| Calcinosis                         | 4*                                       | –            | –           | –            |
| Foci of hyperplasia (regeneration) | 3(*)                                     | –            | –           | –            |
| Embolic abscesses                  | 1  | –            | –           | –            |
| Acute tubular necrosis             | 2  | –            | –           | –            |
| Infarction (recent and healed)     | 4  | 1            | 3           | 1            |
| Pyelonephritis                     | –  | 1            | –           | –            |

<sup>a</sup> Values differ significantly from the control treatment, \*\* $P < 0.01$ , \* $P < 0.05$ ; (\*) $0.05 < P < 0.1$ . PCTs, proximal convoluted tubuli; DCTs, distal convoluted tubuli; OVC, oval cells.

cellular or cholangiocellular carcinomas developed at the higher dosage levels used (10 and 25 FB<sub>1</sub>/kg). The neoplasms detected in the rats in this study are considered to be incidental age-associated lesions as they occurred in various organs and also in the control group of animals. There was no obvious dose-response relationship between the neoplasms seen in the various groups and the level of FB<sub>1</sub> ingested. Septicemia was also significantly increased in highest dosage group. Whether this effect could be related to the adverse effects of FB<sub>1</sub> on the immune system as described by Martinova et al. (1995) is not known at present.

The lesions induced by FB<sub>1</sub> in rats in the present experiment were similar but far less severe than those seen in short- and long-term experiments in which higher dosages were used (Gelderblom et al., 1996a). The clinical pathology data correlated with the presence and extent of the lesions seen histopathologically in the liver and kidney. The changes that occurred in the liver of the FB<sub>1</sub>-treated rats, such as fibrosis, proliferation of bile duct (oval) epithelial cells, lipid accumulation and hepatocyte nodules have been described in detail elsewhere (Gelderblom et al., 1988, 1991, 1992, 1993, 1994). In this study, the peri-acinar fatty changes and cell swelling also occurred in the control rats and these changes appear to be a consequence of the diet rather than the ingestion of FB<sub>1</sub>. The histological lesions caused by FB<sub>1</sub> in the kidneys, also reflected by an increase in serum creatinine levels were present in the rats fed the 10 and 25 mg FB<sub>1</sub>/kg diet. These findings are in agreement with those reported in studies, where renal lesions were detected in male and female Sprague–Dawley rats fed 15 and 50 mg FB<sub>1</sub>/kg, respectively, for 4 weeks (Voss et al., 1993, 1995). The changes that can be ascribed to FB<sub>1</sub> in this study are those of chronic, low-grade renal toxicity in which the lesions are limited to the proximal and distal tubules. Many of the lesions seen in the kidneys of the rats in this study were those of an endemic, age-associated glomerulopathy. The severity of these age-associated lesions that appeared in terms of severity, to reflect a dose-response effect, did appear to be enhanced by exposure to the FB<sub>1</sub> in the diet. A

focal hyperplastic (or regenerative response) was seen in only three of the rats at a dosage level of 25 mg FB<sub>1</sub>/kg in the diet. In some instances, interpretation of induced lesions was hampered by the changes that could be ascribed as incidental as they were caused by embolic abscessation, acute tubular necrosis, pyelonephritis and infarction due to septicemia and disseminated abscessation in a number of rats in the advanced stage of the experiment.

With respect to the lowest observed effects, it would appear that under the present experimental conditions, a level of 1 mg FB<sub>1</sub>/kg diet still caused minimal toxic changes (histological) in the liver and kidneys of BD IX rats fed a marginally deficient diet over a period of 2 years. The clinical pathology data also reflects significant to marginal effects in serum creatinine and AST. The only significant change obtained in the liver with 1 mg FB<sub>1</sub>/kg dietary level was the induction of ground glass foci. The low dietary levels of FB<sub>1</sub> used in the present study did not induce significant ( $P > 0.05$ ) feed refusal and a reduction in body weight gain as was observed in short-term studies using high dosage levels of the toxin (Gelderblom et al., 1994). However, the relative liver weight was significantly decreased (Table 4), probably due to the inhibitory effect of FB<sub>1</sub> on hepatocyte cell proliferation (Gelderblom et al., 1994, 1996b) and the induction of apoptosis (Lemmer et al., 1999).

The current study further supports the hypothesis that a relationship exists between FB<sub>1</sub>-induced hepatotoxicity and hepatocarcinogenicity. In the short-term cancer initiating promoting assays, dietary levels of 100 mg FB<sub>1</sub>/kg and less, which only induce mild toxic effects failed to initiate cancer (Gelderblom et al., 1994). Cancer promotion occurs at lower dietary levels (up to 50 mg FB<sub>1</sub>/kg diet) in the absence of marked toxicity (Gelderblom et al., 1996b). These data suggest that a threshold exists for cancer initiation and promotion. This threshold may be a function of the level of FB<sub>1</sub>, the severity of the lesions induced and the duration of exposure. The hepatocyte nodules noticed in the rats that received 25

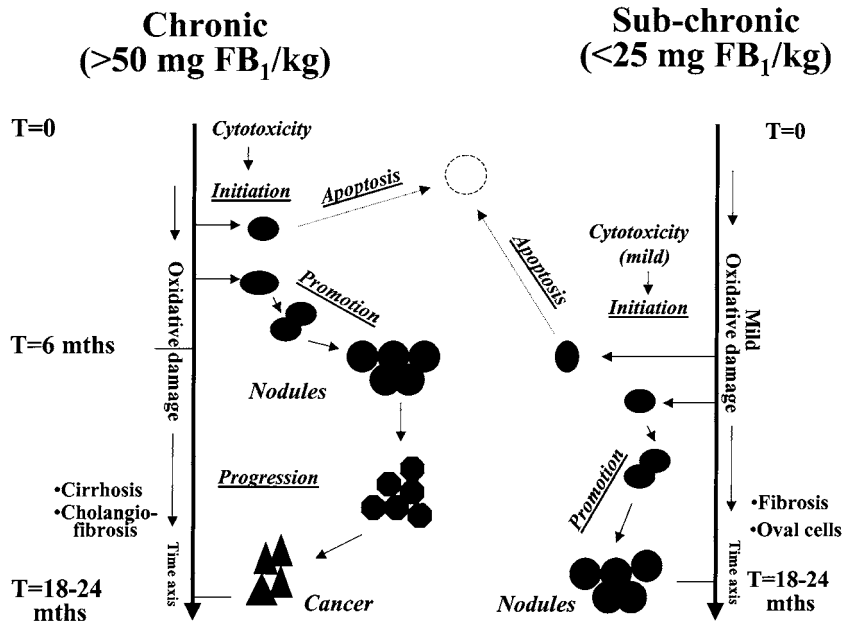


Fig. 3. Diagram illustrates the kinetics of the induction of preneoplastic and neoplastic lesions in the liver of male BD IX rats as a function of time and different dietary levels of  $FB_1$ . A dietary level of  $> 50$  mg  $FB_1$ /kg (Gelderblom et al., 1991) induces hepatocyte nodules after 6 months ( $T = 6$  months) while HCC are induced between 18 and 24 months ( $T = 18-24$  months). Dietary levels  $< 25$  mg  $FB_1$ /kg induces mild toxic effects and hepatocyte nodules between 18 and 24 months ( $T = 18-24$  months).

mg  $FB_1$ /kg (9/17) in the present experiment, indicate that the mild hepatotoxic effects induced by  $FB_1$  is associated with their development. It can be argued that the presence of only mild toxic effects caused ingestion of low levels of  $FB_1$ , delay the progression of these lesions to cancer. This is also in agreement with the threshold effect with respect to cancer promoting potential of the fumonisins (Gelderblom et al., 1996b). A cytotoxic/proliferative threshold, therefore, seems to exist with respect to induction of hepatocyte nodules and their progression into cancer. This conclusion is in agreement with the mechanism proposed for cancer induction by non-genotoxic carcinogens by Cohen and Ellwein (1990).

The induction of hepatocyte nodules and/or foci in the present study was similar with respect to GSTP and GGT staining and the histological appearance of the changes when compared with the earlier studies in which these lesions developed into neoplasms. The chronic hepatotoxicity caused by the higher levels of  $FB_1$  used in the

earlier study (Gelderblom et al., 1991) is likely to have played a critical role in the induction and progression of these early lesions into neoplasia. The mild hepatotoxicity induced in the present study resulted in less regenerative proliferation and oxidative stress than would have been the case at higher dosages. This would suggest that the kinetics of cancer induction and the subsequent development of neoplasms occur at a far slower rate when only mild hepatotoxic effects are present (Fig. 3).

Short-term studies in rats showed that the fumonisins are slow cancer initiators (Gelderblom et al., 1994). Cancer promotion, on the other hand, is effected at relative low dietary levels in the absence of excessive hepatotoxicity (Gelderblom et al., 1996b). The role of 'spontaneously' initiated cells has been considered to play a role when animals are chronically exposed to the non-genotoxic carcinogens such as nafenopin and phenobarbital (Schulte-Hermann et al., 1981; Ward and Henneman, 1990; Kraupp-Grasl et al., 1991).

The association of these pre-existing putative initiated cells in normal animals and their subsequent development into cancer appears to be unlikely as there is very little association between enzyme-altered foci and the development of the ultimate cancer, especially those that occur in older animals (Ghoshal and Farber, 1993). Recent studies suggested that the fumonisins (Gelderblom et al., 1992, 1994) and the peroxisome proliferators, ciprofibrate (Rao and Reddy, 1991) and clofibrate (Nagai et al., 1993) exhibit cancer-initiating potential. This implies that the role of 'spontaneous' initiated cells in fumonisin-induced hepatocarcinogenesis or the induction of hepatocyte nodules in the present study cannot be proven beyond doubt. This becomes evident in recent studies, which indicate that oxidative damage induced by FB<sub>1</sub> in vitro and in vivo in the liver is likely to be involved in the cancer initiating activity of the compound (Abel and Gelderblom, 1998; Sahu et al., 1998; Yin et al., 1998). The high dosage FB<sub>1</sub>-treated rats also exhibited enhanced lipid peroxidation in the liver in the present study.

The presence of the early preneoplastic lesions (GSTP<sup>+</sup> foci and/or nodules) needs to be discussed in relation to the carcinogenic properties of the fumonisins and the determination of risk assessment parameters. These lesions are generally considered as the site from which cancer develops in the liver of rats (Farber, 1992). The ability of carcinogens to induce these early, 'potentially' preneoplastic lesions has been frequently used to assess the carcinogenic potential of naturally occurring compounds, which are important in risk determination (Tsuda and Farber, 1980). A model for estimating the risk of carcinogen exposure in humans was developed by incorporating information on both intermediate lesions, such as altered hepatocyte foci, as well as malignant tumours (Moolgavkar and Luebeck, 1995) into the assessment. Such risk assessment parameters have been calculated (Gelderblom et al., 1996a) for the carcinogenic potential of FB<sub>1</sub> based on the data obtained from long-term studies in BD IX rats (present study, Gelderblom et al., 1991). The dietary level of 25 mg FB<sub>1</sub>/kg was considered to be the No Observed Effect Level (NOEL) for cancer induction of the fumonisins as a diet of 50 mg

FB<sub>1</sub>/kg resulted in 66% of the rats developing cancer under similar experimental conditions. The corresponding Tolerable Daily Intake (TDI) value for cancer induction in humans, using a safety factor of 1000 was 0.8 µg FB<sub>1</sub>/kg body weight per day (Gelderblom et al., 1996a). Based on the current data, the Lowest Observed Effect Level (LOEL) in male BD IX rats with respect to the induction of GSTP/GGT<sup>+</sup> nodules and/or foci, is between 10 and 25 mg FB<sub>1</sub>/kg or a FB<sub>1</sub> intake of between 0.3 and 0.8 mg FB<sub>1</sub>/kg body weight per day. The NOEL for the induction of hepatocyte nodules in male BD IX rats is < 10 mg FB<sub>1</sub>/kg diet (0.3 mg FB<sub>1</sub>/kg body weight). The NOEL of FB<sub>1</sub> for hepato- and nephrotoxicity in male BD IX rats appear to be in the region of 1 mg FB<sub>1</sub>/kg diet or an intake of 0.03 mg FB<sub>1</sub>/kg body weight per day.

The interpretation of these risk assessment parameters are complicated, as accurate FB intake profiles have not been established to determine Probable Daily Intakes (PDI) in humans. In addition, FB-intake depends not only on the level of corn contamination but also on the pattern of consumption of corn by the population at risk (Gelderblom et al., 1996a). Other important parameters include the pharmacokinetics, biochemical mode of action, threshold effects, as well as the dependence on cell proliferation and dietary deficiencies (Butterworth and Goldsworthy, 1991; Gelderblom et al., 1991). These factors have to be considered against the background that FB<sub>1</sub> is carcinogenic to rats only at dosages that cause overt hepatotoxic lesions that may culminate in cirrhosis. As the fumonisins co-occur with aflatoxin B<sub>1</sub> (Chamberlain et al., 1993; Chu and Li, 1994), the synergistic interaction between these carcinogens, as well as other carcinogenic stimuli such as a chronic hepatitis B virus infection have to be critically evaluated to assess the risk of the fumonisins to humans. This is of particular interest as the ingestion of the fumonisins is implicated as a possible risk factor in the development of primary liver cancer in endemic high liver cancer areas in China (Ueno et al., 1997).

Based on the present findings, important parameters such as the modulation of cell prolifer-



eration and dietary deficiencies, which could enhance the progression of early pre-malignant lesions into neoplasia need to be considered in future studies to establish relevant risk assessment parameters for the fumonisins in humans.

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Toxicology 171 (2002) 161–173

**TOXICOLOGY**

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## Interaction of fumonisin B<sub>1</sub> and aflatoxin B<sub>1</sub> in a short-term carcinogenesis model in rat liver

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Received 29 June 2001; received in revised form 21 September 2001; accepted 12 November 2001

### Abstract

The co-existence of the fumonisin and aflatoxin mycotoxins in corn merited studies to investigate their possible synergistic toxicological and carcinogenic effects. When utilising a short-term carcinogenesis model in rat liver, both the compounds exhibited slow cancer initiating potency as monitored by the induction of foci and nodules stained positively for the placental form of glutathione-*S*-transferase (GSTP<sup>+</sup>). However, when rats were treated in a sequential manner with AFB<sub>1</sub> and FB<sub>1</sub> the number and size of GSTP<sup>+</sup> lesions significantly increased as compared to the separate treatments. Histopathological analyses indicated that the individual treatments showed far less toxic effects, including occasional hepatocytes with dysplastic nuclei, oval cell proliferation and, in the case of FB<sub>1</sub>, a few apoptotic bodies in the central vein regions. The sequential treatment regimen induced numerous foci and dysplastic hepatocyte nodules, and with oval cells extending from the periportal regions into the centrilobular regions. This would imply that, in addition to the cancer promoting activity of FB<sub>1</sub> of AFB<sub>1</sub>-initiated hepatocytes, the AFB<sub>1</sub> pre-treatment enhanced the FB<sub>1</sub> initiating potency, presumably by rendering the liver more susceptible to the toxic effects of FB<sub>1</sub>. The co-occurrence of AFB<sub>1</sub> and FB<sub>1</sub> in corn consumed as a staple diet could pose an increased risk and should be included in establishing risk assessment parameters in humans. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

*Keywords:* Fumonisin B<sub>1</sub>; Aflatoxin B<sub>1</sub>; Rat liver

### 1. Introduction

The bulk of our knowledge regarding the adverse effects of naturally occurring mutagens and carcinogens and their possible effects on human health are obtained by investigating the biological effects of purified compounds in experimental ani-

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mals. In contrast very little is known about the interaction of complex mixtures in biological systems. A single compound may effect different reactions within one biological system while it may also exhibit additive, antagonistic or synergistic interactions with other compounds (Carpenter et al., 1998). The co-occurrence of mycotoxins together with various endogenous and exogenous risk factors such as nutrition, viral infections, alcohol and tobacco that may modulate cell proliferation and drug metabolism, are known to act synergistically with respect to the development of cancer in animals and possibly in humans (Yu et al., 1996). The multiplicity and complexity of these interactions need to be elucidated in order to clarify the adverse health and environmental effects of a mixture of compounds and their subsequent regulation.

Numerous studies have been conducted on the aflatoxins, secondary metabolites produced by species in the genus *Aspergillus*, that are natural contaminants of human foods and animal feeds. They are known to be potent liver carcinogens in laboratory animals (Wogan, 1973, 1992; Wogan et al., 1974). Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) has been implicated as one of the etiological factors, apart from hepatitis B virus, in the development of human liver cancer (Van Rensburg et al., 1985; Peers et al., 1987; JECFA, 1998). In transgenic mice it was shown that the chronic liver damage due to the over expression of the hepatitis B virus large peptide envelope acts synergistically with AFB<sub>1</sub> with respect to neoplastic development (Sell et al., 1991). The recently discovered fumonisin B mycotoxins (Gelderblom et al., 1988) are produced by *Fusarium* spp. that contaminate corn worldwide (Rheeder et al., 1992; Shephard et al., 1996). The fumonisins are non-mutagenic in different bacterial assays (Gelderblom and Snyman, 1991a; Knasmuller et al., 1997) and lack genotoxicity in short-term DNA repair assays in vivo and in vitro (Gelderblom et al., 1992). However, a recent study indicated that the fumonisins exhibited clastogenic effects in primary hepatocytes (Knasmuller et al., 1997). Long-term studies indicated that fumonisin B<sub>1</sub> (FB<sub>1</sub>) is hepatocarcinogenic in male BD IX rats (Gelderblom et al., 1991b, 2001a) while a recent study reported on the nephrocarcinogenicity and

cancer promoting activity in male Fischer rats and the liver of female B6C3F<sub>1</sub> mice, respectively (Howard et al., 2001). The occurrence of FB<sub>1</sub> in corn is associated with the incidence of oesophageal (Rheeder et al., 1992; Chu and Li, 1994) and liver cancer (Ueno et al., 1997) in humans.

Several studies reported on the co-occurrence of aflatoxin B<sub>1</sub>, the fumonisins and the trichothecenes deoxynivalenol, nivalenol and zearalenone (Gao and Yoshizawa, 1997; Ueno et al., 1997; Zhang et al., 1997; Ali et al., 1998). At present little is known about the interaction between AFB<sub>1</sub> and FB<sub>1</sub> with regard to their toxic and carcinogenic properties. FB<sub>1</sub> is known to exhibit different kinetics with respect to cancer initiation as compared to genotoxins, presumably due to the inhibitory effect on hepatocyte proliferation (Gelderblom et al., 1994, 1996a, 2001b) and induction of apoptosis (Lemmer et al., 1999). On the other hand the inhibitory effect on cell proliferation is suggested to be the major mechanism for the cancer promoting property of the fumonisins (Gelderblom et al., 1995, 1996b, 2001c). Cancer initiation by AFB<sub>1</sub> in rat liver is similar to that seen with FB<sub>1</sub>, and is not readily effected by a single toxic dosage, but rather by a low-level prolonged feeding regimen (Godoy et al., 1976; Neal and Cabral, 1980). The cytotoxicity and inhibitory effect on hepatocyte proliferation effected by AFB<sub>1</sub>, prohibited the induction of a population of 'resistant' hepatocytes with a reduced sensitivity to the cytotoxic effects of AFB<sub>1</sub>. In general it would appear that the toxic and/or carcinogenic response to AFB<sub>1</sub> and FB<sub>1</sub> in terms of the induction and rate of progression of hyperplastic lesions depend on the time of exposure, the dose levels and the rate of cell proliferation.

The present paper reports on the interaction of AFB<sub>1</sub> and FB<sub>1</sub> in a short-term cancer model in rat liver.

## 2. Materials and methods

### 2.1. Chemicals

FB<sub>1</sub> was purified according to the method described by Cawood et al. (1991) to a purity of

90–95%. A major impurity (3–4%) was the mono methyl ester of FB<sub>1</sub>, which is known to exhibit similar properties with respect to cancer initiation (Gelderblom et al., 1993). Diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF) were obtained from Sigma Chemical Company and dissolved in dimethylsulfoxide (DMSO) while AFB<sub>1</sub> was obtained from Makor Chemicals Ltd and dissolved in DMSO:H<sub>2</sub>O (1:1). Glutathione-S-transferase placental form (GSTP) and alpha smooth muscle ( $\alpha$ SMA) antibodies were obtained from DAKO and the OV-6 antibody was a generous gift from Professor S. Sell, Albany, NY.

## 2.2. Animals and diets

The FB<sub>1</sub> diet (250 mg FB<sub>1</sub>/kg) used was prepared as described previously (Gelderblom et al., 1994). In short, the FB<sub>1</sub> was first dissolved in methanol and added to a sub sample (200 g) of the diet and dried overnight. The sub-sample was diluted with the AIN-76 diet (American Institute of Nutrition, 1980) to obtain the desired FB<sub>1</sub> concentration (250 mg/kg diet) and stored under nitrogen at –20 °C for the duration of the experiment. Male Fischer rats, weighing approximately 150 g, were fed a modified AIN-76 diet when weaned. They were randomised (5–8 animals/group) and housed separately prior to the start of the experiment and weighed three times weekly.

## 2.3. Treatment models

Two different protocols were used to investigate the cancer potency of AFB<sub>1</sub> and FB<sub>1</sub>. (Fig. 1) The first protocol compared the cancer initiation potency of AFB<sub>1</sub> and FB<sub>1</sub> with that of DEN, utilising the resistant hepatocyte model with 2-AAF/partial hepatectomy (PH) as the promoting stimuli (Semple-Roberts et al., 1987). Initiation was effected by either a single initiating dose of DEN (200 mg/kg; i.p.) or a daily per gavage dose (17  $\mu$ g/kg bw) of AFB<sub>1</sub> over a period of 14 days to obtain a total dosage of 0.25 mg AFB<sub>1</sub>/kg bw or by feeding FB<sub>1</sub> (250 mg FB<sub>1</sub>/kg) over a period of 21 days as described previously (Gelderblom et al., 1994). Promotion commenced

three weeks after initiation. In the case of the 2-AAF/PH promoting regimen the rats received a gavage dosage of 2-acetylaminofluorene (20 mg/kg bw) on three consecutive days followed by partial hepatectomy on day four. Control groups included 2-AAF/PH and PH groups for cancer promotion and DMSO and DMSO:H<sub>2</sub>O (1:1) as the initiation controls for DEN and AFB<sub>1</sub> treatments respectively. In the second protocol, referred to as the sequential treatment model, the combined effects of AFB<sub>1</sub> and FB<sub>1</sub> were monitored. Rats were treated with AFB<sub>1</sub> (17  $\mu$ g AFB<sub>1</sub>/kg bw/day per gavage) over a period of 14 days followed by FB<sub>1</sub> treatment (250 mg/kg diet) 3 weeks later over a period of 21 days. Control groups consisted of a 14 day DMSO:H<sub>2</sub>O (1:1) treatment (0.2 ml/100g bw), a 3 week FB<sub>1</sub> dietary treatment and the AFB<sub>1</sub> gavage treatment over a 14-day period.

## 2.4. Necropsy and histopathology

Rats were killed 3 weeks following the 2-AAF/PH promoting treatment and immediately after the FB<sub>1</sub> treatment when using the sequential treatment model (Fig. 1). Rats were killed by decapitation, liver weights recorded and liver slices were fixed in neutral buffered formalin (pH 7.4) for routine processing, staining with haematoxylin and eosin (H&E) and immunohistochemical staining as described below. The sirius red stain was used to confirm the presence of fibrous tissue in livers that appeared to be cirrhotic. For the purpose of histopathological examination a ‘focus’ of hepatocyte dysplasia was defined as <1 mm (Wanless et al., 1992).

## 2.5. Immunohistochemistry

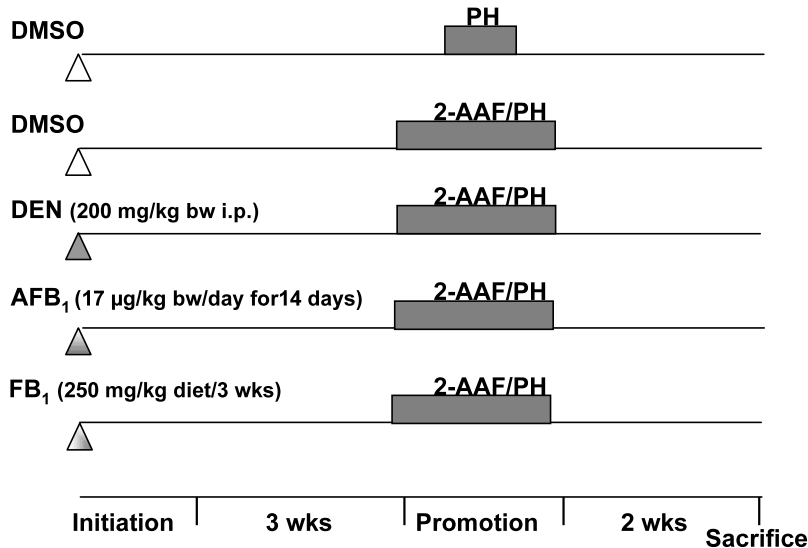
Dewaxed sections were immunostained using a standard three stage indirect streptavidin–biotin technique; peroxidase activity was developed using the chromagen substrate diaminobenzidine and GSTP<sup>+</sup> staining was used to identify initiated hepatocytes (Ogawa et al. 1980). The number and size (area) of the ‘enzyme altered’ foci were quantified microscopically ( $\times 10$  magnification) and categorised according to the number of cells

per focus or ‘minifocus’ (< 5, < 20 and > 20 cells per focus) and expressed as GSTP<sup>+</sup> lesions per cm<sup>2</sup> of the liver section.

The mouse monoclonal antibody OV-6, origi-

nally raised against the hepatocytes isolated from nodules from the liver of 2-AAF treated rats also stains bile ducts (Dunsford and Sell, 1998). In rats OV-6 recognises a cytokeratin with epitopes

### Resistant hepatocyte model



### Sequential treatment model

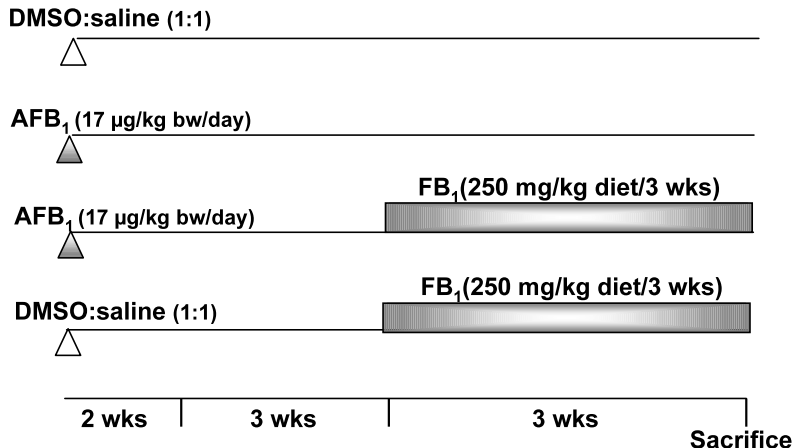


Fig. 1. A schematic diagram illustrating the different experimental and control treatments. The cancer initiating and promoting treatment with FB<sub>1</sub> consisted of the 3 week feeding period (250 mg FB<sub>1</sub>/kg diet), while with AFB<sub>1</sub> a daily gavage dosage over a period of two weeks was administered. Initiation by diethylnitrosamine (DEN) consisted of a single i.p. dosage. In all the cases a 3 week recovery period was allowed before the promoting regimen commenced.

shared on both CK 14 and CK 19 (Bisgaard et al., 1993). The amount of oval cell proliferation was graded according to how far the cells extended from the periportal regions into the acini—grade 1, oval cells limited to the periportal region; grade 2, oval cell extending through zone 1 into zone 2; and, grade 3, oval cells extending through all zones of the acinus. The  $\alpha$ -smooth muscle antibody ( $\alpha$ -SMA) antibody (Dako A/S Copenhagen, Clone 1A4 code M0851) was used to demonstrate stellate (Ito) cells.

### 2.6. Statistical analyses

Statistical analyses were performed by the analyses of variance (ANOVA) using the SAS program while the Tukey Studentized Range method was used to test for significant differences between the means. The interaction between  $\text{FB}_1$ ,  $\text{AFB}_1$  and 2-AAF/PH treatments was also determined. The Kolmogorov–Smirnov test was used to test the data for normality (5% significance level). As there were only two groups, which needed to be tested for group differences, and the data were normally distributed, the Student's  $t$ -test was used ( $P < 0.05$ ). Tests for testing the equality of variances were done beforehand. Depending on whether the variances were equal or unequal, the Pooled or Satterthwaite methods were used for the  $t$ -test. The three interactive plots are the mean GSTP values for each of  $\text{FB}_1$  and  $\text{AFB}_1$  when comparing it with the YES and NO groups for each of the 2-AAF/PH and  $\text{AFB}_1$  variables.

## 3. Results

The relative liver weight of the  $\text{AFB}_1$ ,  $\text{FB}_1$ , and the sequentially treated ( $\text{AFB}_1/\text{FB}_1$ ) rats was significantly ( $P < 0.01$ ) reduced (Table 1) compared to the control treatment. However, the relative liver weight of the rats receiving  $\text{AFB}_1$  and the combined  $\text{AFB}_1/\text{FB}_1$  treatments were significantly ( $P < 0.05$ ) higher as compared to the  $\text{FB}_1$  treatment group. These changes are reflected in the body weight gain of the rats subjected to the different treatment regimens. No dietary intake

was conducted but a daily intake of 15 mg  $\text{FB}_1/\text{kg}$  bw was reported from a feeding study using a dietary level of 250 mg  $\text{FB}_1/\text{kg}$  diet over a period of 21 days carried out under similar experimental conditions (Gelderblom et al., 1994).

### 3.1. GSTP<sup>+</sup> foci and nodules

When the  $\text{AFB}_1$  and  $\text{FB}_1$ -treated rats were subjected to the 2-AAF/PH promoting treatment (resistant hepatocyte model, Fig. 1), a significant ( $P < 0.001$ ) increase in the number of the larger GSTP<sup>+</sup> lesions was noticed with no significant differences between the two regimens (Table 1). The total  $\text{AFB}_1$  intake during the 14 day period was 0.25 mg/kg body weight while dietary studies showed that the  $\text{FB}_1$  intake of the rats over the 21 day treatment period is in the order of 308 mg/kg body weight/day (Gelderblom et al., 1994). The induction of GSTP<sup>+</sup> lesions was also significantly enhanced in rats treated either with  $\text{AFB}_1$  or  $\text{FB}_1$  without the 2-AAF/PH promoting stimuli. The sequential treatment (sequential treatment model, Fig. 1) of the rats with  $\text{AFB}_1$  and  $\text{FB}_1$  was more effective in the induction of GSTP<sup>+</sup> lesions into foci and/or nodules than the 2-AAF/PH promoting treatment of the individual treatments. The  $\text{AFB}_1/\text{FB}_1$  treatment not only increased the size of the foci but also the number by a factor of at least fivefold indicating that the sequential treatment exceeded the additive effect of the separate treatments even after 2-AAF/PH selection (Table 1). The different interactions of  $\text{FB}_1$  and  $\text{AFB}_1$ , with each other, or with the 2-AAF/PH promoting treatment are illustrated in Fig. 2. A highly significant interaction ( $P = 0.001$ ) is noticed between  $\text{AFB}_1$  and  $\text{FB}_1$  in the sequential treatment model. Under the present experimental conditions a higher interaction ( $P = 0.03$ ) was obtained between  $\text{FB}_1$  and the 2-AAF/PH promoting regimen than with the  $\text{AFB}_1$  initiating treatment ( $P = 0.07$ ) using the resistant hepatocyte model.

### 3.2. Histopathology

The livers from some control rats showed an occasional mitotic figure but no abnormal changes were seen in any of the livers. The livers

Table 1  
Induction of GSTP<sup>+</sup> foci by single and combined treatment of AFB<sub>1</sub> and FB<sub>1</sub> utilising different carcinogenesis models in rat liver

| Group                             | Initiation             | Promotion                                | Liver weight (% of bw <sup>b</sup> ) | GSTP <sup>+</sup> foci and/or nodules |                           |                           |                                   |
|-----------------------------------|------------------------|--|--------------------------------------|---------------------------------------|---------------------------|---------------------------|-----------------------------------|
|                                   |                        |  |                                      | <5 cells/cm <sup>2</sup>              | <20 cells/cm <sup>2</sup> | >20 cells/cm <sup>2</sup> | Total (>5 cells/cm <sup>2</sup> ) |
| <i>Resistant hepatocyte model</i> |                        |  |                                      |                                       |                           |                           |                                   |
| Control (DMSO)                    | 0.2 ml/100 g bw        | PH                                       | ND                                   | –                                     | –                         | –                         | –                                 |
| Control (DMSO)                    | 0.2 ml/100 g bw        | 2-AAF/PH                                 | ND                                   | 1.18 ± 0.65a                          | –                         | –                         | 0.04 ± 0.13a                      |
| FB <sub>1</sub>                   | 250 mg/kg diet/21 days | 2-AAF/PH                                 | ND                                   | 0.73 ± 0.25a                          | 0.18 ± 0.19a              | 3.97 ± 1.65a              | 4.16 ± 1.55A                      |
| AFB <sub>1</sub> <sup>a</sup>     | 1.8 µg/100g bw/day     | 2-AAF/PH                                 | ND                                   | –                                     | 2.86 ± 2.46a              | 9.17 ± 8.00a              | 12.03 ± 8.20A                     |
| DEN                               | 200 mg/kg bw           | 2-AAF/PH                                 | ND                                   | 2.60 ± 1.30A                          | 8.99 ± 4.25b              | 29.61 ± 14.34B            | 38.60 ± 17.86B                    |
| <i>Sequential treatment model</i> |                        |  |                                      |                                       |                           |                           |                                   |
| Control(DMSO)                     | 0.2 ml/100 g bw        | –  | 4.0 ± 0.26a<br>(29.5 ± 5.8)          | 0.14 ± 0.36a                          | 0.04 ± 0.13a              | –                         | 0.04 ± 0.13a                      |
| AFB <sub>1</sub> <sup>a</sup>     | 1.8 µg/100g bw/day     | –  | 3.32 ± 0.19Ab<br>(19.7 ± 7.6)        | –                                     | 1.40 ± 0.94a              | 3.62 ± 3.00a              | 5.02 ± 2.95b                      |
| AFB <sub>1</sub> <sup>a</sup>     | 1.8 µg/100g bw/day     | FB <sub>1</sub> (250 mg/kg diet/21 days) | 3.01 ± 0.21Ab(20.3 ± 8.6)            | –                                     | 4.15 ± 1.84A              | 67.61 ± 32.28A            | 71.76 ± 32.69B                    |
| DMSO/Saline <sup>a</sup>          | 0.2 ml/100g bw/day     | FB <sub>1</sub> (250 mg/kg diet/21 days) | 2.50 ± 0.31Ac<br>(8.5 ± 7.5)         | 0.10 ± 0.23a                          | 0.92 ± 0.79a              | 0.73 ± 0.92a              | 1.65 ± 1.47b                      |

Data represent the means ± SD. Means followed by the same letter do not differ significantly, if the letter differ then  $P < 0.05$ , if the cases differ then  $P < 0.001$ .

<sup>a</sup> Dosed on a daily basis for a period of 14 days.

<sup>b</sup> Values in brackets indicate the body weight gain. ND, not determined.



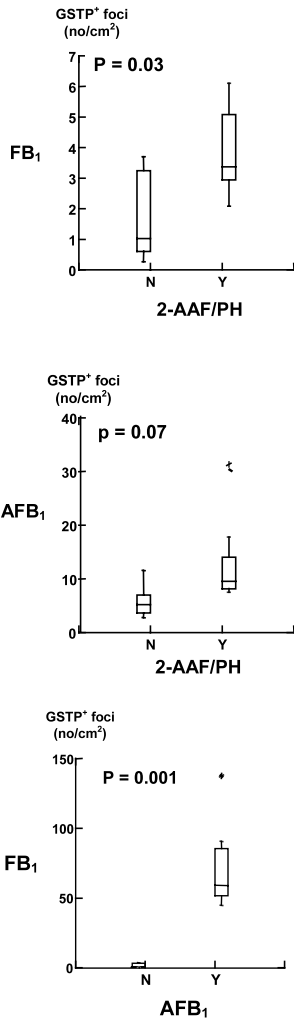


Fig. 2. Statistical box plots representation of the interaction between FB<sub>1</sub> (Fig. 2a), AFB<sub>1</sub> (Fig. 2b) and the cancer promoting 2-AAF/PH treatment with respect of the induction of GSTP<sup>+</sup> foci in the liver. The interaction between FB<sub>1</sub> and AFB<sub>1</sub> is illustrated in Fig. 2c. Date represents the median, 75 percentile (upper limit) and 25 percentile (lower limit). N, No and Y, Yes and reflects the absence or presence of the variable.

of rats that had a partial hepatectomy showed increased numbers of mitoses indicating regeneration. Rats that received the DMSO showed slightly increased numbers of mitoses suggesting regeneration after minor liver injury. Rats that received the combined DMSO and 2-AAF/PH promoting treatment showed occasional apoptotic cells, and 6/7 of the livers showed increased num-

bers of mitotic figures, some of which were abnormal. Four rats showed occasional single dysplastic-appearing hepatocytes. All of the livers showed oval cell proliferation, which ranged from grade 1 to grade 2, i.e. the oval cells extended from the periportal zone 1 region, or through zone 1 into zone 2, respectively. The oval cells formed cords that intermingled with individual hepatocytes in a 'lace-like' pattern. No nodules were identified. However, no oval cell proliferation, foci or nodule formation were noted in any of the control groups of animals. FB<sub>1</sub> treatment resulted in liver cell death mainly through apoptosis. Hepatocyte regeneration was seen as increased numbers of mitoses. The apoptotic cells were predominantly found in zone 3 around the central veins and there was an impression of a minor degree of zone 3 collapse. Four livers showed an occasional single dysplastic hepatocyte, while the liver from one rat showed grade 1 oval cell proliferation confined to the periportal (zone I) region. Treatment with AFB<sub>1</sub> alone did not cause an increase in the number of apoptotic cells or mitoses. In 3/8 animals occasional dysplastic hepatocyte nuclei were observed. These nuclei were enlarged, hyperchromatic and pleomorphic with a coarse chromatin pattern (Fig. 3). Oval cell proliferation, extending through zones 1 and 2 of the acinus (grade 2), was seen in the livers of 5/8 animals. Although no nodules were identified, a focus of small hepatocytes was seen in one rat.

The sequential AFB<sub>1</sub>/FB<sub>1</sub> administration resulted in increased numbers of both apoptotic cells and mitoses. The livers of all animals were cirrhotic with the majority of the regenerative nodules being less than 1 mm in diameter (Fig. 4). In each animal some lobes showed more architectural disturbance than others. Thin bands of fibrous tissue, confirmed by staining with sirius red, were seen encircling the nodules of hepatocytes and large numbers of oval cells were seen within the fibrous tissue (Fig. 5a, 5b). Sheets of OV-6 positive oval cells also surrounded single hepatocytes. Scattered hepatic stellate cells, immunoreactive for  $\alpha$ -SMA and OV-6 negative, were also demonstrated in the bands of fibrous tissue amongst the sheets of oval cells. In the cirrhotic livers the distinction between regenera-

tive nodules and dysplastic hepatocyte nodules was difficult. The presence of mitoses, abnormal nuclei and cytoplasmic eosinophilic changes in the hepatocytes in the nodules, which measured over 1mm in size, favoured the interpretation that most of the nodules were dysplastic. The largest dysplastic nodule identified measured 3 mm in diameter. In addition, multiple foci that showed eosinophilic cell and large cell change, were seen in all animals. Occasional single cells with dysplastic nuclei were also identified in these foci. Occasional dysplastic nuclei were also seen in the

proliferating oval cell-derived ductular structures. No hepatocellular carcinomas were identified but several dysplastic hepatocyte nodules showed a focal spindle cell pattern, the significance of which was not known.

In the AFB<sub>1</sub> treated group subjected to the 2-AAF/PH promoting regimen apoptotic cells were not prominent, but again increased numbers of mitoses were identified. The liver of one rat showed a cirrhotic appearance. All livers showed oval cell proliferation although there was some variation in the number and distribution of the

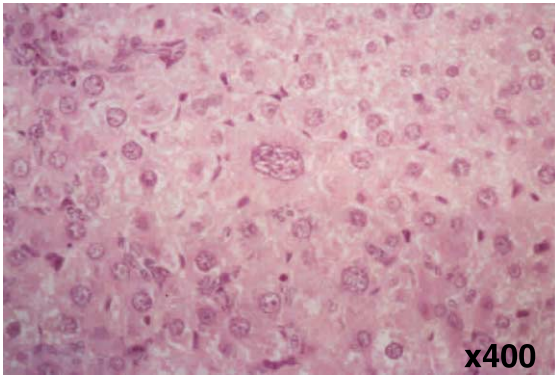
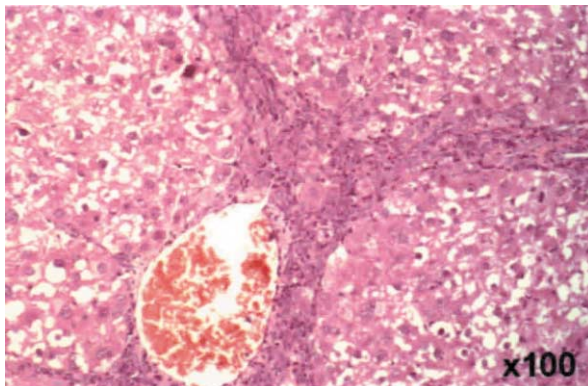


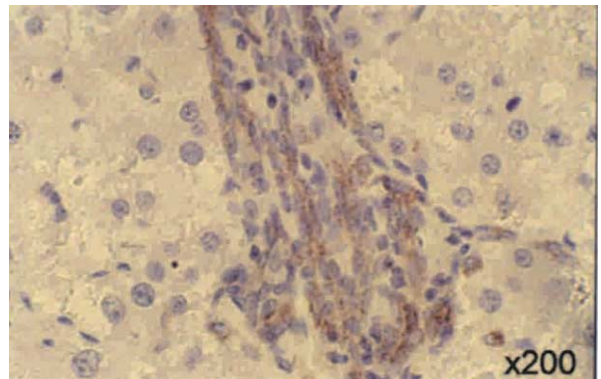
Fig. 3



Fig. 4



(a)



(b)

Fig. 5

Fig. 3. Hepatocellular dysplasia in an AFB<sub>1</sub> treated rat. The hepatocyte nucleus (centre) displays hyperchromatic and pleomorphic features with a coarse chromatin pattern H&E × 400.

Fig. 4. Cirrhotic liver of a rat that received sequential AFB<sub>1</sub>/FB<sub>1</sub> treatment. The features are those of a micronodular cirrhosis, many regenerative nodules being less than 1 mm in diameter. H&E × 25.

Fig. 5. (a) Proliferating oval cells surrounding regenerative nodules in a rat that received the sequential AFB<sub>1</sub>/FB<sub>1</sub> treatment. H&E × 100. (b) Proliferating oval cells between parts of two regenerative nodules in a rat that received the sequential AFB<sub>1</sub>/FB<sub>1</sub> treatment. Immunostained for OV-6 × 200.

cells ranging from grade 1 to grade 3. The oval cells formed cords, intermingling between individual hepatocytes in a lace-like pattern. Some cells with the morphology of hepatocytes were noted within ductule walls. In addition occasional small groups of small basophilic cells with a morphological appearance of cells in transition from biliary cells to hepatocytes, so called transitional hepatocytes, were identified. Single dysplastic hepatocytes were identified in the livers of six animals. In five animals foci and dysplastic hepatocyte nodules were identified, the largest of which was 3.1 mm in size. Some basophilic foci were seen and the dysplastic hepatocyte nodules showed mixed eosinophilic and large cell dysplasia and clear cell change. Oval cells were observed forming a rim around and penetrating the nodules and penetrating small foci of eosinophilic hepatocytes. In addition a small cholangiofibroma was observed in one rat. In the rats that received FB<sub>1</sub> and subjected to the 2-AAF/PH-stimulus, apoptotic cells were not prominent but increased numbers of mitoses were identified. All animals showed oval cell proliferation extending from the periportal to the central areas, i.e. grade 1- grade 3. The oval cells formed cords, intermingling between individual hepatocytes in a lace-like pattern. Single dysplastic hepatocytes were identified in the livers of 6/6 animals. In 3/6 animals foci were seen and in 4/6 animals dysplastic hepatocyte nodules were identified, the largest of which was 3.4 mm in size. The dysplastic nodules showed mixed large cell dysplasia, eosinophilic and clear cell change.

#### 4. Discussion

Information regarding the potential synergistic interaction between AFB<sub>1</sub> and FB<sub>1</sub> could be important for risk assessment analyses for liver cancer development in humans. A recent study in growing barrows showed that diets containing both AFB<sub>1</sub> and FB<sub>1</sub> induce liver disease more effectively than the individual toxins (Harvey et al., 1995). In a similar study, FB<sub>1</sub> and deoxynivalenol (DON) induced a more toxic response regarding clinical performances, serum biochemical,

hematologic and immunologic values than that induced by either toxin separately (Harvey et al., 1996). Therefore, sequential or combined administration of mycotoxins may give additive and sometimes synergistic results. Induction of metabolic enzymes related to aflatoxin metabolism (Eaton and Gallagher, 1994) as well as changes in the rate of cell proliferation in the liver are known to alter carcinogenesis induced by AFB<sub>1</sub> (Neal and Cabral, 1980; Hiruma et al., 1996).

The present study investigated the interaction of FB<sub>1</sub> and AFB<sub>1</sub> by comparing their separate and combined effects on the cancer initiating and promoting phases of hepatocarcinogenesis in rats. The effective dosage level (EDL) for cancer initiation by FB<sub>1</sub> treatment (per gavage) over a period of 14 and a feeding study over 21 days in male Fischer rats is  $0.39 < \text{EDL} < 0.83$  and  $0.7 < \text{EDL} < 1.5$  mg FB<sub>1</sub>/100g bw per day, respectively (Gelderblom et al., 1994). When compared to the gavage treatment over 14 days using AFB<sub>1</sub> (present study), FB<sub>1</sub> is between 200 and 400 times less potent as a cancer initiator, further emphasizing the weak cancer initiating potential of FB<sub>1</sub>. The differences in the cancer initiating potencies could be related to the mechanisms involved during the initiating phase of cancer induction. FB<sub>1</sub> lacks genotoxicity and mutagenic effects although one study indicates that FB<sub>1</sub> induces clastogenic effects in primary hepatocytes (Knasmüller et al., 1997). Oxidative damage, secondary to the hepatotoxicity of FB<sub>1</sub>, was proposed as a possible mechanism for cancer initiation in rat liver (Gelderblom et al., 1996b; Abel and Gelderblom, 1998; Abado-Becongnee et al., 1998). The hepatotoxicity and subsequent lipid peroxidation could explain the delayed response and dose-dependent threshold effect associated with FB<sub>1</sub>-induced cancer initiation as compared to the genotoxic carcinogens that interact directly with the DNA. In this regard, AFB<sub>1</sub> exhibits potent mutagenic and genotoxic properties in different *in vitro* and *in vivo* test systems (Eaton and Gallagher, 1994). Similar to FB<sub>1</sub>, AFB<sub>1</sub> also effects oxidative damage in primary rat hepatocytes and in rat liver *in vivo* (Shen et al., 1995a,b), which has been suggested to be involved during cancer initiation of the latter mycotoxin.

FB<sub>1</sub> significantly reduced the relative liver weight similar to that reported previously (Gelderblom et al., 1995, 1996a; Lemmer et al., 1999), indicating that the resultant hepatotoxicity, apoptosis and inhibitory effect on cell proliferation, interferes with normal growth related processes and hence the disruption of normal liver homeostasis. The reduction in the relative liver weight by the AFB<sub>1</sub> treatment is possibly due to the high cytotoxicity and inhibition of hepatocyte cell proliferation (Neal and Cabral, 1980). However, the marked increase in the sizes and numbers of hepatocyte nodules in the liver of the rats receiving the sequential treatment resulted in a significant increased liver weight as compared to the separate treatments. Treatment of the rats with either AFB<sub>1</sub> or FB<sub>1</sub> only shows mild toxicological effects including mitoses, apoptosis and occasional hepatocytes with dysplastic nuclei and oval cell proliferation. However, the sequential treatment resulted in cirrhotic livers with numerous regenerative and dysplastic nodules encircled extensively by oval cells. Therefore, the sequential treatment of AFB<sub>1</sub> and FB<sub>1</sub> extensively enhance the susceptibility of the liver to the toxicity and the induction of dysplastic nodules as compared to the separate treatments. However, mechanistically this interaction is extremely complex and appears to involve different biological parameters regarding toxicity, cancer initiation and promotion that facilitate the induction and subsequent development of early pre-neoplastic lesions. The effect of a simultaneous treatment regimen of AFB<sub>1</sub> and FB<sub>1</sub> on the induction of hepatocyte nodules was not monitored under the present experimental design. The inhibitory effect on cell proliferation and induction of apoptosis (Lemmer et al., 1999) by FB<sub>1</sub> is likely to reduce the capacity of AFB<sub>1</sub>-induced initiation as was reported for initiation effected by DEN (Lebepe-Mazur et al., 1995). However, other experimental designs need to be developed to investigate such a treatment regimen.

FB<sub>1</sub> increased GSTP<sup>+</sup> lesions to a similar extent as AFB<sub>1</sub>, even though the AFB<sub>1</sub>-treated rats were killed 5 weeks after the initiating treatment as compared to the FB<sub>1</sub> rats that were terminated immediately after the 3 week treatment. When

utilising 2-AAF/PH promoting treatment the number of large foci (> 20 cells/foci) increased markedly or significantly in the AFB<sub>1</sub> and FB<sub>1</sub> groups, respectively. However, when compared to the rats that received the DEN treatment (single gavage dose), both AFB<sub>1</sub> and FB<sub>1</sub> were weak or slow cancer initiators under the present experimental conditions. As cell proliferation is a prerequisite for initiation (Columbano et al., 1981), the inhibitory effect on hepatocyte cell proliferation and apoptotic effects induced by AFB<sub>1</sub> and FB<sub>1</sub> has been suggested to be responsible for the slow cancer initiating potential of these compounds (Gelderblom et al., 1996b; Neal and Cabral, 1980). Pre-treatment of Sprague-Dawley male rats with dietary FB<sub>1</sub> decreases the persistence of GSTP<sup>+</sup> lesions induced by DEN suggesting that the inhibitory effect of cell proliferation and/or apoptosis could counteract the efficacy of initiation (Lebepe-Mazur et al., 1995). It is evident that, in studies of the synergistic interaction of different compounds with respect to certain biological effects, the time of administration of one compound relative to the other will have an important impact on the outcome of the study. As different parameters appear to be involved in determining the outcome of cancer initiation by FB<sub>1</sub> and AFB<sub>1</sub>, the sequential treatment protocol seems to favour this process in the present study.

Despite the fact that AFB<sub>1</sub> and FB<sub>1</sub> were administered three weeks apart when utilising the sequential model, they seem to act synergistically with respect to cancer initiation as the number of hepatocyte nodules and foci was significantly increased. It seems likely that the marked toxicity induced by AFB<sub>1</sub>, still evident at 5 weeks following the exposure, could render the liver more susceptible to the cancer initiating potency of FB<sub>1</sub> when treated after three weeks (sequential model). In this regard it is known that treatment with hepatotoxins, such as CCl<sub>4</sub>, that effect regenerative cell proliferation, markedly enhanced the cancer initiating potential of carcinogens such as AFB<sub>1</sub> (Hiruma et al., 1996) and FB<sub>1</sub> (Gelderblom et al., 2001b). The underlying mechanism that resulted in the significant ( $P < 0.001$ ) increase in the size of GSTP<sup>+</sup> foci and nodules during the successive AFB<sub>1</sub>/FB<sub>1</sub> treatment regimen could be

ascribed to the potent cancer promoting potential of FB<sub>1</sub>, a property used in its original isolation and purification (Gelderblom et al., 1988). A study in rainbow trout failed to indicate any cancer initiating activity by FB<sub>1</sub>, while it enhances tumor formation following AFB<sub>1</sub> initiation presumably solely through a cancer promoting mechanism (Carlson et al., 2001).

Of interest is the effectiveness of the FB<sub>1</sub> promoting regimen as compared to the 2-AAF/PH, which is regarded as a very strong selection stimulus (Semple-Roberts et al., 1987) as indicated when using DEN as a cancer initiator. The reason why FB<sub>1</sub> exhibited a far stronger promoting stimulus, under the present conditions, could be due to (i) as stated above, the additional cancer initiating potency of FB<sub>1</sub>; and (ii) the selection of a broader spectrum of initiated cells induced by AFB<sub>1</sub> when compared to 2-AAF/PH. AFB<sub>1</sub> has long been known to induce different types of hepatic lesions of which the growth of subsets of the lesions could be stimulated by different growth promoting and/or inhibitory signals (Kalengayi and Desmet, 1975; Kalengayi et al., 1975). The interaction between FB<sub>1</sub> and 2-AAF/PH was also significantly higher than the AFB<sub>1</sub>/2-AAF/PH interaction in the resistant hepatocyte model suggesting the 2-AAF/PH promoting protocol is not very effective in stimulating the growth of AFB<sub>1</sub>-initiated hepatocytes.

A threshold exists with respect to the cancer initiating and promoting activity of FB<sub>1</sub> (Gelderblom et al., 1994, 1996a,b). As a result, it has been postulated that due to the relatively low levels in naturally contaminated corn, FB<sub>1</sub> could be regarded solely as a cancer promoter (Gelderblom et al., 1996b). However, the present study indicated that the co-occurrence of AFB<sub>1</sub> and FB<sub>1</sub> could pose a threat when consumers, particularly in Southern Africa, are exposed to both toxins. Under specified conditions that mimic cancer initiation and promotion, the two mycotoxins can interact synergistically with respect to the induction of enzyme altered foci and nodules. Future studies need to explore the interaction of low dosage levels as well as other exposure regimens involving these carcinogens in different model systems. As stated above, the

synergistic interactions between different natural occurring toxins and carcinogens are very complex as a recent study indicated that the trichothecene mycotoxin, nivalenol, also promotes the development of GSTP<sup>+</sup> foci induced by AFB<sub>1</sub> (Ueno et al., 1992). As AFB<sub>1</sub> was shown to occur in the endemic areas for liver cancer it was suggested that the fumonisins could modulate the incidence of liver cancer together with hepatitis B virus. Studies in southern Africa also reported on the natural occurrence of AFB<sub>1</sub> in maize and groundnuts in regions of a high incidence of primary liver cancer (Van Rensburg et al., 1985). In the Transkei region of the Eastern Cape Province the incidence of esophageal cancer is very high when compared to liver cancer and it is not known whether any association exists between FB<sub>1</sub> and liver cancer development (Rheeder et al., 1992). Other mycotoxins such as nivalenol and deoxynivalenol also occur naturally in home-grown in this endemic regions for oesophageal cancer (Sydenham et al., 1990). The co-occurrence of FB<sub>1</sub> with other natural occurring cancer promoting and initiating food contaminants such as AFB<sub>1</sub>, the trichothecenes and HBV could therefore be of relevance in the development of liver cancer in high incidence regions. The present study indicates that more emphasis should be placed on the synergistic interaction between carcinogenic principles rather than investigating single biological effects representing only certain phases of cancer development.

### Acknowledgements

The authors are indebted to P. Snijman for the purification of fumonisin B<sub>1</sub>.

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# Fumonisin contamination of a corn sample associated with the induction of hepatocarcinogenesis in rats—role of dietary deficiencies

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Received 15 October 2002; accepted 17 October 2003

## Abstract

A corn sample associated with a field outbreak of equine leukoencephalomalacia in Pennsylvania, USA, during 1983/1984 and induced hepatotoxic and hepatocarcinogenic effects when fed to male Fischer rats was analyzed mycologically and chemically for the presence of fumonisins (FB), hydrolysed FB derivatives and aflatoxins (AFB). *Fusarium verticillioides* was found to be the predominant fungal contaminant in the corn sample but *Aspergillus flavus* was also present. Trace amounts (0.1 µg/kg) of AFB<sub>1</sub> and AFB<sub>2</sub> and a total FB level of 33.5 mg/kg (FB<sub>1</sub>:FB<sub>2</sub>:FB<sub>3</sub> ratio of 9:2.3:1) were found. No hydrolysed FB derivatives or AFG<sub>1</sub> and AFG<sub>2</sub> were detected. Based on the chemical stability of the fumonisins in different corn cultures of *F. verticillioides* kept at 4 °C over a period of 13–20 years, a level of approximately 55 mg/kg of total FB is estimated in the original corn sample. A possible role of certain dietary constituents such as the high protein content and deficiencies in certain micronutrients is evaluated to address differences in the organ-specific toxicity of FB<sub>1</sub> in rats using commercial, semi-purified, purified and corn-only diets.

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**Keywords:** Fumonisin; Carcinogenesis; Nutrition deficiencies

## 1. Introduction

Fungal contaminated corn (*Zea mays*) obtained from households in areas with a high incidence of oesophageal cancer (OC) in the Transkei region of the Eastern Cape Province, South Africa, was shown to cause toxic and preneoplastic lesions in the liver of male BD IX rats when fed for a prolonged period of time (Purchase et al., 1975). The carcinogenic potential of corn cultures of *Fusarium verticillioides* (previously known as *F. moniliforme*), a major fungal contaminant identified in the home-grown corn, was first described experimentally by Marasas et al. (1984) when fed at levels ranging from 2 to 4% in the diet to male BD IX rats over a period of approximately 15 months. The majority (80%) of rats developed hepatocellular carcinoma while 63% developed cholangiocarcinoma and/or cholangiofibrosis. These

cancers developed in livers that were severely cirrhotic and contained lesions such as nodular hyperplasia and bile duct proliferation. A commercial rat diet (EPOL Ltd, South Africa) that sustains breeding and general maintenance of the rats was used as the experimental diet. In a follow-up long-term experiment, using the same fungal culture material but at a much lower dietary level (0.5%), the carcinogenicity was monitored in the male BD IX rat using a semi-purified corn-based diet, marginally deficient in certain vitamins and lipotropes (Jaskiewicz et al., 1987a). Much lower incidences of hepatocellular (2/21) and cholangiocarcinomas (8/21) were noticed after a feeding period of 23 to 27 months. Wilson et al. (1985) showed that corn screenings, known to cause leukoencephalomalacia (LEM) in horses in the USA, also caused severe hepatotoxic and hepatocarcinogenic lesions in male Fischer 344 rats when fed over a period of 6 months. The major lesions reported after feeding the corn-only diet, which included hepatic nodules and large areas of adenofibrosis and cholangiocarcinoma, were remarkably similar to those obtained in BD IX rats fed the fungal cultures irrespective of the diet

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used. Studies in male BD IX and Fischer 344 rats, using naturally contaminated corn (Purchase et al., 1975; Wilson et al., 1985) suggest that nutritional imbalances and specifically the low methionine/choline levels in the corn-only diet may have promoted the formation of the liver tumors observed in these experiments. Studies with purified fumonisin B<sub>1</sub> (FB<sub>1</sub>) in male BD IX rats fed a dietary level of 50 mg/kg using the semi-purified diet described by Jaskiewicz et al. (1987a), prove that this mycotoxin is responsible for the hepatocarcinogenic effects of the fungal culture material (Gelderblom et al., 1991, 2001a). A long-term (2 years) study of FB<sub>1</sub> in male Fischer 344 rats fed dietary levels of 50 and 150 mg FB<sub>1</sub>/kg, using a NIH 33 commercial diet, however, failed to show any toxic and/or carcinogenic effects in the liver but rather produced nephrotoxic and nephrocarcinogenic effects (NTP, 2000; Howard et al., 2001). Some aspects regarding certain dietary deficiencies and dose response effects as possible confounders in FB<sub>1</sub>-induced hepatocarcinogenesis in male Fischer 344 and BD IX rats need to be considered to address differences in the target organ affected.

The present study reports on the fumonisin levels in the corn sample from the USA which was implicated in a field outbreak of LEM and which caused hepatocarcinogenic effects in male Fischer 344 rats when fed over a period of 6 months (Wilson et al., 1985). In addition, the nutritional constituents of some diets used in the long-term fumonisin carcinogenesis studies in different rat species are compared and discussed to provide some new perspectives regarding their possible modulating role in FB<sub>1</sub>-induced toxic and carcinogenic effects in rats.

## 2. Materials and methods

### 2.1. Fungal cultures

Corn cultures of *F. verticillioides* strain MRC 826 (Marasas et al., 1984) were prepared by inoculating autoclaved, moistened whole yellow kernels with lyophilised conidia, incubated at 25 °C for 21 days, freeze dried and ground to a fine meal. Different corn culture batches of *F. verticillioides* MRC 826 were prepared, batch B during 1981 and batches 55 and 57 during 1987. Batch B was used in long-term rat experiments (Marasas et al., 1984; Jaskiewicz et al., 1987a) while batches 55 and 57 were used in long-term vervet monkey experiments (Jaskiewicz et al., 1987b; Fincham et al., 1992; Gelderblom et al., 2001b). The different cultured batches were kept in airtight containers at 4 °C until analysed for the fumonisins in 1990 and again in 2000. The culture material of *F. verticillioides* MRC 826 batch B was prepared at approximately the same time as the outbreaks of LEM in the USA (see below).

### 2.2. Corn sample

A sample of corn screenings that caused LEM in horses on a farm in southeastern Pennsylvania during 1983–1984 and caused hepatotoxic and carcinogenic effects in male Fischer 344 rats (Wilson et al., 1985; Thiel et al., 1986a) was obtained from the late Prof PE Nelson, Pennsylvania State University, Pennsylvania, USA. The sample, consisting of broken kernels, pieces of cobs and stalks was finely ground and stored in an airtight container at 4 °C until analysed for fumonisins in 2000, i.e. approximately 17 years after it was naturally infected.

### 2.3. Mycological analyses

Fungal counts of the corn sample were performed by weighing out 1 g of the finely ground sample into the first tube of each of two dilution series of 1:10<sup>1</sup> to 1:10<sup>6</sup> in sterile distilled water. Aliquots (1 ml) of each dilution were dispensed into individual sterile 9-mm-diameter Petri dishes, mixed with molten sterilised malt extract agar (15 ml), allowed to cool and incubated at 25 °C in the dark for 7 days. The developing fungal colonies were microscopically identified and the number of colonies expressed as colony forming units per gram of the ground sample (cfu g<sup>-1</sup>).

### 2.4. Chemical analyses

#### 2.4.1. Fumonisin

The corn sample (20 g) or fungal culture material (5 g) were extracted by homogenisation with 100 ml of a mixture (3:1) of methanol (MeOH) and distilled water (H<sub>2</sub>O) in duplicate. Aliquots (10 ml) were adjusted to pH 2.5 with the addition of 0.1 M HCl and fractionated on Bond Elute C<sub>18</sub> solid phase extraction (SPE) cartridges, which had been preconditioned with MeOH (5 ml) and H<sub>2</sub>O (5 ml). After the sample was applied the cartridge was washed with H<sub>2</sub>O/MeOH mixture (3:1) and the fumonisins and aminopolyols eluted in MeOH (15 ml). The eluates were evaporated to dryness and reconstituted in MeOH (200 µl) and retained for chromatographic analyses. Aliquots (50 µl) of the reconstituted residues were derivatised with *o*-phthalaldehyde (OPA) as previously described (Shephard et al., 1990). The derivatised sample was injected via a Reodyne injector (Cotati, CA) onto a Phenomex (Torrance, CA) column (150×4.6 mm i.d.) packed with 5 µm Ultracarb ODS 20 material. A mobile phase of MeOH:0.1 M sodium dihydrogen phosphate (77:23) was adjusted to pH 3.35 with phosphoric acid and used at a flow rate of 1 ml/min. The fumonisins and aminopolyols were monitored by fluorescence at an excitation wavelength of 335 nm and an emission wavelength of 440 nm. Detection limit of the method is 50 µg/kg.

### 2.4.2. Aflatoxins

The corn sample (20 g) was extracted by homogenization with 100 ml MeOH/H<sub>2</sub>O (7:3). Extract was diluted (4×) with distilled water and applied (25 ml) to an AflaTest (Vicam, Watertown, MA) immuno-affinity column. The column was washed with distilled water (2×10 ml) and allowed to run dry for 15 minutes. The aflatoxins were eluted with HPLC grade MeOH (1 ml) and the eluates evaporated to dryness under nitrogen and dissolved in MeOH (200 µl). Samples (20 µl) were injected by means of a Waters U6K injector onto a reversed-phase column (Phenomenex Ultracarb 3 µ ODS (20) 4.6 mm i.d.×75 mm) using a mobile phase of 0.01 M KH<sub>2</sub>PO<sub>4</sub>/acetonitrile/MeOH (690:130:75) delivered by a Waters Model 510 pump at a flow rate of 1 ml/min. Post-column derivatisation for the enhancement of fluorescence was achieved with aqueous iodine delivered by a Waters Model 510 pump via a T-junction at a flow rate of 0.4 ml/min (Thiel et al., 1986b). The combined flow was passed through a Teflon reaction coil (0.3 mm×ID×20 m). The temperature of the chromatographic column, T-junction and reaction coil was maintained at 65 °C by means of an oven (Millipore, South Africa). The aflatoxins were detected using a Perkin Elmer (Norwalk, CT) Fluorescence Detector (Model 650S) at an excitation wavelength of 365 nm and an emission wavelength of 440 nm. The presence of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) was confirmed by making sequential injections with and without post-column reagent addition. The limit of detection was 0.05 µg/kg (0.05 ppb) based on a signal to noise ratio of 3:1.

### 2.5. Stability of FB<sub>1</sub> standard and fumonisins in fungal cultures

The corn cultures of *F. verticillioides* MRC 826 batches B, 55 and 57 were analysed for the fumonisin B mycotoxins in 1990 and 2000 in order to assess their stability. Two batches of FB<sub>1</sub> standards (0.5 mg solid), stored in closed glass vials at 4 °C during 1990, were also included and analysed in 2000.

### 2.6. Nutritional constituents of different diets used in FB<sub>1</sub>-carcinogenesis studies

Dietary constituents of the semi-purified diets used in carcinogenicity studies of FB<sub>1</sub> in male BD IX rats were calculated using the MRC Food Composition Table (Langenhoven et al., 1991). The nutritional values of yellow corn were obtained from the United States Department of Agriculture (USDA) Nutrient Database for Standard Reference (USDA, 2001). The dietary formulation of the NIH-31 diet used in the long-term carcinogenicity study of FB<sub>1</sub> in Fischer rats was obtained from the National Toxicology Program (NTP) Technical Report, NTP TR 496 (NTP, 2000). The dif-

ferent dietary constituents were compared to the minimal requirements for normal rat growth (Keenan et al., 2000).

## 3. Results

### 3.1. Mycological analyses

*Fusarium verticillioides* was the predominant fungal species in the sample of corn screenings while *Aspergillus flavus* and *Penicillium spp.* were also found to be present (Table 1).

### 3.2. Chemical analyses

The major fumonisins (FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub>) were detected in the corn screenings with trace amounts of AFB<sub>1</sub> and AFB<sub>2</sub> (Table 1). Low levels (1–1.5% of total FB) of the hydrolysed FB<sub>1</sub> (aminopentol) were also detected in the different fungal cultures of *F. verticillioides* strain MRC 826 (data not shown) while no hydrolysed FB derivatives were detected in the corn sample.

### 3.3. Stability of fumonisins

The analytical standards of FB<sub>1</sub> remained stable during a 10-year storage period at 4 °C with a recovery of 94% (Table 2). The original fumonisin levels in the fungal cultures prepared in 1981 and 1987 are unknown, but during the last 10 years in storage (1990–2000) the mean recovery of the total FB was 69%.

Table 1  
Mycological and chemical analyses of the corn sample associated with LEM in horses and hepatocarcinogenicity in rats

| Fungi                           | Mycological analyses (cfu/g)           |
|---------------------------------|--|
| <i>Fusarium verticillioides</i> | 2300                                   |
| <i>Aspergillus flavus</i>       | 400                                    |
| <i>Penicillium spp</i>          | 100                                    |
| Mycotoxin                       | Chemical analyses (mg/kg) <sup>a</sup> |
| <i>Fumonisin</i>                |  |
| FB <sub>1</sub>                 | 24.3                                   |
| FB <sub>2</sub>                 | 6.4                                    |
| FB <sub>3</sub>                 | 2.8                                    |
| Total FB                        | 33.5                                   |
| Aflatoxins                      | (µg/kg) <sup>b</sup>                   |
| AFB <sub>1</sub>                | 0.1                                    |
| AFB <sub>2</sub>                | 0.1                                    |

No hydrolysed FB derivatives, AFG<sub>1</sub> and AFG<sub>2</sub> detected.

<sup>a</sup> Detection limit: 50 µg/kg.

<sup>b</sup> Detection limit 0.05 µg/kg.

Table 2  
Stability of fumonisin B<sub>1</sub> analytical standard and total fumonisins in culture material of *Fusarium verticillioides* MRC 826 stored at 4 °C

| Sample   | 1990    | 2000    | Stability<br>(% recovered) |
|--|---------|---------|----------------------------|
| <i>FB<sub>1</sub> standard (mg)</i>                            |         |         |                            |
| Batch A  | 0.53    | 0.49    | 93                         |
| Batch D  | 0.63    | 0.59    | 95                         |
|  |         |         | Mean = 94                  |
| <i>F. verticillioides cultures<sup>a</sup> (g total FB/kg)</i> |         |         |                            |
| MRC 826-B <sup>a</sup>   | 4.9±0.5 | 3.6±1.0 | 73.5                       |
| MRC 826-55 <sup>b</sup>  | 2.6±0.1 | 1.7±0.5 | 65.4                       |
| MRC 826-57 <sup>b</sup>  | 3.7±0.3 | 2.5±0.8 | 67.6                       |
|  |         |         | Mean = 68.9                |

<sup>a</sup> Fungal cultures were prepared during 1981 and kept at 4 °C in airtight containers.

<sup>b</sup> Fungal cultures were prepared during 1987 and kept at 4 °C in airtight containers.

### 3.4. Dietary constituents of various diets used in FB<sub>1</sub> short- and long-term studies

Different diets have been used in long-term toxicological studies of the fungal cultures and the purified fumonisins in various rat species. These included a corn-only diet (Purchase et al., 1975; Wilson et al., 1985), a commercial diet (Masasas et al., 1984), semi-purified basal diet (Jaskiewicz et al., 1987a, b; Gelderblom et al., 1991, 2001a) and the NIH-31 (NTP, 2000). The dietary constituents of these diets are summarised (Table 3) and compared to the minimum dietary requirements (Keenan et al., 2000) and a standard purified diet (AIN-76) developed by the American Institute of Nutrition (AIN, 1980) in order to meet the growth requirements of adult rats. In spite of differences in the constituents of the semi-purified and corn-only diets used, the protein, fat and carbohydrate content, provide a similar energy content (KJ) to that proposed by the AIN-76 diet (AIN, 1980). The protein content (g/100 g diet) of the AIN 76, NIH-31 and commercial diets varied between 21, 20, and 18%, respectively. The semi-purified and corn-only diet contained 10 and 9% protein, respectively.

When considering the choline level, the corn-only and the semi-purified diets are similar to the estimated requirements for rats (Keenan et al., 2000) as compared to the level in the NIH-31 diet, which is 4.5 times higher than the estimated requirements. The methionine levels of the corn-only and the semi-purified diets are between 40 and 60% of that of the standard AIN 76, NIH-31 and commercial diets (Table 3). Biotin, vitamins B<sub>12</sub>, D and K were absent in the corn while riboflavin and vitamin E were approximately 50 and 30% lower respectively, than the estimated requirements. In the semi-purified diet used in the BD IX studies, vitamins B<sub>6</sub>, B<sub>12</sub> and D constituted 20, 15 and 25%, respectively while thiamine and riboflavin about 75% and folate and biotin about 60% of the estimated requirements. The

NIH-31 diet used in the long-term FB<sub>1</sub> study (NTP, 2000), contained most of the vitamins in excess of the estimated requirements and to some extent to that of the levels in the AIN-76 diet (AIN, 1980). These increased levels include thiamine (10 times), nicotinic acid (8 times), vitamins A (7 times), vitamin D (4 times), vitamin E (2 times), vitamin B<sub>6</sub> (2 times), riboflavin (2 times), folate (2.5 times) and pantothenate (2 times).

Relative to the estimated nutritional requirements of rats, the semi-purified and corn-only diets contained approximately 12 and 2% calcium, 13 and 25% magnesium and 40 and 60% phosphate, respectively. In contrast the NIH-31 and the commercial Epol diet contained approximately three times more calcium and phosphate than the minimum requirements while the magnesium level was about 50% of the required amount. The semi-purified diet contained about 23% of the required amount while the NIH-31 and the commercial diet contained about seven times and three times, respectively, more dietary iron. Of the micronutrients, manganese constituted about 5% when using the corn-only diet while the zinc level was approximately two times higher than the required level. Both the NIH-31 and the commercial diet contained excess zinc (5–8 times) and similar manganese levels with respect to the minimal requirements.

## 4. Discussion

The present study confirmed that *F. verticillioides* was the predominant fungus present in the corn sample associated with the field outbreak of LEM field outbreak in the USA as well as causing hepatotoxic and hepatocarcinogenic effects in male Fischer rats (Wilson et al., 1985). However, other fungal species, *A. flavus* and some *Penicillium* spp are reported, for the first time, to be present as fungal contaminants of the corn sample. Of the mycotoxins analysed, the presence of fusarin C and moniliformin was reported previously by Thiel et al. (1986b) while no aflatoxins were detected (Wilson et al., 1985). Thiel et al (1986b) suggested that it was highly unlikely that either moniliformin or fusarin C was involved in the aetiology of either LEM or the hepatocarcinogenicity in rats. They further emphasized the importance of identifying the active compound(s) in corn culture material of *F. verticillioides* MRC 826 which was shown to cause LEM in horses (Kriek et al., 1981) as well as liver cancer in rats (Marasas et al., 1984). This goal was achieved in 1988 with the isolation and characterization of the fumonisins from corn cultures of the fungus (Gelderblom et al., 1988).

The trace amounts (two times above the detection limit) of AFB<sub>1</sub> and AFB<sub>2</sub> detected in the corn sample may be ascribed to the high sensitivity (approximately 20 times) of the method used in the present study as

Table 3  
Comparison of dietary composition of AIN, NIH-31, semi-purified, commercial and corn only diets used in fumonisin B<sub>1</sub> feeding studies

|                             | ER     | AIN-76 | NIH-31 <sup>a</sup> | Comm. diet | Semi-purified     | Yellow Corn |
|-----------------------------|--------|--------|---------------------|------------|-------------------|-------------|
| Protein (g/kg)              | 50–100 | 214.3  | 201                 | 180        | 102               | 94          |
| Soy protein                 | –      | –      | –                   | –          | 15                | –           |
| Casein                      | –      | 200    | –                   | –          | 10                | –           |
| Egg albumin                 | –      | –      | –                   | –          | 10                | –           |
| Corn meal                   | –      | –      | –                   | –          | 66.7              | –           |
| Methionine                  | –      | 3      | 3.7                 | 3.9        | 1.5               | 2           |
| CHO (g/kg)                  | NR     | 605.1  | –                   | –          | 785.4             | 740.3       |
| Corn meal                   | –      | 150    | –                   | –          | 612               | –           |
| Sucrose                     | –      | 500    | –                   | –          | –                 | –           |
| Glucose                     | –      | –      | –                   | –          | 111.3             | –           |
| Dextrin                     | –      | –      | –                   | –          | 54.3              | –           |
| Fat (g/kg)                  | 40–60  | 46.4   | 35.5                | 40         | 49.2              | 47.4        |
| Saturated                   | –      | 5.9    | –                   | –          | 6.3               | 6.67        |
| MUFA                        | –      | 9.0    | –                   | –          | 11.4              | 12.51       |
| PUFA                        | –      | 29.3   | –                   | –          | 26.4              | 21.63       |
| Sunflower oil               | –      | 44     | –                   | –          | 30                | –           |
| <i>Energy</i>               |        |        |                     |            |                   |             |
| KJ                          | NR     | 15 235 | 18 125              | 10 892     | 14 426            | 15 270      |
| Fibre (g/kg)                | NR     | 54.8   | –                   | 70.2       | 26.7              | 34          |
| <i>Vitamins<sup>a</sup></i> |        |        |                     |            |                   |             |
| Thiamine (mg)               | 4      | 6      | 40                  | 3.94       | 3.0               | 3.9         |
| Riboflavin (mg)             | 4      | 6      | 8.54                | 4.95       | 3.18              | 2.0         |
| Nicotinic acid (mg)         | 15     | 30     | 122.3               | 50         | 30.5              | 36.27       |
| Vitamin B6 (mg)             | 6      | 7      | 11.4                | 6          | 1.1               | 6.2         |
| Folate (mg)                 | 1      | 2      | 2.48                | 9.5        | 0.62              | 0.19        |
| Vitamin B12 (µg)            | 50     | 10     | 43                  | 11         | 8                 | –           |
| Panto. (mg)                 | 8.8    | 16     | 33.6                | 12         | 6                 | 4.24        |
| Biotin (mg)                 | 0.2    | 0.2    | –                   | –          | 0.13              | –           |
| Vitamin A (IU)              | 2300   | 4005   | 17 000              | 11 000     | 16 672            | 4690        |
| Vitamin D (IU)              | 1000   | 1000   | 3881                | 2750       | 250               | –           |
| Vitamin E (mg)              | 27     | 80     | 53.5                | 60         | 34.6              | 7.5         |
| Vitamin K (mg)              | 1      | 5      | –                   | 2.86       | 2.95              | –           |
| Choline (g)                 | 0.75   | 2      | 3.38                | –          | 0.7               | 0.89        |
| <i>Minerals<sup>b</sup></i> |        |        |                     |            |                   |             |
| Calcium                     | 5000   | 5123   | 14 300              | 9000       | 590               | 70          |
| Iron                        | 35     | 50.6   | 260                 | 100        | 8                 | 27.1        |
| Magnesium                   | 5000   | 514.5  | 2470                | 75         | 643.5             | 1270        |
| Phosphate                   | 3600   | 4059   | 9370                | 7400       | 1467 <sup>#</sup> | 2100        |
| Potassium                   | 3600   | 3707   | 6900                | 8768       | 3343              | 2870        |
| Sodium                      | 50     | 1083   | 2470                | 2156       | 990               | 350         |
| Zinc                        | 12     | 44.9   | 92.2                | 64         | 10.4              | 22.1        |
| Copper                      | 5      | 5.7    | 11.7                | 10         | 1.9               | 3.14        |
| Manganese                   | 100    | 54     | 131.3               | 75         | –                 | 4.85        |
| Selenium                    | 0.15   | 0.1    | 0.5                 | –          | –                 | 0.16        |

Abbreviations: CHO = carbohydrate, MUFA = monounsaturated fatty acids, PUFA = polyunsaturated fatty acids. AIN-76 (AIN, 1980); Semi-purified diet (Marasas, et al., 1984; Jaskiewicz et al., 1987a). ER = estimated requirements (Keenan et al., 2000), Comm. = commercial; NR = not reported. “–” No data.

<sup>a</sup> mg/kg or units/kg.

<sup>b</sup> mg/kg.

compared to the method used by Wilson et al. (1985). It is highly unlikely, however, that these trace amounts of AFB could have played a role in the hepatocarcinogenic effect of the corn sample despite the fact that a recent study

indicated a synergistic effect between AFB<sub>1</sub> and FB<sub>1</sub> in a short-term liver carcinogenesis model (Gelderblom et al., 2002). The presence of the fumonisins (33.5 mg FB/kg) in the corn sample is the first report on the natural

occurrence in a naturally infected US corn sample involved in the development of LEM in horses and to exhibit hepatocarcinogenic effects in male Fischer 344.

When considering the stability of the total FB in the corn cultures, the retrospective estimation of the total FB in the corn sample (Wilson et al., 1985) fed to the male Fischer rats was likely to be in the order of between 47.8 and 55.8 mg FB/kg corn. This calculation is based on the fact that almost 70% of FB was recovered in corn cultures kept for 13–20 years and analysed over a 10 year period (1990–2000) and an assumption that at least between 60 and 70% of the total FB (33.5 mg FB/kg) is likely to be recovered in the corn sample after 17 years. This dietary level closely simulates the level of purified FB<sub>1</sub> fed to male BD IX rats (Gelderblom et al., 1991) when remarkably similar hepatic lesions were reproduced. These include hepatocyte nodules, adenofibrosis and cholangiocarcinoma that developed in the liver after a period of 5–6 months. Similar hepatic lesions were reported in long-term studies conducted by Marasas et al. (1984) and Jaskiewicz et al. (1987a) in male BD IX rats fed low dietary levels of corn culture material of *F. verticillioides* MRC 826 over a period of 24 months. The mean daily FB intake (mg/kg bw) of the rats was between 6.9 (288 days) and 3.2 (606 days) in the study of Marasas et al. (1984) and varied between 0.4 (211 days), 0.9 (311 days), 1.3 (81 days) and 0.9 (266 days) mg/kg bw/day in the study of Jaskiewicz et al. (1987a) (JECFA, 2001). These calculations were based on an average daily feed intake of BD IX rats over this period of between 30 and 35 g/kg bw. If a similar assumption is made for the male Fischer rats fed the corn screenings (Wilson et al., 1985), a daily FB intake of between 2.3 and 3.2 mg FB/kg bw is estimated (JECFA, 2001). These FB intake values are in the same order (1.6 mg FB<sub>1</sub>/kg bw) to those of male BD IX rats using purified FB<sub>1</sub> at a dietary level of 50 mg/kg diet (Gelderblom et al., 1991). A daily FB<sub>1</sub> intake of 1.6 mg/kg diet and 0.8 mg FB<sub>1</sub>/kg resulted in liver cancer or the induction of hepatocyte nodules in male BD IX rats, respectively (Gelderblom et al., 2001a). In contrast, studies in male Fischer rats fed purified FB<sub>1</sub> for 2 years, at dietary levels of 50 and 150 mg FB<sub>1</sub>/kg, representing a daily intake of 2.2 and 6.6 mg FB<sub>1</sub>/kg bw, respectively, did not cause any marked pathological lesions in the liver (NTP, 2000; JECFA, 2001).

Short-term studies in male Fischer rats indicated that a daily intake of 3.5 mg FB<sub>1</sub>/kg bw using the AIN-76 diet showed mild changes in the liver after 21 days (Gelderblom et al., 1996). However, other studies in male Fischer rats showed that FB<sub>1</sub> intakes up to 12 and 20 mg FB<sub>1</sub>/kg bw/day over a period of 28 days (NTP, 2000; JECFA, 2001) using the NIH-31 diet, or a daily intake up to 5.7 FB<sub>1</sub>/kg bw over a period of 90 days using the NIH 07 diet (Voss et al., 1995a) did not show any liver lesions. With respect to the kidneys, dietary

levels 0.7 mg FB<sub>1</sub>/kg bw/day over 21 days (JECFA, 2001; Gelderblom et al., 1996) and 2 years (NTP, 2000; JECFA, 2001) were shown to effect degenerative changes. A study by Voss et al. (1995b) also showed kidney lesions in male Fischer rats at a dietary level of 0.6 mg FB<sub>1</sub>/kg bw/day over a period of 90 days. When considering the long-term study when feeding naturally contaminated corn sample to male Fischer rats for 6 months (Wilson et al., 1985) with an estimated daily exposure of between 2.3 and 3.2 mg FB/kg bw (NTP, 2000; JECFA, 2001), liver damage was effected while no kidney damage was reported (Wilson et al., 1985). It would appear that major differences exist with respect to dose response toxic effects in the liver of male Fischer rats while these differences do not exist with respect to the induction of degenerative changes in the kidneys. The outcome of the 2-year study performed in male Fischer rats also provides the first evidence that the fumonisins induced kidney tumours in the absence of liver damage (NTP, 2000). This must be considered against the background that long-term feeding studies using naturally infected corn (Purchase et al., 1975; Wilson et al., 1985), corn cultures of *F. verticillioides* (Marasas et al., 1984; Jaskiewicz et al., 1987a) and purified FB<sub>1</sub> (Gelderblom et al., 1991, 2001a) induced liver damage in both BD IX and Fischer 344 rats in the absence of any prominent kidney lesions.

Feeding of two samples of maize screenings associated with field cases of LEM in horses in the USA during 1986 to male Sprague–Dawley rats over a period of 4 weeks induced similar pathological changes in the liver as described above for the fungal culture material in BD IX and Fischer rats, which included single cells necrosis, mild fibrosis, bile duct hyperplasia, adenofibrosis and apoptosis (Voss et al., 1989). Based on the FB levels and the daily feed intake, a FB intake of between 10.2–13.5 and 1.2–1.4 mg FB/100g bw/day could be calculated (Plattner et al., 1990; JECFA, 2001). Only mild changes were observed in the liver of the rats exposed to the low FB<sub>1</sub>-containing latter sample while both samples induced lesions in the kidneys. When fungal culture material was fed in the diet over a period of 21 days, mild liver changes were noticed at a dietary level of 2.7–4.5 mg FB/kg bw in male Sprague–Dawley rats (JECFA, 2001; Voss et al., 1998). Studies in male Sprague–Dawley rats using purified FB<sub>1</sub> showed mild liver changes when fed daily levels of 4.1 mg FB<sub>1</sub>/kg bw over a period of 28 days (JECFA, 2001; Voss et al., 1993, 1995b).

In view of the above toxicological studies, it would appear that differences exist with respect to the induction of toxicological effects by FB<sub>1</sub> in the liver and kidney of rats that cannot be ascribed to strain differences because both male BD IX, Sprague–Dawley and Fischer rats, fed similar dietary levels of FB<sub>1</sub> using different experimental diets, developed mild hepatic changes. Interestingly

comparative toxicity experiments have shown that Fischer 344 are more sensitive than Sprague–Dawley rats to 1,2 dichlorobenzene-mediated hepatocellular oxidative stress when fed the same diet (Younis et al., 2000). As diet could play a major role in the susceptibility of rats to toxins, an evaluation of the different diets used to investigate the toxicological effects of FB<sub>1</sub> is required. It is not known at present whether differences in the nutritional status of the different diets could have modulated the sensitivity of the target organ to the effects of FB<sub>1</sub>. Dietary analyses could therefore provide important information on the differential effects of FB<sub>1</sub> with respect to its carcinogenicity in the kidney and liver of rats.

Detailed comparison of the corn-only and the semi-purified diets used in the studies in male BD IX and Fischer rats showed that the protein content is well within the minimal requirements for rats and approximately 50% lower when compared to the AIN-76 and the NIH 31 diets. Recently the NIH-07 diet, containing 22% protein and used in the National Toxicology Program's (NTP) toxicological and carcinogenesis studies was replaced by the NTP 2000 diet containing much lower protein levels (14.5%) due to chronic diet and age related changes such as nephropathy (Rao et al., 2001). In male Fischer 344 rats the severity of nephropathy, of which the presence of focal to multifocal regenerative renal tubule is an indicator, was reduced markedly. In the long-term study of FB<sub>1</sub> in Fischer rats, using the NIH-31 diet with a protein content of 20.1%, nephropathy up to 80–90% was reported in the male and female Fischer rats (NTP, 2000). Renal tubular hyperplasia was reported to be significantly higher in the high dose groups (50 and 150 mg FB<sub>1</sub>/kg diet) with non-typical dose response effects. The high protein diet could have predisposed the kidney to the carcinogenic effects of FB<sub>1</sub> in male Fischer rats while the low protein semi-purified diet protects against nephrocarcinogenic effects in the male BD IX rats (Gelderblom et al., 1991, 2001a). In this regard no kidney pathology was reported in the male Fischer rats fed the corn sample that caused major hepatic lesions (Wilson et al., 1985). However, high protein diets enhance the susceptibility of the liver for the induction of preneoplastic lesions (Youngman and Campbell, 1991). The finding in the NTP study (NTP, 2000) that the high protein content of the NIH-31 diet and FB<sub>1</sub> levels 3X higher than that used in the studies in male BD IX (Gelderblom et al., 1991, 2001a) and Fischer 344 rats (Wilson et al., 1985) did not markedly affect the liver is, therefore, difficult to explain.

The corn-only (Purchase et al., 1975; Wilson et al., 1985) and semi-purified diets used (Jaskiewicz et al., 1987a and b, Gelderblom et al., 1991, 2001a) were proposed to be marginally deficient in vitamins and micronutrients, such as choline, methionine and certain vitamins (Wilson

et al., 1985). However, when considering the minimal nutritional requirements for rats (Keenan et al., 2000) both the semi-purified and corn-only diets contained sufficient quantities of choline and 50–70% of the methionine levels when compared to the AIN-76 diet. Both the AIN-76 and the NIH-31 diets contained choline levels far in excess (2–4×) of the required amount. As sufficient levels of these feed constituents were present an increased the sensitivity of the liver to FB<sub>1</sub>-induced pathological effects in the liver seems unlikely. However, with respect to certain vitamins, both the corn only and the semi-purified diets contained far less than the required levels. Of interest is the absence of vitamins B<sub>12</sub> and D and low levels of vitamins E in the corn-only diet. The semi-purified diet also contained low levels of vitamins B6 and B12. The low levels of folate in both these diets appear not to be a factor as the intestinal bacteria serves as a source (Keenan et al., 2000). In contrast the NIH-31 diet contained folate levels far in excess of the required levels. However, recent studies showed that FB<sub>1</sub> disrupts folate transport (Stevens and Tang, 1997) and induces developmental abnormalities in mice embryos in culture, which are partially counteracted by the addition of folate (Sadler et al., 2002). It is not known at present whether the uptake of dietary folate is impaired in rats. As oxidative damage has been implicated in FB<sub>1</sub>-induced liver carcinogenesis (Abel and Gelderblom, 1998) the low levels of dietary vitamin E and selenium in the corn-only diet could have played an enhancing role in the induction of hepatotoxic effects by the fungal contaminated corn (Purchase et al., 1975; Wilson et al., 1985).

With respect to the minerals both the corn-only and the semi-purified diets contained extremely low calcium levels resulting in incorrect calcium to phosphorus ratio which is very important in the maintenance of rat growth (Keenan et al., 2000). The phosphorus in plant products is present as phytate which is largely unavailable to the animal due to a lack of absorbance from the gut. In both the semi-synthetic and corn-only diets the calcium level is lower than the phosphate and therefore below the 1:3 molar ratio of calcium-to-phosphorus, suggested to prevent nephrocalcinosis (Keenan et al., 2000). In a chronic feeding study in male BD IX rats, nephrocalcinosis has been reported to occur, but it was only noticed in the rats fed the high dose of FB<sub>1</sub> (Gelderblom et al., 2001a).

The dietary modulation of FB<sub>1</sub>-induced carcinogenesis in rats is likely to play an important role in the sensitivity of rats within and between different strains. When considering the long-term carcinogenesis experiments of fumonisins in rats and the nutritional content of the diets used, certain dietary components most likely affected the dose–response effects monitored. Future studies need to be conducted focusing on the effect of different dietary components as possible modulators of

fumonisin-induced carcinogenesis in different rat strains and/or different target organs in the same rat strain.

## Acknowledgements

A special tribute to the late Professor Paul E. Nelson for his unselfish contribution to *Fusarium* mycotoxicology and for sending us the sample of corn screenings: "... a small feed sample from one of the LEM incidents—Terry has been feeding this to rats for about 6 months and is finding severe liver damage...". 7 September 1984. Unpublished letter from Prof. Paul E. Nelson to Professor W.F.O. Marasas.

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## LEUKOENCEPHALOMALACIA IN TWO HORSES INDUCED BY ORAL DOSING OF FUMONISIN B<sub>1</sub>

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### ABSTRACT

KELLERMAN, T. S., MARASAS, W. F. O., THIEL, P. G., GELDERBLOM, W. C. A., CAWOOD, M. & COETZER, J. A. W., 1990. Leukoencephalomalacia in two horses induced by oral dosing of fumonisin B<sub>1</sub>. *Onderstepoort Journal of Veterinary Research* 57, 269-275 (1990).

Leukoencephalomalacia (LEM) was induced by the oral administration of fumonisin B<sub>1</sub> (FB<sub>1</sub>) to 2 horses: a filly received 59.5 mg/kg of a 50 % preparation of FB<sub>1</sub>, administered in 21 doses of 1.25-4 mg/kg over 33 days; a colt, 44.3 mg/kg of 95 % pure FB<sub>1</sub> in 20 doses of 1-4 mg/kg in 29 days. Both animals developed nervous signs such as apathy, changes in temperament, inco-ordination, walking into objects, and one showed paralysis of the lips and tongue. Characteristic lesions of LEM were present in the brains. These trials proved conclusively that FB<sub>1</sub> can induce LEM in horses.

### INTRODUCTION

The equine neurotoxicosis, leukoencephalomalacia (LEM), was first reproduced experimentally with naturally contaminated mouldy maize in the United States of America by Butler (1902), with pure cultures of the fungus *Fusarium moniliforme* Sheldon by Wilson & Maronpot (1971), and with a pure compound, fumonisin B<sub>1</sub> (FB<sub>1</sub>) isolated from cultures of *F. moniliforme*, by Marasas, Kellerman, Gelderblom, Coetzer, Thiel & Van der Lugt (1988). In the experiment with FB<sub>1</sub>, a horse was injected intravenously 7 times with 0.125 mg of FB<sub>1</sub>/kg live mass/day (total dose: 276 mg of FB<sub>1</sub>). Typical clinical signs of LEM, including nervousness followed by apathy, a wide-based stance, trembling, ataxia, reluctance to move, paresis of the lower lip and tongue, and inability to eat or drink, appeared on Day 8. Euthanasia was performed on the horse on Day 10 while the animal was in a tetanic convulsion. The principle lesions were severe oedema of the brain and early, bilaterally symmetrical, focal necrosis of the medulla oblongata (Marasas *et al.*, 1988). Advanced brain lesions of LEM (Badiali, Abou-Youssef, Radwin, Hamdy & Hildebrand, 1968; Wilson & Maronpot, 1971; Marasas, Kellerman, Pienaar & Naudé, 1976; Buck, Haliburton, Thilsted, Lock & Vesonder, 1979; Haliburton, Vesonder, Lock & Buck, 1979; Pienaar, Kellerman & Marasas, 1981; Marasas, Nelson & Toussoun, 1984; Kellerman, Coetzer & Naudé, 1988; Wilson, Nelson, Marasas, Thiel Shephard & Sydenham, 1990a) were not present in this horse.

The induction of the typical clinical signs and characteristic brain lesions of LEM in 2 horses by the oral administration of FB<sub>1</sub>, is reported in this paper.

### MATERIALS AND METHODS

#### *Fumonisin B<sub>1</sub> preparation*

FB<sub>1</sub> was isolated from maize cultures of *F. moniliforme* MRC 826 as previously described (Gelderblom, Jaskiewicz, Marasas, Thiel, Horak, Vleggaar & Kriek, 1988) with some modifications (M. Cawood, W. C. A. Gelderblom, R. Vleggaar, P. G. Thiel & W. F. O. Marasas, unpublished data). The

purity of FB<sub>1</sub> was determined by high performance liquid chromatography (HPLC) according to the method described by Alberts, Gelderblom, Thiel, Marasas, Van Schalkwyk & Behrend (1990). The FB<sub>1</sub> preparation used in the pilot trial (Horse 1) had a purity of c. 50 %, the other 50 % being inorganic matter that co-eluted during the purification on silica gel (Gelderblom *et al.*, 1988). The FB<sub>1</sub> preparation used in the second experiment (Horse 2) had a purity of 95 to 98 %, and is referred to as pure FB<sub>1</sub> in this paper.

#### *Dosing regimens*

The dosing regimens for Horse 1 and 2 are summarized in Table 1 and Fig. 1.

*Horse 1.* In a pilot trial, the FB<sub>1</sub> preparation (8 925 mg) dissolved in water was administered per stomach tube to a 9-month-old filly of 150 kg live mass. Based on the purity of the FB<sub>1</sub> preparation (50 %), the effective total dose was 29.7 mg FB<sub>1</sub>/kg live mass.

*Horse 2.* In a follow-up experiment, pure FB<sub>1</sub> (8 417 mg) was similarly administered to a 14-month-old colt of 190 kg live mass. Based on a purity of 95 % for FB<sub>1</sub> used in this experiment, the total dose was 42.1 mg FB<sub>1</sub>/kg live mass.

#### *Clinical observations and chemical pathology*

The horses were kept under close observation and all clinical signs noted. In order to monitor the effects of FB<sub>1</sub> administration on liver function, the following chemical pathological determinations were performed on serum samples collected periodically during dosing: aspartate transaminase (AST)<sup>1</sup>, gamma glutamyl transferase (GGT)<sup>1</sup>, lactate dehydrogenase (LD)<sup>1</sup> and total bilirubin (Tietz, 1982). Enzyme activities were measured at 25 °C.

#### *Pathology*

The horses were necropsied immediately after euthanasia, carried out by the intravenous injection of an overdose of pentobarbitone sodium. The brain and spinal cord, and specimens of the lungs, myocardium, skeletal muscles, spleen, lymph nodes, liver, kidneys, adrenals and gastrointestinal tract were fixed in 10 % buffered-formalin. After fixation, serial coronal sections c. 5 mm in thickness of the brain were cut and examined macroscopically. Selected blocks of the tissues were routinely processed, embedded in paraffin-wax, sectioned and stained with haematoxylin and eosin.

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LEUKOENCEPHALOMALACIA IN TWO HORSES INDUCED BY ORAL DOSING OF FUMONISIN B<sub>1</sub>

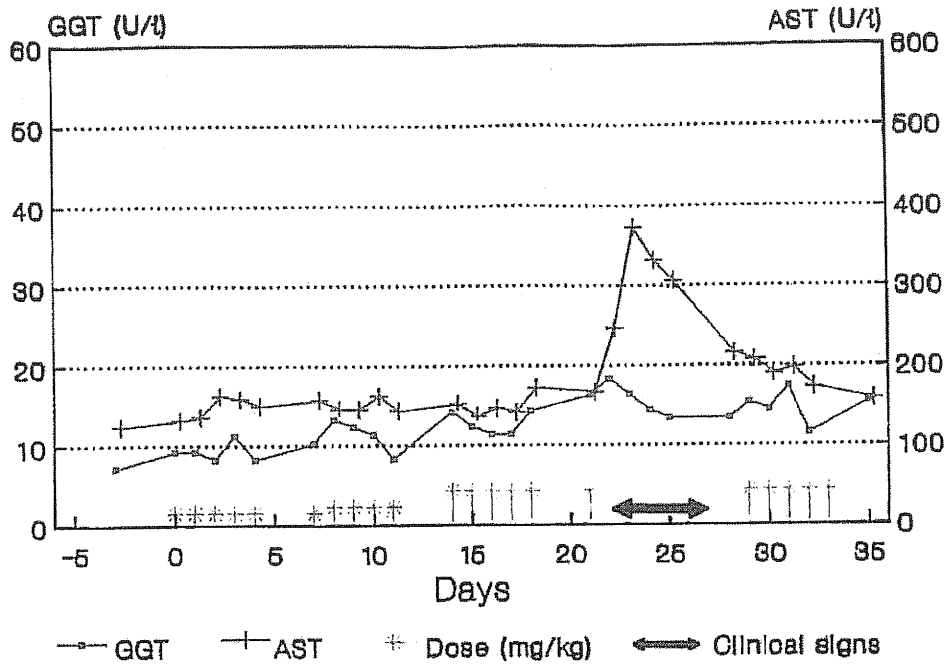


FIG. 1 Clinical pathological changes in the serum of Horse 1 dosed *per os* with a preparation (50 % pure) of fumonisin B<sub>1</sub>

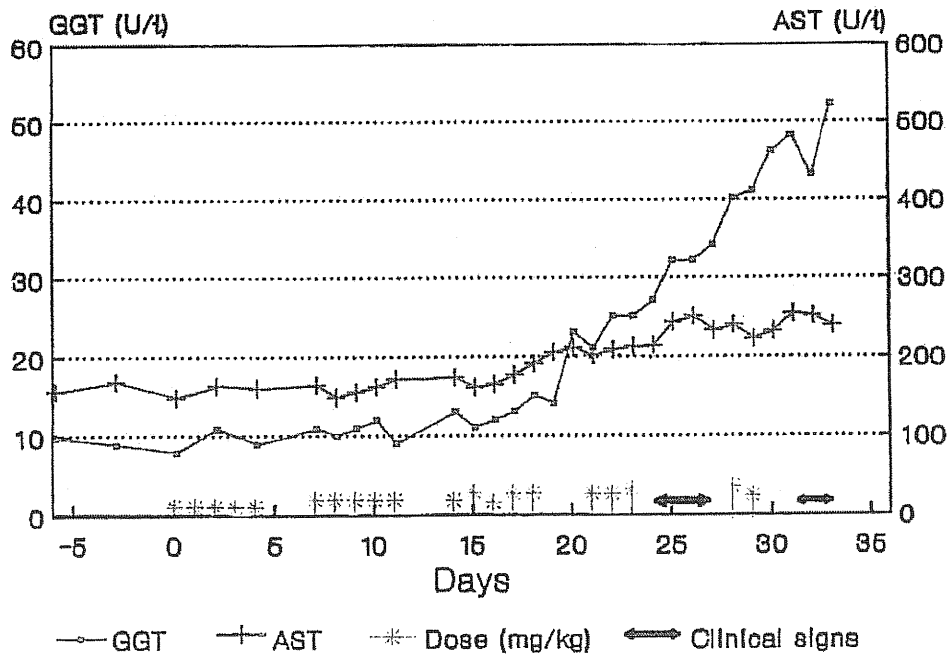


FIG. 2 Clinical pathological changes in the serum of Horse 2 dosed *per os* with 95 % pure fumonisin B<sub>1</sub>

RESULTS

Clinical signs

*Horse 1.* Clinical signs were manifested from Day 22 to 27 (Table 1) and consisted of a change in temperament such as apathy and apparent stupidity; the filly stood wide-eyed, with ears pricked, staring uncomprehendingly at a person directly in front of it, or would almost walk into an obstacle before veering at the last moment to avoid collision. It was incoordinated, walking with a shuffling gait, hocks flexed

and rump down, taking short steps, apparently lame in the left fore limb, occasionally stumbling and sometimes dragging the feet and knocking-on. When forced to turn suddenly it would lift both fore feet off the ground and pivot clumsily on the hind-limbs. It also had difficulty in judging distances and would sometimes bump its mouth against the crib while attempting to eat. Eventually it could neither eat or drink; hay hung unchewed from the mouth, and it could not swallow water.

The nervous signs improved progressively from about the 2nd day after they were first noticed until by the 6th day (Day 27) the foal was essentially normal. Despite the administration of 5 further doses of 4 mg/kg of FB<sub>1</sub> on Days 29–33, this apparent state of normality, during which the foal was perhaps slightly wilder and more clumsy than before, lasted until euthanasia was carried out on Day 35.

*Horse 2.* Clinical signs were manifested from Day 24 to 26 and again from Day 31 to 33 (Table 1). On the first occasion, Horse 2 stumbled and fell while getting up from the sternal position. Mild tremors were evident in the hind quarters, flanks and shoulders, and it made sporadic pawing motions with the right fore limb. It appeared to have undergone a subtle change in temperament, becoming uncharacteristically apathetic and docile, even allowing humans to come close before it moved away.

After a 4-day period of apparent normality, and 2 doses of FB<sub>1</sub> on Days 28 and 29, the signs reappeared, this time lasting until Day 33 (Table 1). At first the colt was merely apathetic and stood motionless with the head down and neck extended, showing little interest in the environment. If disturbed, it walked about restlessly without purpose, stumbling over the watertrough or bumping against the railings of the circular pen in which it was kept. The animal appeared rapidly to lose cognisance of its surroundings. It walked into objects with increasing frequency, and once, after a minor collision, reeled back and fell. Eventually it stood motionless for long periods, sometimes with its head pressed against the railings. Although the horse at this stage could still be led by the halter, it would stand immobile immediately upon release, as if incapable of voluntary movement. Its gait was not noticeably affected. Euthanasia was carried out on Day 33, when it could no longer eat or drink.

**Chemical Pathology**

The chemical pathological changes in Horse 1 and Horse 2 are illustrated in Fig. 1 and 2, respectively. In Horse 1 dosed with the 50 % pure preparation of FB<sub>1</sub>, there was an elevation of AST activity in the serum between Days 22 and 31, with a maximum of 365 U/l on Day 23. In contrast, there was a marked elevation of GGT activity in Horse 2 dosed with pure FB, between Days 20 and 33, with a maximum of 52 U/l on Day 33.

**Pathological changes**

*Horse 1.* At necropsy a sunken area, c. 2 cm in diameter, was evident in the lateral part of the anterior frontal lobe of the left cerebral hemisphere (Fig. 3). The cerebrospinal fluid beneath the meninges in this area was slightly increased and tinged yellowish-brown. On the cut section of this focus the white matter was softer than normal and reddish-brown (Fig. 4). No lesions were seen in other parts of the brain. The synovial fluid was slightly increased in both carpal joints.

Microscopically, the focal lesion in the brain revealed liquefactive necrosis of the white matter, numerous macrophages (Fig. 5), aggregates of mineralization, and a few small haemorrhages. Some of the blood vessels in and at the periphery of the necrotic area showed marked hypertrophy and hyperplasia of endothelial cells, fibrinoid changes of their cell walls and mild perivascular mononuclear cell infiltration. Mild to moderate fibrosis was evident around some of these blood vessels (Fig. 6) and a fibrin mural thrombus occurred in a blood vessel in

TABLE 1 Induction of leukoencephalomalacia by the oral administration of fumonisin B<sub>1</sub> and pure fumonisin B<sub>1</sub> to horses

| No. | Sex   | Live mass (kg) | Age (yrs) | Dose (mg/kg x n)   | Days on which dosed   | Total dose (g) | Day of euthanasia | Clinical signs   | Clinical pathology   | Results  |
|-----|-------|----------------|-----------|--|---|----------------|-------------------|--|--|--|
| 1   | Filly | 150            | 0,75      | 1,25 x 5*<br>1,25 x 1*<br>2,00 x 4*<br>4,00 x 5*<br>4,00 x 1*<br>4,00 x 5*   | 0-4<br>7<br>8-11<br>14-18<br>21<br>29-33                          | 8,925*         | 35                | Apathy, stupidity, inco-ordination, walking into objects, paralysis of lips and tongue (Day 22-27) followed by apparent recovery (Day 28-35)                                   | Marked elevation of AST (maximum 365 U/l) activity in the serum between Days 21 and 30 | Leukoencephalomalacia, left frontal lobe               |
| 2   | Colt  | 190            | 1,3       | 1,00 x 5<br>2,00 x 5<br>2,00 x 1<br>3,00 x 1<br>1,84 x 1<br>3,00 x 2<br>3,00 x 2<br>3,56 x 1<br>4,00 x 1<br>2,90 x 1 | 0-4<br>7-11<br>14<br>15<br>16<br>17-18<br>21-22<br>23<br>28<br>29 | 8,417          | 33                | Apathy, docility, tremors, pawing motions (Day 24-26). Recovery or remission (Day 27-30). Apathy, bumping into objects, sporiferousness, inability to eat or drink (Day 31-33) | Marked elevation of GGT (maximum 52 U/l) activity in the serum between Days 20 and 33  | Leukoencephalomalacia, left frontal to occipital lobes |

\* Preparation of fumonisin B<sub>1</sub> (50 % pure)

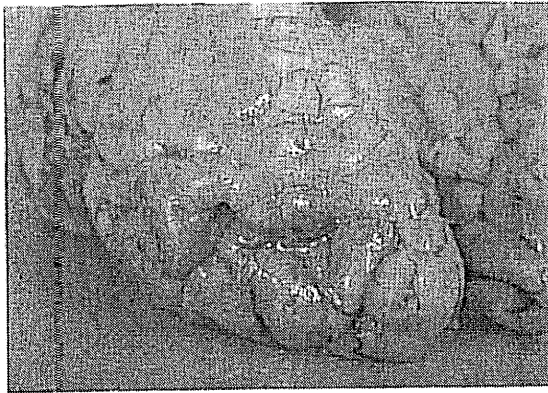


FIG. 3 Horse 1: Sunken malacic area in frontal part of the left cerebral hemisphere

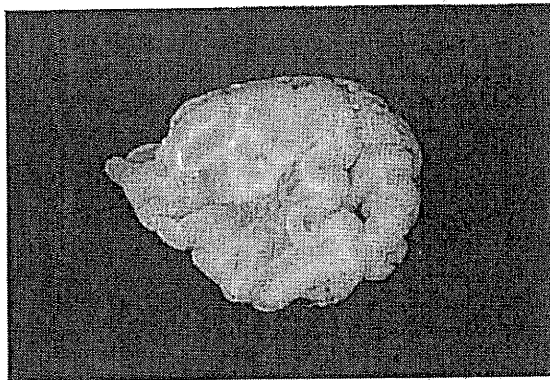


FIG. 4 Horse 1: Coronal section of malacic area

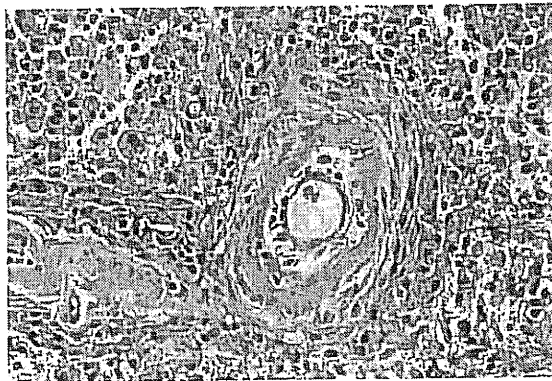


FIG. 5 Numerous macrophages arranged around an affected blood vessel in the brain of Horse 1. Not fibrinoid changes in the vascular wall.

the malacic area. The white matter in close proximity to the focal lesion revealed mild status spongiosus and mild to moderate proliferation of astrocytes.

Apart from diffuse cloudy swelling and hydropic degeneration of hepatocytes no other lesions were evident in the tissue collected for examination.

*Horse 2.* The left cerebral hemisphere was diffusely swollen and the gyri were somewhat flattened. On cut section a yellowish-brown, less-dense, gelatinous, irregularly-shaped focus, c. 1-2 cm in width

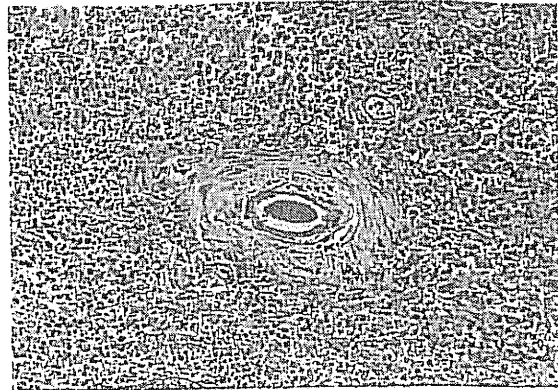


FIG. 6 Marked perivascular fibrosis in a necrotic area in the brain

and c. 4 cm in length, in the subcortical white matter of the left dorsal frontal lobe, extended posteriorly from just anterior of the middle of the hemisphere to the occipital lobe (Fig. 7). Apart from a small gelatinous focus, c. 1 cm in diameter, in the white matter of the right occipital lobe, no other lesions were seen in the brain and spinal cord.

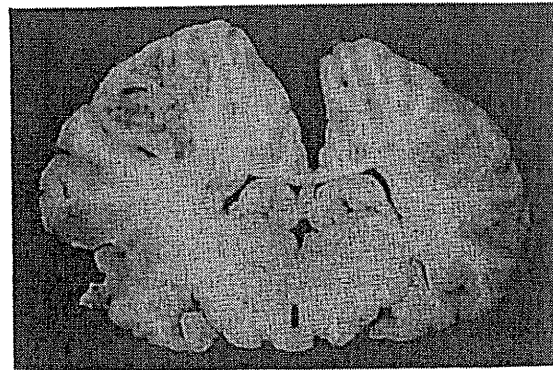


FIG. 7 Horse 2: Severe swelling of left cerebral hemisphere and liquifactive necrosis of subcortical white matter

The kidneys were moderately swollen and diffusely greyish-yellow. The other organs and tissues showed no significant macroscopic lesions.

Microscopically, the grossly discernible focal lesions revealed moderate to severe rarefaction of the neuropil, partial loss of cellular detail of the white matter, swelling and proliferation of astrocytes, infiltration of moderate numbers of macrophages, and the presence of swollen axons (Fig. 8). Many of the blood vessels in and on the periphery of these foci revealed moderate hyperplasia and hypertrophy of endothelial cells, and haemorrhage and/or accumulation of oedematous fluid or homogeneous, eosinophilic droplets of varying size in dilated perivascular spaces (Fig 9).

Apart from these focal lesions the white and grey matter of the remainder of the left side of the brain showed changes referable to a moderate oedema.

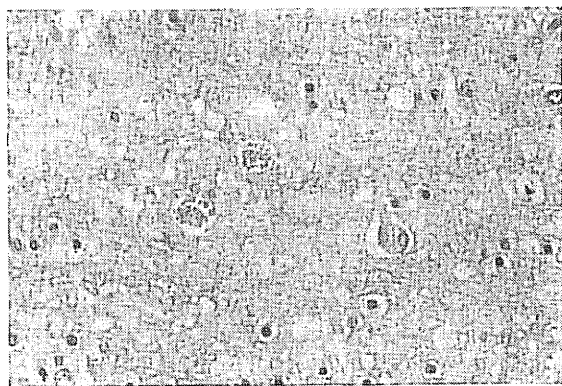


FIG. 8. Rarification of white matter with infiltration by a few macrophages

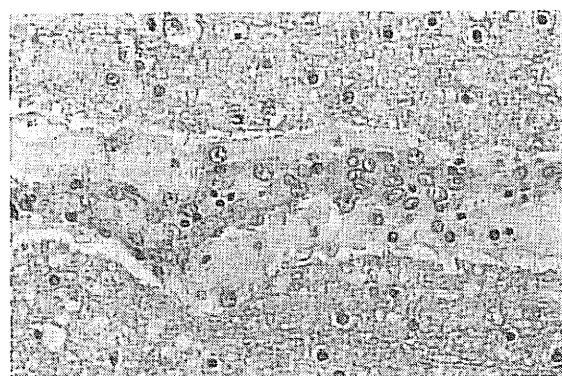


FIG. 9. Marked endothelial hyperplasia and perivascular oedema in an area of rarification in the brain

The right side of the brain showed only a mild oedema.

The epithelium of the proximal convoluted tubules in the kidneys revealed cloudy swelling and hydropic degeneration.

#### DISCUSSION

Natural outbreaks of LEM in South Africa have, as far as can be established, been recorded only in adult horses. Experimental evidence reported in this study that a 9-month-old foal contracted the condition may, therefore, be of some diagnostic significance. Knowing that young horses are susceptible may also have bearing on the selection of animals for future research. Young animals are good subjects, easy to handle and require less  $FB_1$  for the induction of LEM. Since LEM can be induced by either the oral or the intravenous routes (Marasas *et al.*, 1988), injection of fumonisins by the latter route would result in a further reduction of the dose. The fact that grossly discernible malacic areas were not evident in the brain of that horse injected intravenously with  $FB_1$  (Marasas *et al.*, 1988), was probably related to the level, route and rate of administration of the toxin. In the case of the present 2 orally-dosed horses, the courses of the toxicoses were remarkably similar with respect to the onset of clinical signs (Day 22–24) and the number of dosages (16 to 18). However, the total dose administered prior to the

appearance of the first clinical signs differed widely, i.e. 19.7 and 31.6 mg/kg live mass in Horse 1 and Horse 2, respectively. Possible reasons for this discrepancy may be the differences in sex, age and individual variation between the 2 horses. The clinical signs of LEM may vary according to the size and siting of the lesion in the brain. The commonest signs observed in experimental cases induced with either culture material of *F. moniliforme* or  $FB_1$  were changes in temperament, ranging from frenzy to depression with or without locomotory disturbance, walking into objects and paralysis of the lips and tongue. It should be borne in mind that the nervous signs are not always associated with liquefactive necrosis of the brain: experimental cases have been produced in which only oedema of the brain was evident (Marasas *et al.*, 1988). The apparent recovery of both horses after the first manifestation of signs may have been due to relief of intracerebral pressure after the diminution of brain oedema. Horse 1 recovered remarkably well despite the presence of a focal necrotic lesion in its left cerebral hemisphere. These observations indicate that LEM may not always be fatal and that horses may function reasonably well even with severe cerebral damage.

Poisoning of horses with  $FB_1$  is unusual in that both the brain and liver lesions are caused by the same toxin. Considerable evidence has been produced that small doses of culture material over long periods culminate in LEM, whereas high doses over shorter periods result in hepatosis (Kellerman, Marasas, Pienaar & Naudé, 1972; Marasas *et al.*, 1976; Marasas *et al.*, 1988). LEM is not easy to reproduce experimentally as too high doses might lead to acute fatal liver failure while too small doses have no effect. This fact is illustrated by the failure of Laurent, Platzner, Kohler, Sauviat & Pellegrin (1989) to induce LEM in a horse despite the *per os* administration of 11.4 g of "macrofusine", a compound evidently similar to  $FB_1$ . The difficulties encountered in inducing LEM are exacerbated by the lack of reliable parameters for measuring nervous damage. As nervous signs are often preceded by elevations in the activities of liver enzymes such as AST and GGT in the serum, small but progressively higher doses of  $FB_1$  were given until the activities of one or both of these enzymes started to rise. This indicated that a neurotoxic dose had possibly been given. Extreme care must nevertheless be exercised in the interpretation of chemical pathological data as nervous signs have been known to appear 5 days after dosing had ceased, without notable elevations of liver enzymes having been recorded (Marasas *et al.*, 1988). The effects of the interrupted administration of  $FB_1$ , compared to the continuous ingestion of maize naturally contaminated with  $FB_1$ , and other fumonisins, on the pathogenesis of LEM, remain to be determined.

Analytical techniques to detect and quantify  $FB_1$  and fumonisin B<sub>2</sub> ( $FB_2$ ), in feeds have been developed recently (Shephard, Sydenham, Thiel & Gelderblom, 1990; Wilson, Ross, Rice, Osweiler, Nelson, Owens, Plattner, Reggiardo, Noon & Pickrell, 1990b). This has made it possible to compare the dosage rates of  $FB_1$  employed in the present study with the levels of fumonisins in naturally contaminated feeds implicated in field outbreaks of LEM. The conclusion that  $FB_1$  is a causative factor of LEM has been substantiated by the fact that this compound, together with  $FB_2$ , have been detected in every incriminated feed sample thus far analyzed. Norred, Plattner, Voss, Bacon & Porter (1989)

detected both FB<sub>1</sub> and FB<sub>2</sub> in a sample of maize screenings associated with a field outbreak of LEM in the USA. This finding was confirmed by Voss, Norred, Plattner & Bacon (1989), who detected both FB<sub>1</sub> and FB<sub>2</sub> in two maize samples implicated in separate field outbreaks of LEM in the USA. Although these authors confirmed the presence of FB<sub>1</sub> and FB<sub>2</sub> in the naturally contaminated feed samples by mass spectrometry, the levels were not quantified.

In South Africa, Shephard *et al.* (1990) detected 8,85 and 3,00 µg/g FB<sub>1</sub> and FB<sub>2</sub>, respectively, in a commercial mixed feed sample associated with a field outbreak of LEM. Thiel, Shephard, Sydenham, Marasas, Nelson & Wilson (1990) reported that 14 feed samples from confirmed cases of LEM in the USA contained FB<sub>1</sub> and FB<sub>2</sub> at levels ranging from 1,30 to 27,00 µg/g (mean = 7,70 µg/g) and 0,10 to 12,60 µg/g (mean = 3,10 µg/g), respectively. It was not possible to determine what proportion of the diet of the horses that developed LEM was contributed by the contaminated feeds. Consequently the dosage rates of naturally occurring fumonisins required to induce LEM under field conditions could not be calculated.

Wilson *et al.* (1990b) found both FB<sub>1</sub> and FB<sub>2</sub> in 3 samples of maize screenings which formed part of the diet of 66 horses. Of these 18 contracted LEM and 14 died. The fumonisins were present in damaged kernels and cob parts but not in undamaged kernels. The 3 samples contained 37, 56 and 122 µg/g FB<sub>1</sub> and 2, 11 and 23 µg/g FB<sub>2</sub>, respectively. These authors calculated a daily dose rate of FB<sub>1</sub> for each of 13 of the horses that died by estimating the daily intake of contaminated maize screenings, assuming an average concentration of 72 µg/g FB<sub>1</sub> in the maize screenings and using the known live mass of each individual animal. Their estimates of the daily intake of FB<sub>1</sub> varied from 0,6 to 2,1 mg/kg/day. These dosage rates agree well with the dosage rates used in this study (1,25–4,00 mg/kg/day) to induce LEM in horses by *per os* administration of FB<sub>1</sub>, especially since the possible contribution of FB<sub>2</sub> to the toxicogenic potential of the feeds was not taken into account by Wilson *et al.* (1990b) in estimating the daily dosage rates. No information is available on the toxicity of FB<sub>2</sub> to horses, but it may be similar to that of FB<sub>1</sub>. Until data become available, a similar toxicogenic potential will have to be assumed for FB<sub>1</sub> and FB<sub>2</sub> in risk assessment, especially since FB<sub>2</sub> invariably forms a sizeable part of the total fumonisin concentration in contaminated feeds implicated in outbreaks of LEM (Shephard *et al.*, 1990; Thiel *et al.*, 1990; Wilson *et al.*, 1990b). When comparing the daily dosage rates used to induce LEM experimentally in this investigation (Horse 1: 0,625–2,0 and Horse 2: 1,0–4,0 mg/kg/day) with those estimated by Wilson *et al.* (1990b) (0,6–2,1 mg/kg/day), it should also be kept in mind that lower rates might also induce the disease. This investigation was, however, not designed to assess to lowest experimental dosage rates needed to induce LEM.

The study unequivocally proves that FB<sub>1</sub> can induce LEM in horses. Comparison of dosage rates furthermore indicates that the levels of FB<sub>1</sub> and FB<sub>2</sub> detected in feeds associated with confirmed cases of LEM, could account for the development of the disease in affected animals.

#### ACKNOWLEDGEMENTS

We are indebted to Mr B. P. Maartens and other staff members of the Toxicology Section of the Veter-

inary Research Institute, Onderstepoort, and to Mrs M. Schlechter, Miss S. Stockenström and Mrs P. Smith of the Medical Research Council, Tygerberg, for competent technical assistance.

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## The effects of dietary iron overload on fumonisin B<sub>1</sub>-induced cancer promotion in the rat liver

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Received 17 May 1999; received in revised form 12 July 1999; accepted 13 July 1999

### Abstract

The present study was performed to determine whether excess hepatic iron modulates the cancer-initiating and promoting properties of FB<sub>1</sub>. Thirty-eight male F344 rats were divided into four dietary treatment groups: (i) control diet (AIN,  $n = 8$ ); (ii) FB<sub>1</sub> 250 mg/kg diet (FB<sub>1</sub>,  $n = 10$ ); (iii) 1–2% carbonyl iron (CI,  $n = 10$ ); or (iv) FB<sub>1</sub> plus iron loading (FB<sub>1</sub>/CI,  $n = 10$ ) for 5 weeks (2 × 2 factorial design). Hepatic iron concentrations in iron-loaded animals at 5 weeks were  $444 \pm 56$  (CI) and  $479 \pm 80$  μmol/g dry weight (FB<sub>1</sub>/CI) (mean ± SEM). All the FB<sub>1</sub>-fed rats, in the presence or absence of CI, developed a toxic hepatitis with a 4-fold rise in serum alanine transaminase (ALT) levels. FB<sub>1</sub> appeared to augment iron-induced hepatic lipid peroxidation, as measured by the generation of thiobarbituric acid reacting substances (TBARS) in liver homogenates ( $P < 0.0001$ ). Morphometric analysis showed that FB<sub>1</sub> caused a significantly greater mean ± SEM number of ‘enzyme-altered’ foci and nodules per cm<sup>2</sup> ( $5.34 \pm 1.42$  vs.  $1.50 \pm 0.52$ ,  $P < 0.05$ ), as well as a greater area (%) of liver occupied by foci and nodules ( $0.33 \pm 0.12\%$  vs.  $0.05 \pm 0.03\%$ ,  $P < 0.001$ ), compared with FB<sub>1</sub>/CI. The addition of FB<sub>1</sub> to dietary iron loading caused a shift in distribution of iron from hepatocytes to Kupffer cells, probably due to phagocytosis of necrotic iron-loaded hepatocytes. In conclusion, (i) FB<sub>1</sub> appears to cause toxicity in the liver independently from effects on lipid peroxidation; (ii) FB<sub>1</sub> has a potentiating effect on iron-induced lipid peroxidation; and (iii) dietary iron loading appears to protect against the cancer promoting properties of FB<sub>1</sub>, possibly due to a stimulatory effect of iron on hepatocyte regeneration. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Fumonisin B<sub>1</sub>; Iron overload; Rat liver; Hepatic lipid peroxidation; Cancer initiation and promotion

### 1. Introduction

Fumonisin B<sub>1</sub> (FB<sub>1</sub>), a food-borne mycotoxin produced by the fungus *Fusarium moniliforme* [1], causes a variety of naturally occurring toxicoses in animals, including fatal illnesses in horses and pigs [2,3]. Human dietary consumption of *Fusarium-*

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contaminated corn products has been linked epidemiologically to increased rates of esophageal cancer [4,5] and, perhaps, hepatocellular carcinoma (HCC) [6], in regions of the world in which corn is the staple grain, such as South Africa and China.

FB<sub>1</sub> is hepatotoxic and hepatocarcinogenic in rats. Short-term feeding with FB<sub>1</sub> causes zone 3 liver injury, marked apoptosis, oval cell proliferation, and hepatic fibrosis [7]. Chronic feeding with FB<sub>1</sub> leads to chronic toxic hepatitis, cirrhosis, and HCC [8]. FB<sub>1</sub> acts as a strong promoter (and possibly weak initiator) of 'enzyme-altered' hepatic foci and nodules in short-term cancer studies [9,10]. The mechanisms of FB<sub>1</sub>-induced toxicity and cancer induction in the liver are unclear, but there is some evidence that peroxidation of lipid membranes [11,12] and oxidative DNA damage [13] might play a role.

Dietary iron overload is common in sub-Saharan Africa, and hepatic iron concentrations rival those occurring in genetic hemochromatosis. Recent studies suggest that dietary iron overload may be a risk factor for HCC in black Africans [14,15]. The development of HCC in humans with iron overload (genetic or dietary) usually occurs in the setting of iron- or alcohol-induced cirrhosis [16], and it is not clear whether iron plays a direct role in the induction of liver tumors or whether the increased cancer risk arises solely from the cirrhotic process. Although mechanisms of iron-induced hepatotoxicity are incompletely understood, free radical-mediated peroxidative damage to cellular lipids, proteins, and DNA is likely [17,18]. Experimentally, the potentiation of cancer induction due to polyhalogenated hydrocarbons by iron overload in rodents [19] would suggest that iron may act as a promoter of initiated hepatocytes. Studies carried out to test this hypothesis have, however, shown conflicting results, with both promoting and inhibiting effects on cancer induction by excess hepatic iron [20,21].

Both FB<sub>1</sub> and excess hepatic iron may thus cause peroxidation of membrane lipids and oxidative liver injury [11,17]. Furthermore, both agents may, either directly or indirectly, affect the induction of liver tumors. The aims of this study were to determine whether dietary iron loading modulates the cancer initiating and promoting properties of FB<sub>1</sub>.

## 2. Materials and methods

### 2.1. Chemicals

FB<sub>1</sub> was purified from corn cultures of *Fusarium moniliforme* strain MRC 826 according to the method described by Cawood et al. [22] to a purity of 92–95%. Carbonyl iron (CI), an extremely pure form of elemental iron (>98% iron with < 0.8% carbon, < 0.3% oxygen and < 0.9% nitrogen), was purchased from Sigma Chemical Company (St. Louis, MO).

### 2.2. Animals and diet

The study was approved by the Ethics and Research Committee of the University of Cape Town, and the experiments were conducted in accordance with the laws and regulations controlling experiments on live animals in South Africa. Thirty-eight male Fischer 344 rats were fed a modified AIN diet [23], which has an iron content of 50 mg/kg feed [24]. The diets containing FB<sub>1</sub> 250 mg/kg were prepared as described previously [23] and stored under nitrogen at –20°C for the duration of the study. Animals that were randomized to receive dietary iron loading, were initially fed 2% (w/w) CI when weaned, but this had to be temporarily discontinued after 1 week (for 1 week) due to growth retardation, whereafter iron supplementation was resumed with 1% (w/w) carbonyl iron until the completion of the study.

### 2.3. Experimental design

The 5-week FB<sub>1</sub>-feeding experiment (250 mg/kg diet) was commenced when the rats weighed approximately 155–165 g (group 1, 160.9 ± 5.0 g; group 2, 165.2 ± 1.9 g; group 3, 153.2 ± 4.6 g; group 4, 155.7 ± 4.8 g). The 38 animals were divided into four treatment groups according to a 2 × 2 factorial design, i.e. controls (group 1, *n* = 8); FB<sub>1</sub> 250 mg/kg diet (group 2, *n* = 10); dietary CI 1–2% (group 3, *n* = 10); and FB<sub>1</sub> plus CI (group 4, *n* = 10). The quantities of feeds in the different groups were adjusted to match the average intake of animals in group 4 (FB<sub>1</sub>/CI). Two animals from each treatment group and one animal from the control group were sacrificed at weeks 3 and 4 for analysis of hepatic histopathology, and the remainder of the animals (*n* = 6, each group) were sacrificed at week 5. The

rats were weighed daily and feed intake and wastage were carefully determined. At sacrifice, animals were anaesthetized by the intraperitoneal injection of a sodium pentobarbitone solution (6% m/v). Blood was drawn by cardiac puncture for measurement of alanine transaminase (ALT) levels for biochemical assessment of liver injury, and animals were terminated by exsanguination. The livers were harvested and weighed. A slice of liver was taken from the left, right, and median lobes of each animal, and these were fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin for light microscopy. The remaining liver was snap frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$ .

#### 2.4. Light microscopy and immunohistochemistry

Liver sections ( $5\ \mu\text{m}$ ) were stained with hematoxylin and eosin (HE) for routine light microscopy, and with sirius red for collagen. Perls' Prussian blue stain for trivalent iron was used to assess hepatic iron content [25]. Stainable iron in hepatocytes was graded 0–4, using a modification of the scale devised by Scheuer et al. [26]. Staining with rabbit polyclonal GST pi (Novacastra, Newcastle-Upon-Tyne, UK) was performed for 'enzyme-altered' hepatic foci and nodules. After sequential layering with swine anti-rabbit, (Dako, Copenhagen, Denmark) link antibody (1:250 dilution), peroxidase-conjugated with streptavidin-biotin (Dako, Copenhagen, Denmark) 1:500, was applied for 30 min at room temperature.

#### 2.5. Morphometric analysis of hepatic foci and nodules

A total of 18 liver sections (three sections per rat in six rats sacrificed at week 5) each from rats in group 2 ( $\text{FB}_1$ ) and group 4 ( $\text{FB}_1/\text{CI}$ ) were stained with GST pi for determination of the number and size of 'enzyme-altered' foci and 'pre-malignant' nodules. One section from each of the left, right, and median lobes was examined for each rat. The number of GST pi-positive hepatic lesions (foci and nodules) per  $\text{cm}^2$  were counted, and were considered an estimate of the cancer initiating effects of  $\text{FB}_1$  with or without iron loading [27,28]. For the purposes of this study, a group of GST pi-positive cells was classified as a 'focus' if it had an area of less than  $100\ \mu\text{m}^2$ , and a 'nodule' if it had an area of  $100\ \mu\text{m}^2$  or greater. The

percentage area of liver occupied by GST pi-positive foci and nodules was determined using video image analysis (Optimas, Bothell, WA), and was considered a measurement of the cancer promoting effects of  $\text{FB}_1$  with or without iron loading [27,28].

#### 2.6. Hepatic iron concentration

Liver tissue for determination of iron content was dried for 24 h at  $105^{\circ}\text{C}$ , weighed, digested in 50% nitric acid at  $70^{\circ}\text{C}$  for 1 h, and diluted in 0.2 M sodium acetate buffer (pH 4.5) [29]. All glassware was rendered iron-free and rinsed with iron-free water [29]. Iron concentration was determined using a Roche Unimate 5 Iron Kit (Roche Diagnostic Systems, Basel, Switzerland), on a Roche Cobas Fara II Centrifugal Analyser.

#### 2.7. Assessment of oxidative damage

Malondialdehyde (MDA) is formed when polyunsaturated fatty acids of membrane phospholipids undergo peroxidation. Lipid peroxidation was measured by the thiobarbituric acid (TBA) assay for MDA concentration on samples of liver homogenate, as described by Esterbauer and Cheeseman [30]. Related substances such as sucrose, non-ferrous metal ions and whole tissue homogenates may also react with TBA or influence the assay procedure, and the term thiobarbituric acid reacting substances (TBARS) more accurately describes the product of this assay [30]. Addition of EDTA to the liver homogenate and butylated hydroxytoluene (BHT) to the TBA reagent prevent further oxidative changes during the assay procedure. Lipid peroxidation was expressed as nmol MDA equivalents per mg protein, using a molar extinction coefficient of  $1.56 \times 10^5\ \text{M}^{-1}\text{cm}^{-1}$  at 532 nm for MDA [31].

#### 2.8. Statistics

The data are presented as the mean  $\pm$  SEM. Outcome measurements between the treatment groups were compared by one way analysis of variance (ANOVA). Individual comparisons were made using Scheffé's test.  $\text{FB}_1$ -induced GST pi-positive hepatic lesions in groups 2 and 4 were compared using the Mann-Whitney *U*-test for non-parametric data. Significance was set at a *P*-value of 0.05.

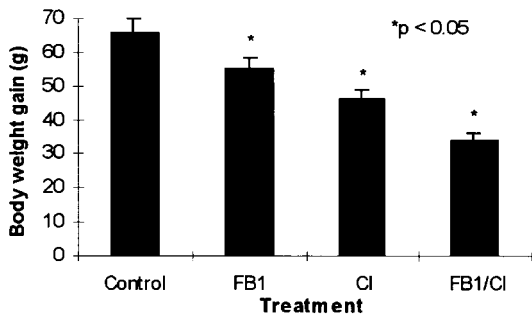


Fig. 1. Body weight gain (g) of rats over 35 days according to treatment group.

### 3. Results

#### 3.1. Body weight gain and liver weight/body weight ratio

Total feed intake was  $222.35 \pm 14.26$  g/100 g body wt., and equivalent total FB<sub>1</sub> intake was calculated as  $55.59 \pm 3.56$  mg/100 g body wt. Daily feed intake was  $6.57 \pm 0.40$  g/100 body wt. and daily FB<sub>1</sub> intake was  $1.64 \pm 0.10$  mg/100 g body wt. Despite control of feed intake there were significant differences in weight gain in the treatment groups, presumably reflecting differential toxic effects (Fig. 1). The liver weight/body weight ratio was reduced only in rats from group 2 (FB<sub>1</sub>;  $P < 0.0001$ ) (Fig. 2).

#### 3.2. Liver injury analysis

Serum levels of ALT in animals that received control AIN diet was  $42 \pm 2$  units/l at 5 weeks. There was a marked increase in week 5 ALT levels in rats fed FB<sub>1</sub> (group 2,  $200 \pm 28$  units/l) and FB<sub>1</sub>/CI (group 4,  $191 \pm 10$  units/l), reflecting significant hepatotoxicity of FB<sub>1</sub>-containing regimens. Serum ALT levels in animals given CI (group 3) were mildly raised ( $66 \pm 7$  units/l) at 5 weeks, indicating minimal hepatocellular injury caused by dietary iron loading alone.

#### 3.3. Liver histopathology

Pathological changes in rat liver caused by feeding of FB<sub>1</sub> 250 mg/kg diet for 5 weeks have been described elsewhere [7], and include severe zone 3 injury with collapse of the reticulin framework,

frequent hepatocyte mitoses, and apoptotic bodies, seen after 1 week. These initial changes are followed by the development of 'enzyme-altered' foci and nodules, marked oval cell proliferation, and hepatic fibrosis by week 3. Sequential liver sections from weeks 3–5 in group 2 animals (FB<sub>1</sub>) from the present study showed milder liver injury, with cell loss and collapse in zone 3, mitoses, minimal fibrosis, and multiple small GST pi-positive foci and nodules. Even though the FB<sub>1</sub> content of the diet was the same compared with the earlier study [7], total intake of FB<sub>1</sub> in the present study was reduced because of averaged feeding. Similarly, liver sections of group 4 rats (FB<sub>1</sub>/CI) showed evidence of mild liver injury histologically, and there appeared to be fewer hepatic GST pi-positive foci in these animals. Liver sections from control animals and group 3 rats (CI) showed no evidence of liver injury.

Perls' Prussian blue staining of sequential liver sections showed progressive hepatic iron loading from weeks 3–5 in animals that received iron supplementation (groups 3 and 4). There was a striking difference in the pattern of iron distribution between animals in group 3 (CI) and group 4 (FB<sub>1</sub>/CI). At week 5, livers from group 3 rats (CI) showed grade 3–4 parenchymal iron loading and a zonal gradient of iron deposition, with maximum deposition in zone 1. Lesser amounts of iron was detected in Kupffer cells (Fig. 3A). Livers from group 4 rats (FB<sub>1</sub>/CI) showed evidence of marked iron deposition mainly in the Kupffer cells in zone 3, occurring in association with hepatocyte death in this region. (Fig. 3B).

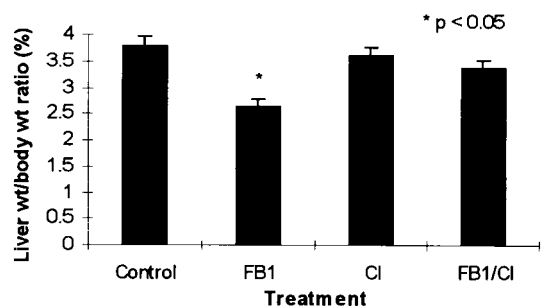


Fig. 2. Liver weight to body weight (LW/BW) ratio of rats according to treatment group.

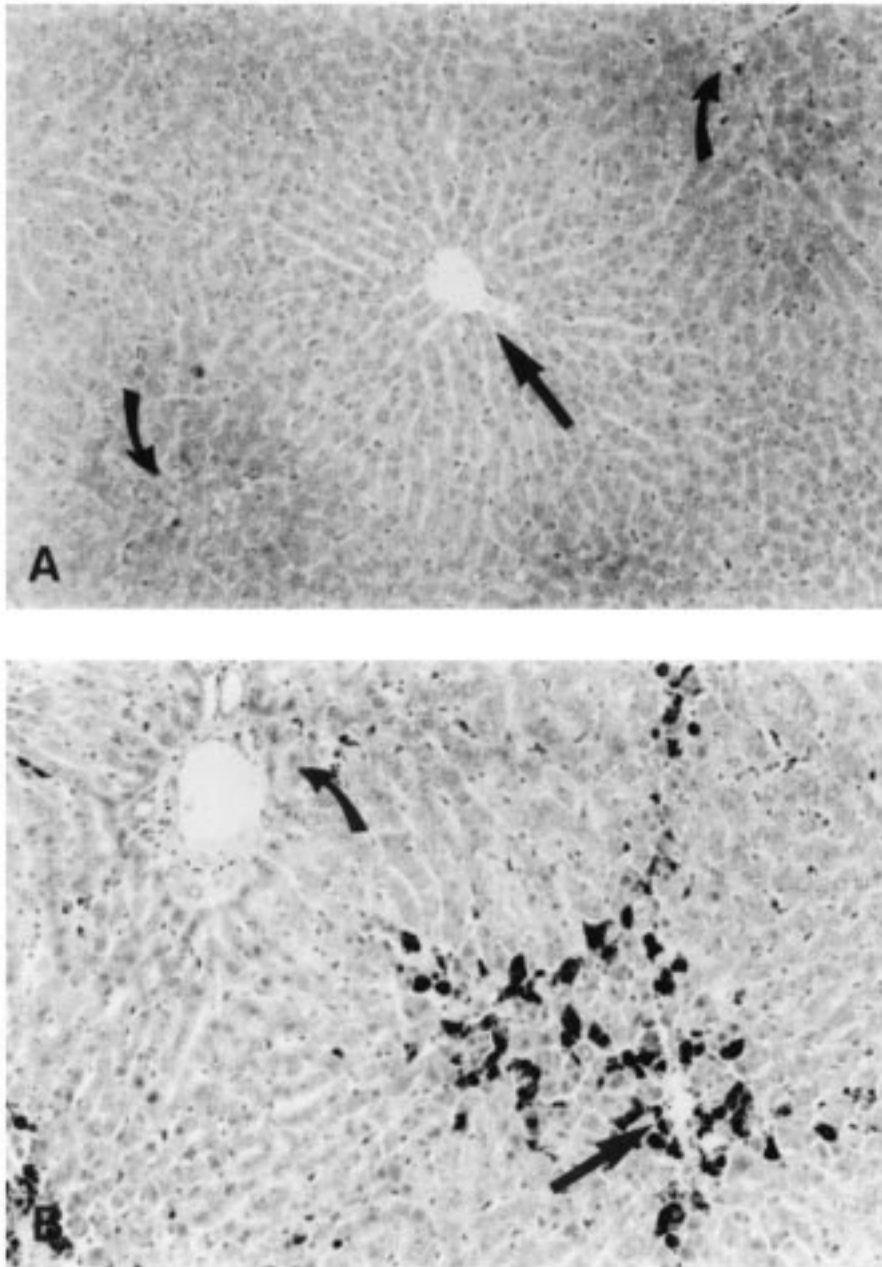


Fig. 3. Stainable iron in livers from rats in groups 3 and 4. (A) Liver section from a rat in group 3 (CI), showing grade 3 parenchymal iron deposition with maximal deposition in zone 1. (B) Liver section from a rat in group 4 (FB<sub>1</sub>/CI), showing loss of hepatocytes in zone 3 and abundant iron in Kupffer cells, with lesser amounts of parenchymal iron deposition. Perls' stain for iron, objective  $\times 10$ . Straight arrows indicate central veins; curved arrows indicate portal tracts.

Table 1

Glutathione S-transferase (GST) pi-positive hepatic lesions found at week 5 in rats treated with FB<sub>1</sub> (group 2) and FB<sub>1</sub>/CI (group 4) (*n* = 6, each)

|                                     | FB <sub>1</sub> (group 2) | FB <sub>1</sub> /CI (group 4) |
|-------------------------------------|---------------------------|-------------------------------|
| No. of rats with foci <sup>a</sup>  | 4/6                       | 3/6                           |
| No. of rats with nodules            | 6/6                       | 4/6                           |
| No. of lesions per cm <sup>2b</sup> | 5.34 ± 1.42 <sup>c</sup>  | 1.50 ± 0.52                   |
| Area (%) of lesions <sup>b</sup>    | 0.33 ± 0.12 <sup>c</sup>  | 0.05 ± 0.03                   |

<sup>a</sup> A GST pi-positive lesion was classified as a 'focus' if it had an area of less than 100 μm<sup>2</sup>, and as a 'nodule' if it had an area of 100 μm<sup>2</sup> or greater.

<sup>b</sup> Data presented as mean ± SEM.

<sup>c</sup> *P* < 0.05.

### 3.4. Morphometric analysis of GST pi-positive foci and nodules

Rats given dietary iron loading alone (CI) did not develop any GST pi-positive hepatic foci or nodules. At 5 weeks, 4/6 rats treated with FB<sub>1</sub> (group 2) had developed hepatic foci and 6/6 rats from this group had developed hepatic nodules. In contrast, 3/6 rats treated with FB<sub>1</sub>/CI (group 4) had developed foci by 5 weeks, and 4/6 rats from this group had developed nodules. At week 5, corresponding liver sections (3 sections/rat from 6 rats in each group) from rats in group 2 contained an average of 5.34 ± 1.42 GST pi-positive lesions per cm<sup>2</sup>, as compared with 1.50 ± 0.52 GST pi-positive lesions per cm<sup>2</sup> in livers of rats from group 4 (*P* < 0.05; Table 1). Furthermore, the area (%) of liver sections occupied by GST pi-positive lesions in rats from group 2 was 0.33 ± 0.12%, as compared with 0.05 ± 0.03% in sections of rats from group 4 (*P* < 0.05; Table 1). These data indicate that there were more, and larger, lesions in the livers of rats that received FB<sub>1</sub> only (group 2).

### 3.5. Hepatic iron concentration

Hepatic iron concentration (μmol/g dry weight of liver) in the livers of rats treated with CI (group 3) and FB<sub>1</sub>/CI (group 4) was 444 ± 56 and 479 ± 80 μmol/g dry weight, respectively. In contrast, liver iron concentration in control animals (group 1) and rats treated FB<sub>1</sub> was 48 ± 4 and 48 ± 5 μmol/g dry weight, respectively.

### 3.6. Hepatic lipid peroxidation

Generation of TBARS in liver homogenates differed between the treatment groups (Fig. 4), and these differences persisted when using differences in weight gain as the covariant (*F* ratio = 5.24, *P* < 0.01). Although treatment with FB<sub>1</sub> (group 2) slightly increased TBARS generation above control levels, these changes were not significant (*P* < 0.3). Treatment with CI (group 3), however, caused a significant increase in TBARS generation (*P* < 0.001). Contrast coefficient analysis revealed that hepatic TBARS generation due to FB<sub>1</sub>/CI (group 4) was significantly more than the sum of TBARS generation by CI (group 3) plus FB<sub>1</sub> (group 2) (*P* < 0.0001), indicating a potentiating effect of FB<sub>1</sub> on iron-induced lipid peroxidation (Fig. 4).

## 4. Discussion

FB<sub>1</sub>-induced hepatotoxicity may be caused, at least in part, by lipid peroxidation and oxidative damage to hepatocytes [11–13]. Abel and Gelderblom [11] recently showed that FB<sub>1</sub> caused a dose-dependent increase in the level of TBARS in rat liver in vivo and in primary rat hepatocytes in vitro. The in vitro effect was further potentiated by the addition of cumene hydroperoxide (CMHP), a potent oxidizing agent. The authors suggested that lipid peroxidation with generation of TBARS appeared to be an end result rather than a cause of FB<sub>1</sub>-induced hepatic injury [11]. Sahu et al. [13] found no effect of metals (iron or copper) on FB<sub>1</sub>-induced peroxidation of lipid

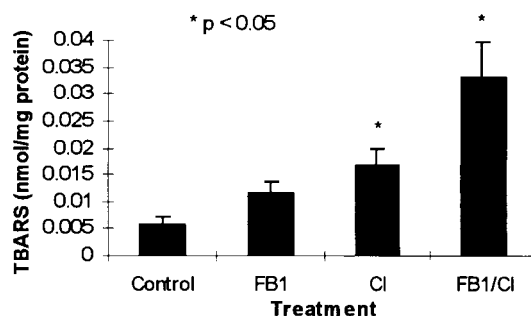


Fig. 4. Generation of thiobarbituric acid reacting substances (TBARS) from liver homogenates according to treatment group (*n* = 6, each).

membranes and oxidative DNA damage of isolated rat nuclei. The present *in vivo* study, however, shows that excess liver iron (mean content 479  $\mu\text{mol/g}$  dry weight) has a significant potentiating effect on  $\text{FB}_1$ -induced lipid peroxidation, measured as generation of TBARS, in liver homogenates. In contrast to the study by Abel and Gelderblom [11],  $\text{FB}_1$  250 mg/kg diet alone did not cause a significant increase in hepatic TBARS generation, although the level appeared to be slightly higher than in controls. This might have been due to the lower total dose of  $\text{FB}_1$  administered in the present study due to averaged feeding of the animals.  $\text{FB}_1$ -induced hepatotoxicity was reflected by a reduction in the liver/body weight ratio and a four-fold rise in serum ALT levels, and was confirmed histologically.

Agents may increase the risk of cancer by causing DNA damage (genotoxicity) and/or by causing increased proliferation (increased DNA replications) in a pluripotential cell population of the tissue [32]. To increase the number of DNA replications, an agent can either increase cell births (direct mitogenesis or toxicity and regenerative proliferation) and/or decrease cells deaths (inhibition of apoptosis) [33].  $\text{FB}_1$  appears to be a unique carcinogen which causes marked TGF- $\beta$ 1-induced apoptosis accompanied by regeneration of hepatocytes and proliferation of oval cells [7], putative precursor cells for liver tumors [34]. Although  $\text{FB}_1$  was found to be non-genotoxic in the Ames mutagenicity test [35,36], recent studies have reported the DNA damaging potential of  $\text{FB}_1$  [13,37,38].  $\text{FB}_1$ -induced lipid peroxidation might result in oxidative damage to DNA and errors in replication. These effects might be enhanced by oxidative injury due to iron loading. However, in the present study, iron overload significantly enhanced lipid peroxidation in the absence of hepatotoxic injury, whereas  $\text{FB}_1$ -induced hepatocyte injury was only associated with mild changes in lipid peroxidation. These findings are in accordance with the hypothesis of Abel and Gelderblom [11] that lipid peroxidation is secondary to  $\text{FB}_1$ -induced liver injury. With respect to cancer induction, this study showed that moderately severe hepatic iron overload (9-fold increase in liver iron content) decreased the number and size of GST pi-positive foci and nodules in  $\text{FB}_1$ -fed rats. A similar protective effect of hepatic iron loading (6- to 13-fold increase) was reported by Stål et al. [20], who added

or substituted dietary iron loading for the initiating and promoting events in the Solt Faber model of chemical hepatocarcinogenesis. They showed a mild mitostimulatory effect of iron on normal hepatocytes, which may have protected against the promotion of resistant hepatocytes. Although hepatocyte proliferation was not measured in this study (e.g. BrdU labeling, PCNA), the liver weight/body weight ratio was maintained in animals treated with  $\text{FB}_1/\text{CI}$ , suggesting that iron may augment the hepatic regenerative response to  $\text{FB}_1$ -induced loss of hepatocytes and hence may counteract the selection process (mitoinhibition of normal hepatocytes) that effects the outgrowth of initiated cells into GST pi-positive foci and nodules. Under the present conditions, where cancer promotion was apparently impaired, the effect of excess hepatic iron on  $\text{FB}_1$ -induced cancer initiation could not be determined.

There was a striking difference in the pattern of distribution of liver storage iron between group 3 (CI) and group 4 ( $\text{FB}_1/\text{CI}$ ). Dietary supplementation with CI is known to cause predominantly parenchymal (zone 1) hepatic iron deposition, with a zonal gradient, similar to that seen in genetic hemochromatosis [39,40]. Addition of  $\text{FB}_1$  feeding to iron loading resulted in a shift of liver iron from parenchymal cells to Kupffer cells, presumably due to ingestion of dead hepatocytes by liver macrophages. A similar shift in the distribution of iron from hepatocytes to reticuloendothelial cells is seen in alcohol-iron- $\text{CCl}_4$ -treated rats [41], and also occurs in genetic hemochromatosis with episodes of alcoholic hepatitis [42]. Although African dietary iron overload is also characterized by massive deposition of reticuloendothelial iron [43], the pathogenesis of the hepatic siderosis is different, and appears to relate to associated ascorbic acid deficiency with impaired release of iron from Kupffer cells [44,45].

In conclusion,  $\text{FB}_1$  had a potentiating effect on iron-induced lipid peroxidation in the liver. However, the effects on cancer induction by the fumonisin mycotoxins still need to be elucidated, particularly as dietary iron overload appears to protect against the promotion of GST pi-positive hepatic lesions by  $\text{FB}_1$ , probably due to a stimulatory effect on hepatocyte regeneration. These exciting findings provide new avenues of research regarding the mechanisms involved in  $\text{FB}_1$ -induced carcinogenesis and the

modulating role of environmental factors, such as iron overload.

### Acknowledgements

The authors thank Rochelle Barnard, Heather McCleod, and Joy McCarthy for excellent technical assistance, and Tom Bothwell for valuable comments. We are grateful to Sedick Isaacs for performing the statistical analysis and Charon Lambrechts for animal care.

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# Fumonisin B<sub>1</sub>-induced hepatocellular and cholangiocellular tumors in male Fischer 344 rats: potentiating effects of 2-acetylaminofluorene on oval cell proliferation and neoplastic development in a discontinued feeding study

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**Fumonisin B<sub>1</sub> (FB<sub>1</sub>) is a naturally occurring mycotoxin produced by *Fusarium verticillioides*. Dietary exposure to FB<sub>1</sub> has been linked to human cancer in certain parts of the world, and treatment with FB<sub>1</sub> causes oval cell proliferation and liver tumors in rats. To study the potential role of oval (liver progenitor) cells in the cellular pathogenesis of FB<sub>1</sub>-induced liver tumors, we gave male F344 rats prolonged treatment with FB<sub>1</sub> for 25 weeks, followed by return to control diet until 50 weeks ('stop study'). The time course of FB<sub>1</sub>-induced liver lesions was followed by examination of serial liver biopsies at set time intervals and post-mortem liver tissue at the end of the study. The effects of different FB<sub>1</sub> treatment regimens (5 versus 25 weeks), as well as the modulating effect of 2-acetylaminofluorene (2-AAF), on the kinetics of oval cell proliferation and development of liver tumors were compared. Prolonged treatment with FB<sub>1</sub> in normal diet caused persistent oval cell proliferation and generation of both hepatic adenomas and cholangiofibromas (CFs). These liver lesions occurred in the setting of chronic toxic hepatitis and liver fibrosis/cirrhosis, similar to that seen in human hepatocarcinogenesis. Some adenomas and CFs were dysplastic, and one post-mortem liver contained a hepatocellular carcinoma. OV-6<sup>+</sup> oval cells were noted in close relation to proliferative neoplastic liver lesions, and some of these lesions expressed OV-6, suggesting that all these cell types were derived from a common progenitor cell. 2-AAF enhanced the size of FB<sub>1</sub>-induced glutathione S-transferase pi<sup>+</sup> hepatocellular lesions and the incidence of CFs in post-mortem liver specimens, but this was not statistically significant. In conclusion, this study supports the involvement of dietary FB<sub>1</sub> in liver carcinogenesis in male F344 rats. Oval cells**

**Abbreviations:** 2-AAF, 2-acetylaminofluorene; CF, cholangiofibroma; FAH, foci of altered hepatocytes; FB<sub>1</sub>, fumonisin B<sub>1</sub>; GSTP, glutathione S-transferase pi; HA, hepatocellular adenoma; HCC, hepatocellular carcinoma; H&E, hematoxylin–eosin; LTFB, long-term fumonisin B<sub>1</sub> treatment; LTFB/AAF, long-term fumonisin B<sub>1</sub> treatment plus 2-AAF; STFB, short-term fumonisin B<sub>1</sub> treatment; STFB/AAF, short-term fumonisin B<sub>1</sub> treatment plus 2-AAF.

may be the source of both the hepatocellular and cholangiocellular tumors induced by prolonged treatment with FB<sub>1</sub>. 2-AAF appears to have an enhancing effect on FB<sub>1</sub>-induced liver tumors, presumably due to its potent inhibitory effects on hepatocyte regeneration.

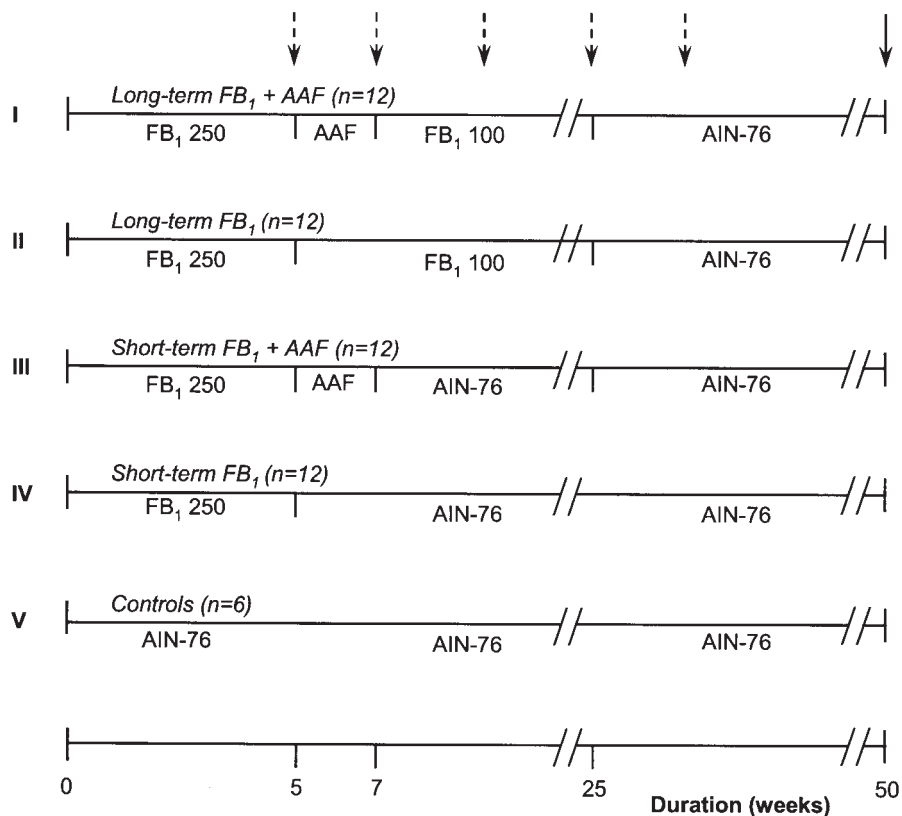
## Introduction

Fumonisin B<sub>1</sub> (FB<sub>1</sub>) is a food-borne mycotoxin produced by the fungus *Fusarium verticillioides* (previously *Fusarium moniliforme*) (1) that occurs worldwide and causes a variety of naturally occurring toxicoses in animals, including fatal illnesses in horses (2) and pigs (3). Human dietary consumption of *Fusarium*-contaminated corn products has been linked epidemiologically to increased rates of esophageal cancer (4,5), and perhaps hepatocellular carcinoma (HCC) (6), in regions of the world in which corn is the staple grain, such as South Africa and China. FB<sub>1</sub> is hepatotoxic and hepatocarcinogenic in rats. Short-term feeding with FB<sub>1</sub> causes severe toxic hepatitis (1), while continued FB<sub>1</sub> administration leads to a chronic toxic hepatitis and fibrosis, which progresses to cirrhosis, and sometimes terminates in HCC or cholangiocarcinoma (7). FB<sub>1</sub> appears to be a unique carcinogen that causes regenerative hyperplasia of hepatocytes and promotion of tumors despite striking pro-apoptotic effects (8,9). The molecular mechanisms underlying FB<sub>1</sub>-induced hepatocarcinogenesis are not known, but recent data implicate the activation of specific apoptotic and oncogenic pathways, including the TGF- $\beta$  pathway (10) and the Akt/cyclinD1 pathway (11).

The cellular origin of HCCs induced by FB<sub>1</sub> is unclear, and most studies have focused on the role of the mature hepatocyte (9). However, we recently showed that short-term treatment of rats with FB<sub>1</sub> resulted in early proliferation of 'oval cells', which coincided with the appearance of foci of altered hepatocytes (FAH) (10). Microscopically, oval cells are small cells with elongated nuclei and scanty cytoplasm (12), which appear to arise from cells in the terminal bile ductules (canals of Hering) or from periductular cells (13). Proliferating oval cells have been noted to appear early in several experimental carcinogenesis protocols, and these cells may represent the progeny of a liver stem cell (14). Oval cells are bipotential and can differentiate into either hepatocytes or bile duct epithelial cells, and they have been shown to have clonogenic potential *in vivo* and *in vitro*. Oval cells are not normally involved in liver regeneration, due to the enormous replicative capacity of hepatocytes, but are induced to proliferate under conditions of severe liver injury when mature hepatocytes are overwhelmed or prevented from proliferating (14). There is now good evidence that bipotential ductular progenitor cells (oval cells) may give rise to HCCs under certain experimental conditions (15).

In order to study the potential role of oval cell proliferation in the generation of FB<sub>1</sub>-induced proliferative and neoplastic

### FB<sub>1</sub> treatment groups



**Fig. 1.** Schema of FB<sub>1</sub>-containing treatment regimens (see text for details of individual treatment groups). Dashed arrow indicate open liver biopsies and solid arrow indicate post-mortem liver tissue.

liver lesions, male F344 rats were treated with FB<sub>1</sub> for 25 weeks, followed by return to control diet ('stop study'). Different FB<sub>1</sub>-containing treatment regimens (5 versus 25 weeks) were compared, and the modulating effect of 2-acetylaminofluorene (2-AAF) on FB<sub>1</sub>-induced oval cell proliferation and tumorigenesis was also assessed. The sequential development of hepatic histopathologic lesions in the different treatment groups was monitored by examination of serial liver biopsies as well as post-mortem liver tissue.

## Materials and methods

### Chemicals

FB<sub>1</sub> was purified from corn cultures of *F.verticillioides* strain MRC 826 according to a method described previously (16). The purity as compared with an analytical standard by high-performance liquid chromatography was in the order of 92–95% (24). The monomethyl ester derivatives of FB<sub>1</sub>, which are artifacts of the purification procedure, constituted the remainder of the FB<sub>1</sub> preparation. 2-AAF stock powder was purchased from Sigma-Aldrich (St Louis, MO).

### Animals and diets

The study was approved by the Animal Ethics and Research Committee of the Faculty of Health Sciences, University of Cape Town and the experiments were conducted in accordance with the laws and regulations controlling experiments on live animals in South Africa. Fifty-four male Fischer 344 rats weighing between 150 and 200 g were used for the experiments. The animals were caged individually in a controlled environment at 23–24°C and 50% humidity with a 12 h artificial light cycle. Food and water were available *ad libitum*, and rats were weighed weekly. All the animals received American Institute of Nutrition (AIN)-76 diet (17) with the following modifications: the corn starch was replaced with glucose/sucrose/corn starch (1:1:1) while sunflower oil was

used instead of corn oil as a fat source. Corn products were excluded from the control diet in order to prevent any possibility of contamination by *F.verticillioides*.

### Treatments

The FB<sub>1</sub>-containing treatments were prepared as described previously (18). The 2-AAF containing treatments were prepared by mixing 2-AAF stock powder into AIN-76 diet to give a concentration of 0.02% (w/w), a dose known to cause effective mitoinhibition of hepatocytes (19,20).

### Experimental

The different FB<sub>1</sub>-containing treatment regimens are shown in Figure 1, and the details of the individual treatment groups (in order of decreasing intensities) are as follows: group I rats (long-term FB<sub>1</sub> plus 2-AAF, LTFB/AAF, *n* = 12) initially received FB<sub>1</sub> 250 mg/kg diet for 5 weeks, a dose shown previously to induce oval cell proliferation and appearance of FAH (10). The animals then received a 2-week course of 2-AAF 0.02% in the diet (weeks 5–7), and were then continued on FB<sub>1</sub> 100 mg/kg until 25 weeks. The maintenance dose of FB<sub>1</sub> was reduced from the initial dose in order to avoid fulminant hepatotoxicity while maintaining a pro-carcinogenic effect (21); group II rats (long-term FB<sub>1</sub>, LTFB, *n* = 12) initially received FB<sub>1</sub> 250 mg/kg diet for 5 weeks, and were then continued on FB<sub>1</sub> 100 mg/kg until 25 weeks; group III rats (short-term FB<sub>1</sub> plus 2-AAF, STFB/AAF, *n* = 12) received FB<sub>1</sub> 250 mg/kg diet for 5 weeks only, followed by 2-AAF 0.02% from weeks 5–7; group IV rats (short-term FB<sub>1</sub>, STFB, *n* = 12) received FB<sub>1</sub> 250 mg/kg diet for 5 weeks only; and group V rats (controls, *n* = 6) received control (modified AIN-76) diet. A treatment group of rats given only a 2-week course of 2-AAF was not included in this study, but this regimen is reported to produce little cellular change (22). Following treatment with the different active (FB<sub>1</sub>-containing) treatment regimens in groups I–IV, all animals were returned to control diet until week 50, in order to assess the reversibility of FB<sub>1</sub>-induced proliferative and neoplastic liver lesions.

### Serial liver biopsies

The sequential development of histopathologic liver lesions was followed by histologic examination of liver tissue obtained by repeated open wedge liver

**Table I.** Scoring systems for hepatic fibrosis, oval cell proliferation and GSTP<sup>+</sup> lesions

|       |  |
|-------|--|
| Score | Fibrosis <sup>a</sup>                                  |
| 0     | None   |
| 1     | Enlarged, fibrotic portal tracts                       |
| 2     | Periportal or portal—portal septa                      |
| 3     | Fibrosis with disturbed architecture, but no cirrhosis |
| 4     | Probable or definite cirrhosis                         |
| Score | Oval cell proliferation                                |
| 0     | None   |
| 1     | Confined to portal tracts                              |
| 2     | Radiating out from portal tracts into fibrous septa    |
| 3     | Infiltrating liver parenchyma                          |
| 4     | Diffuse proliferation                                  |
| Score | GSTP <sup>+</sup> lesions <sup>b</sup>                 |
| 0     | None   |
| 1     | <25% of liver section                                  |
| 2     | 25–50% of liver section                                |
| 3     | >50% but <75% of liver section                         |
| 4     | 75–100% of liver section                               |

<sup>a</sup>Modified from Scheuer (35).

<sup>b</sup>Percentage area of liver section occupied by GSTP<sup>+</sup> lesions was a simple visual assessment by the histopathologist.

biopsies (Figure 1), which were performed under ether anesthesia (to reduce the potential for hepatotoxicity) according to the method described by Cmielewski *et al.* (23). Biopsies were performed via a small (2–3 cm) midline incision, and bleeding from the biopsy site was controlled by diathermy. A different lobe was biopsied each time, commencing with the posteriorly situated left lobe, and rotating to the more accessible median lobe for repeat biopsy. Biopsies were performed at weeks 5 and 7, and then 4-weekly. At each session, two rats from each of the active treatment groups and one control animal were biopsied. Rats receiving biopsies were rotated at each session until all the animals in the different groups had undergone a liver biopsy, thereafter repeat biopsies were performed in the same order. As a result of the high surgical mortality rate (see Results), serial liver biopsies were discontinued at 39 weeks.

#### Post-mortem liver examinations

Post-mortem liver tissue was obtained upon the death of surviving rats at 50 weeks (Figure 1), or following deaths at any time during the course of the study period. The livers were weighed and sectioned. Macroscopic tumor nodules near the peritoneal surface or on the cut surface were recorded. Slices from each of the four liver lobes were processed for histological examination (see below).

#### Light microscopy and immunohistochemistry

Four to five millimeter-thick slices of liver were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4 μm for staining with hematoxylin–eosin (H&E) for routine light microscopy and Sirius red stain for collagen (10). Immunohistochemical staining for desmin (stellate cells), OV-6 (oval cells) and glutathione *S*-transferase pi (GSTP) was performed as described previously (10). Diagnoses of proliferative and neoplastic liver lesions were made according to the criteria of Bannasch and Zerban (24). Oval cells were identified by characteristic light microscopic features (12), and confirmed by staining with OV-6 monoclonal antibody.

#### Scoring of hepatic histopathological lesions

In order to enable semi-quantitative assessments and comparisons between treatment groups, numerical scoring systems were devised for hepatic fibrosis, oval cell proliferation and area (%) occupied by GSTP<sup>+</sup> lesions (Table I). In this study, only GSTP<sup>+</sup> lesions >1 mm were included in the measurements. The liver pathologist examined coded liver sections, and was unaware of the treatment given in each case.

#### Statistical analysis

For comparison of liver biopsy histopathological parameters (scores) amongst the different treatment groups, statistical analysis was performed on the unweighted means of the treatment-time combinations of the groups, using the general linear model of analysis of variance (ANOVA). Histopathological data

from post-mortem livers for the different treatment groups were compared using two-way ANOVA. The level of statistical significance was set at  $P < 0.05$ .

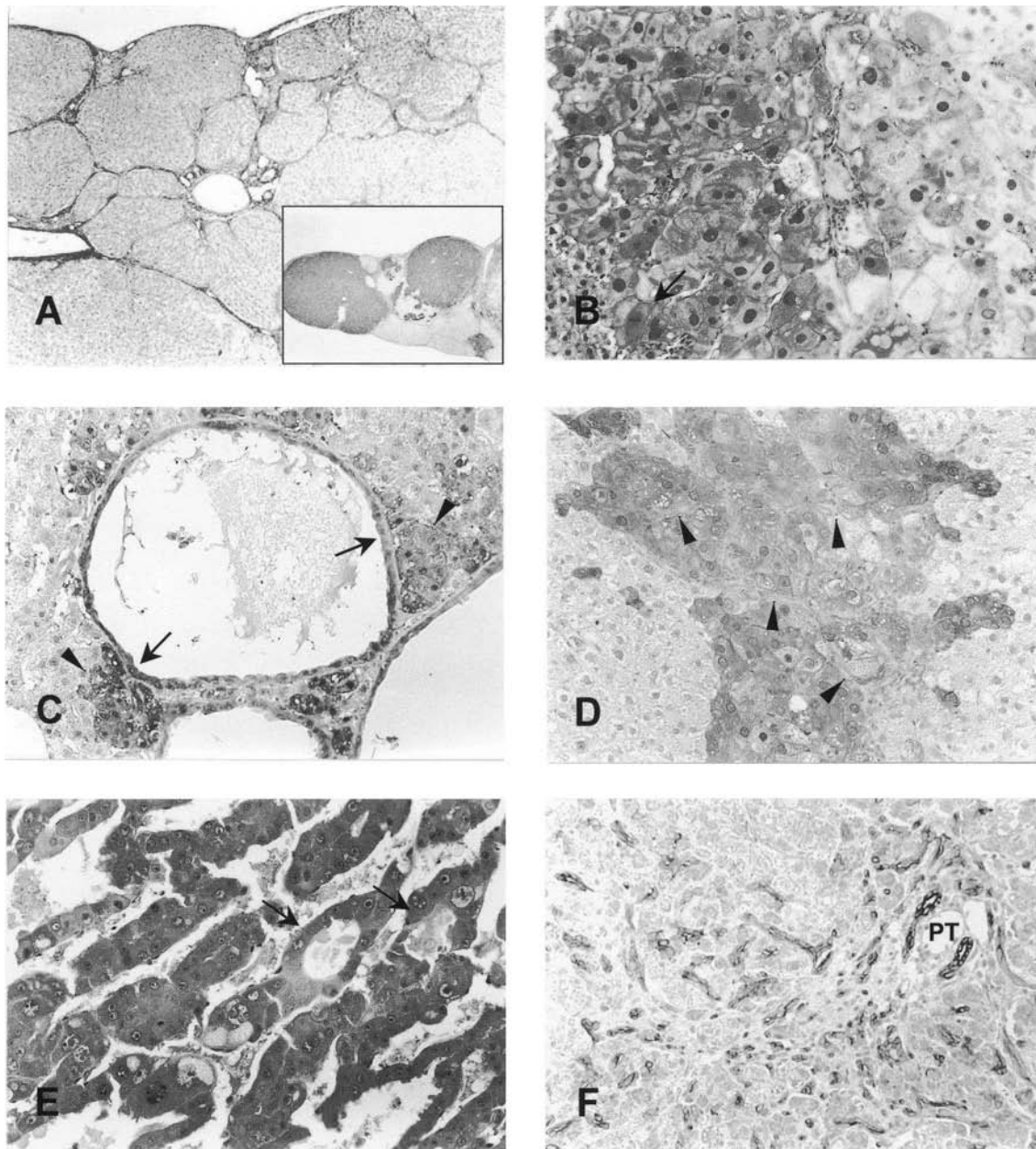
## Results

### Surgical mortality

The 54 rats underwent 84 open liver biopsies at 10 time points during the study period. All 54 rats had one liver biopsy, and 30 rats underwent a second biopsy. The numbers of biopsies performed in the different groups were as follows: group I (LTFB/AAF), 19; group II (LTFB), 18; group III (STFB/AAF), 19; group IV (STFB), 17; and group V (controls), 11. The surgical mortality rates by treatment group were as follows: group I, 5.0% (1/20); group II, 19.0% (4/21); group III, 10.5% (2/19); group IV, 23.3% (5/19); and group V, 11.1% (1/9). Most deaths appeared to be related to general anesthesia and liver surgery in animals with severe chronic liver toxicity, and the single operative death in the control group was an unexpected event related to anesthesia overdose. Because of the high surgical mortality rate encountered, serial liver biopsies were not extended beyond week 39 of the study.

### Hepatic histopathological lesions caused by treatment of male F344 rats with FB<sub>1</sub>-containing feeding regimens

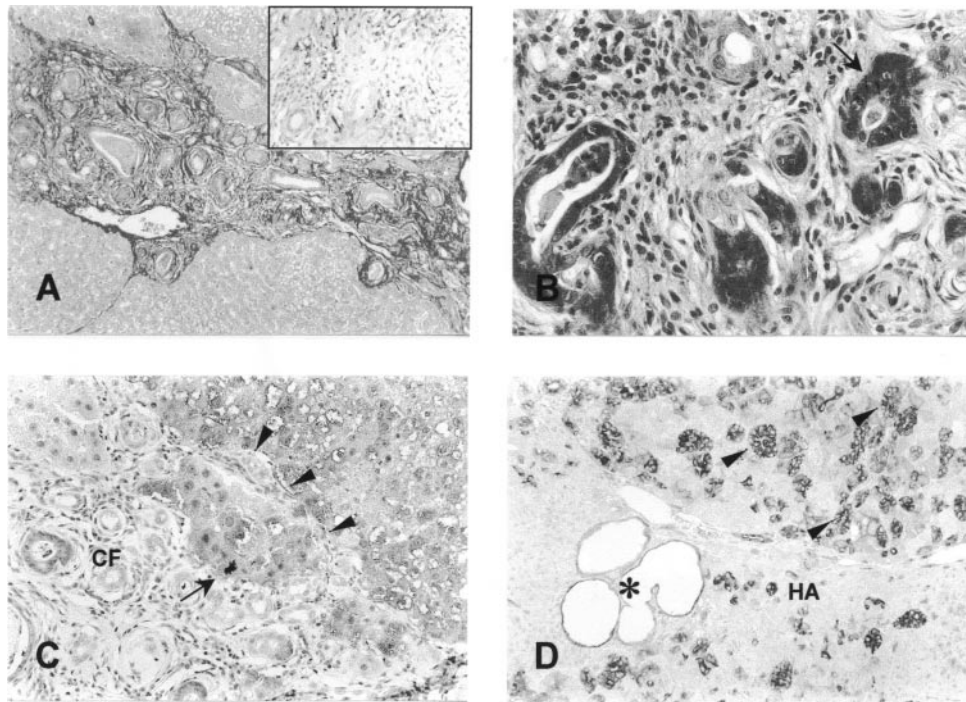
*Group I rats (LTFB/AAF).* The early hepatic histopathological lesions induced by treatment with FB<sub>1</sub> (at 5 weeks) were similar to those described previously (10), although the degree of toxic liver injury and oval cell proliferation appeared to be less severe. Proliferation of hepatic stellate cells was maximal at weeks 5–7 (during treatment with 2-AAF), and desmin-positive stellate cells and portal fibroblasts were seen to radiate out from portal tracts into the surrounding hepatic parenchyma (data not shown). By week 7, there was established hepatic fibrosis, resulting in portal–portal linkage and architectural distortion. Thereafter, liver fibrosis progressed more gradually, eventuating in septal fibrosis or (less commonly) cirrhosis (Figure 2A) during the study period. Many of the larger nodules in cirrhotic livers were GSTP<sup>+</sup>, indicating that these nodules were in fact hepatic adenomas (HAs) (Figure 2A, inset). Proliferation of OV-6<sup>+</sup> oval cells and ductules was maximal at 7 weeks and declined thereafter, despite continued treatment with FB<sub>1</sub>. The size of HAs appeared to enlarge progressively, attaining maximal size after completion of FB<sub>1</sub> treatment (~27 weeks). However, several HAs persisted and even enlarged from weeks 25–50, following discontinuation of FB<sub>1</sub>. Histologically, basophilic and clear cell types predominated in the HAs (Figure 2B). Some HA hepatocytes showed features of nuclear atypia (Figure 2B). Proliferating oval cells and ductules were seen within several GSTP<sup>+</sup> (adenomatous) lesions. In addition, several HAs contained curious cystic lesions lined by cuboidal cells. These lining cells had the morphology of hepatocytes, but they expressed OV-6 antigen (Figure 2C). The intimate spatial relationships and common phenotypic markers of these different epithelial cell types suggested that they might have all originated from proliferating oval cells and ductules. Most livers contained typical cholangiofibromas (CFs) (often multiple) by the end of the study, characterized by proliferating bile ducts embedded in dense connective tissue (Figure 3A), presumably laid down by proliferating stellate cells (Figure 3A, inset). Some CFs showed cytologic features of dysplasia (Figure 3B), but no unequivocal cholangiocarcinomas were seen. In post-mortem livers, some GSTP<sup>+</sup> lesions were noted to be located directly



**Fig. 2.** Hepatic histopathological changes in F344 rats treated with FB<sub>1</sub>-containing regimens. (A) Sirius red stain of a liver biopsy from group I rat (LTFB/AAF) at week 23 showing loss of the normal acinar architecture due to the presence of regenerative nodules of hepatocytes, which are partially or completely surrounded by bands of fibrous tissue, indicating established cirrhosis. Sirius red, 40 $\times$ . (Inset) Low power view of liver biopsy from another group I rat at week 23 showing cirrhotic liver with regenerative nodules of different sizes. Several of the larger nodules are composed of GSTP<sup>+</sup> hepatocytes, indicating altered enzyme phenotype and suggesting that these are HAs. GSTP; 40 $\times$ . (B) Post-mortem liver from group I (LTFB/AAF) rat at week 50 death showing a HA with features of dysplasia. The HA contains both basophilic and clear hepatocytes, and these cells show evidence of cytological atypia. A mitotic figure (arrow) is seen in a basophilic hepatocyte. H&E, 200 $\times$ . (C) OV-6 immunostaining of liver from a group I (LTFB/AAF) rat at week 50 death showing large cystic lesions that are lined by cuboidal cells, and which have the morphology of hepatocytes but express OV-6 antigen (arrows). These cystic lesions are located within a HA, and some of the surrounding hepatocytes also express OV-6 (arrowheads). OV-6, 200 $\times$ . (D) Liver biopsy specimen from group II rat (LTFB) at week 19 showing cells with the morphology of oval cells (arrowheads) which are proliferating inside a lesion composed of GSTP<sup>+</sup> hepatocytes. The oval cells do not stain with GSTP, which aids their identification within the GSTP<sup>+</sup> lesion. GSTP, 200 $\times$ . (E) Post-mortem liver from a group II rat (LTFB) at week 50 death showing an unequivocal HCC. The malignant tumor is moderately differentiated and has a predominantly trabecular pattern, but in areas the formation of pseudoglands can be seen (arrows). H&E, 200 $\times$ . (F) Liver biopsy from group III animal (STFB/AAF) at week 7 showing extensive proliferation of OV-6-positive oval cells and ductules, which are radiating out from a portal tract (PT) as ribbons and cords into the hepatic parenchyma. OV-6, 200 $\times$ .

adjacent to CFs (Figure 3C), and proliferating oval cells were intertwined with both of these lesions (Figure 3C). Furthermore, some HAs contained hepatocytes that appeared to form ductular structures, and these cells showed expression of OV-6 in a peripheral pattern (Figure 3D). Multilocular cystic cholangiomas were seen in some livers, and the lining epithelial

cells of these biliary lesions also expressed OV-6 (Figure 3D). Taken together, these close spatial and immunophenotypic relationships raised the possibility that proliferating oval and ductular cells might be involved in the generation of both the hepatocellular and cholangiocellular lesions seen in these animals.



**Fig. 3.** Hepatic histopathological changes in F344 rats treated with FB<sub>1</sub>-containing regimens. (A) Post-mortem liver from a group I rat (LTFB/AAF) at week 50 death showing a CF with dense fibrosis surrounding proliferative bile duct lesions. Strands of fibrotic tissue extend into the surrounding liver, which shows disturbance of the normal acinar architecture and early regenerative nodules. Sirius red, 100 $\times$ . (Inset) Staining of the same liver specimen with desmin showed the presence of proliferating vessels and hepatic stellate cells within the fibrous tissue surrounding the bile duct lesions in a CF. Desmin, 100 $\times$ . (B) Liver biopsy specimen from a group I rat (LTFB/AAF) at week 15 showing a CF containing irregular tapered bile ducts, which show features of cytological atypia, e.g. crowding of cells, nuclear hyperchromasia, and a mitotic figure (arrow). H&E, 400 $\times$ . (C) Post-mortem liver from group I rat (LTFB/AAF) at week 50 death showing a HA containing of GSTP<sup>+</sup> hepatocytes (upper right), and which is directly adjacent to proliferative bile duct lesions of a CF. Furthermore, cells with the morphology of oval cells and ductules are proliferating in close relationship to the GSTP<sup>+</sup> hepatocytes (arrowheads) and proliferative bile duct lesions. Of note is that the proliferating oval cells, ductules, and bile duct lesions do not stain with GSTP. A mitotic figure (arrow) is seen within one of the GSTP<sup>+</sup> hepatocytes. GSTP, 200 $\times$ . (D) OV-6 immunostaining of a post-mortem liver from a group I rat (LTFB/AAF) at week 50 death showing HA-containing hepatocytes that are expressing OV-6 antigen in a peripheral (membranous) pattern. Many of the OV-6-positive hepatocytes within the nodule appear to be forming ductules (arrowheads). A multilobular cholangioma (\*) is seen adjacent to the adenoma, and the flattened epithelial lining cells also express OV-6. OV-6, 200 $\times$ .

**Group II rats (LTFB).** The histopathological changes were similar to those described for group I animals (LTFB/AAF) above. Once again, proliferating oval cells were noted within and around GSTP<sup>+</sup> (Figure 2D) lesions, suggesting a common cell of origin. One post-mortem liver contained a trabecular HCC (Figure 2E), and this was the only animal in the study that had developed an unequivocal HCC.

**Group III rats (STFB/AAF).** 2-AAF appeared to enhance FB<sub>1</sub>-induced proliferation of OV-6<sup>+</sup> oval cells and ductules seen at 7 weeks (Figure 2F), but oval cell proliferation declined rapidly following discontinuation of treatment. HAs were small and no HCCs were found. However, 2-AAF appeared to have a potentiating effect on the incidence and number of CFs induced by short-term treatment with FB<sub>1</sub>.

**Group IV rats (STFB).** Minimal long-term effects, e.g. fibrosis, HAs and CFs were noted in livers from animals that received short-term treatment with FB<sub>1</sub> only.

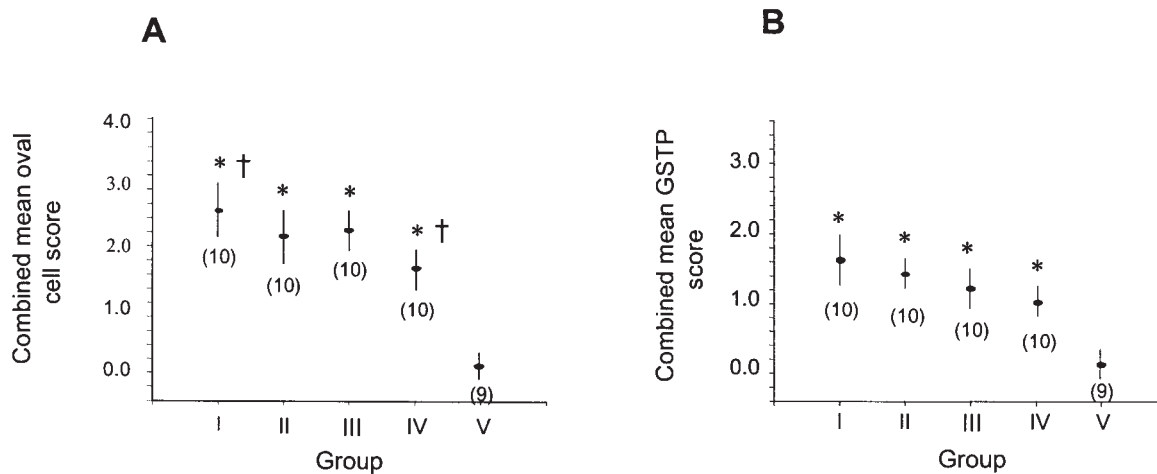
**Group V (controls).** Livers from control rats all showed normal histology, apart from occasional single cell GSTP<sup>+</sup> foci by the end of the study.

**Kinetics of oval cell proliferation and development of liver tumors in rats from the different FB<sub>1</sub>-containing treatment groups**

**Liver biopsy data.** As shown in Figure 4A, mean oval cell scores (treatment-time combinations) were significantly

increased in all active (FB<sub>1</sub>-containing) treatment groups compared with controls. There was no clear increase in oval cell score by either 2-AAF [group I (LTFB/AAF) versus II (LTFB) and III (STFB/AAF) versus IV (STFB)] or prolonged FB<sub>1</sub> treatment [group I (LTFB/AAF) versus III (STFB/AAF) and II (LTFB) versus IV (STFB)]. However, the combination of 2-AAF and prolonged FB<sub>1</sub> (LTFB/AAF) resulted in a significant increase in mean oval cell score when compared with brief FB<sub>1</sub> treatment (STFB) alone (Figure 4A), suggesting an enhancing effect. Despite marked variability at individual time points, there appeared to be a decline in oval cell scores over time in all active treatment groups (data not shown). Biopsy GSTP scores were significantly increased in all treatment groups compared with controls, but there were no differences between the different active treatment groups (Figure 4B). Unlike the sequential biopsy oval cell scores, GSTP scores appeared to remain stable or increase over time in all active treatment groups (data not shown). Scores for fibrosis were increased in all active treatment groups compared with controls, but there were no differences between treatment groups, and no significant increases over time (data not shown).

**Post-mortem liver data.** Scores for oval cell proliferation were increased in all active (FB<sub>1</sub>-containing) treatment groups compared with controls, despite return to control diet after completion of the FB<sub>1</sub>-containing regimens (Table II). Similar to the biopsy findings (see above), there is evidence of



**Fig. 4.** Histopathological scores for liver lesions caused by different FB<sub>1</sub>-containing treatment regimens (liver biopsy data). The combined (treatment-time) mean histological score for each treatment group is shown. (A) Oval cell proliferation. (B) GSTP<sup>+</sup> lesions. The numbers of animals per group are indicated in brackets and vertical bars represent the standard error (SE). \**P* < 0.01, active treatment groups (groups I-IV) versus controls; †*P*(0.05), group I (LTFB/AAF) versus IV (STFB). See Table I for numerical histologic scoring systems and Figure 1 for details of treatment protocols.

**Table II.** Comparisons of hepatic histopathological lesions caused by different FB<sub>1</sub>-containing treatment regimens (post-mortem liver data)<sup>a</sup>

| Group (n) | Treatment | Oval cell score        | % GSTP <sup>+</sup> lesions area | Fibrosis score         | Livers (%) with cirrhosis | Livers (%) with CF | Livers (%) with HCC |
|-----------|-----------|------------------------|----------------------------------|------------------------|---------------------------|--------------------|---------------------|
| I (8)     | LTFB/AAF  | 2.6 ± 0.4              | 49.0 ± 6.9 <sup>b</sup>          | 2.3 ± 0.3              | 2 (25)                    | 7 (88)             | 0 (0)               |
| II (6)    | LTFB      | 2.0 ± 0.5              | 22.9 ± 8.8                       | 2.0 ± 0.4              | 2 (33)                    | 2 (33)             | 1 (17)              |
| III (9)   | STFB/AAF  | 1.6 ± 0.3              | 22.2 ± 6.9                       | 1.8 ± 0.3              | 0 (0)                     | 5 (56)             | 0 (0)               |
| IV (7)    | STFB      | 1.3 ± 0.5              | 6.3 ± 9.8                        | 2.0 ± 0.4              | 1 (20)                    | 1 (14)             | 0 (0)               |
| V (5)     | Controls  | 0.0 ± 0.0 <sup>c</sup> | 0.5 ± 9.8 <sup>d</sup>           | 0.0 ± 0.0 <sup>b</sup> | 0 (0)                     | 0 (0)              | 0 (0)               |

<sup>a</sup>See Table I for details of histological scoring systems and Figure 1 for outline of FB<sub>1</sub> treatment groups.

<sup>b</sup>*P* < 0.05 compared with group IV (STFB).

<sup>c</sup>*P* < 0.01 versus active treatment groups (I-IV).

<sup>d</sup>*P* < 0.05 versus active treatment groups.

enhancement of oval cell scores by either 2-AAF or prolonged FB<sub>1</sub> treatment alone, but in combination (LTFB/AAF) the two treatments had an augmented effect compared with brief FB<sub>1</sub> treatment (STFB) alone. The scores for GSTP<sup>+</sup> lesions were increased in all active treatment groups compared with controls. GSTP<sup>+</sup> scores were increased by 15% for group I (LTFB/AAF) versus group II (LTFB) and by 50% for group III (STFB/AAF) versus group IV (STFB) (data not shown), but these differences were not statistically significant. However, the ranges of GSTP<sup>+</sup> lesion sizes (percentage liver area occupied) that were chosen for the different scoring levels were quite broad (Table I), and could hide marked differences in sizes of lesions. Examination of the raw data for percentage area occupied by GSTP<sup>+</sup> lesions showed that there was clear evidence of considerable enhancement of GSTP<sup>+</sup> lesions caused by both 2-AAF (group I versus II and III versus IV) and prolonged FB<sub>1</sub> treatment (group I versus III and II versus IV) (Table II), which was of borderline statistical significance. Furthermore, the effects of 2-AAF and prolonged FB<sub>1</sub> treatment on GSTP<sup>+</sup> lesions appeared to be augmented, as evidenced by a significant increase in percentage area of GSTP<sup>+</sup> lesions in group I (LTFB/AAF) compared with group IV (STFB). Scores for fibrosis were increased in all treatment groups compared with controls, but there were no significant differences in fibrosis scores (or the development of cirrhosis) between the different active treatment groups. Interestingly, there appeared to be an enhancing effect of 2-AAF (but not prolonged FB<sub>1</sub>) on the development of CFs, as evidenced by a

non-significant increase in the number of post-mortem livers that contained CF in groups I (LTFB/AAF) and III (STFB/AAF) compared with groups II (LTFB) and IV (STFB), respectively. Only one post-mortem liver was found to contain unequivocal HCC, and this liver came from a group II (LTFB) animal.

## Discussion

The present study shows that prolonged treatment with FB<sub>1</sub> in normal diet causes liver tumors (both hepatocellular and cholangiocellular) in rats. Male F344 rats given FB<sub>1</sub> (250 mg/kg diet for 5 weeks followed by 100 mg/kg diet until 25 weeks) developed GSTP<sup>+</sup> hepatic foci (FAH) and adenomas (HA), and these lesions persisted following discontinuation of FB<sub>1</sub>, indicating autonomous (neoplastic) growth. The FB<sub>1</sub>-induced hepatocellular tumors developed in a setting of chronic (toxic) hepatitis and marked hepatic fibrosis, sometimes progressing to cirrhosis. These findings are reminiscent of human HCC, which typically develops in the setting of chronic necroinflammatory liver disease and cirrhosis (25). The FB<sub>1</sub>-fed rat may thus be a useful model to study the histopathogenesis of tumor development in human HCC. Prolonged FB<sub>1</sub> treatment also resulted in the appearance of CFs, and some hepatocellular and cholangiocellular tumors showed cytological features of dysplasia. However, only one post-mortem liver (from a rat that received prolonged FB<sub>1</sub> without 2-AAF) showed a trabecular

HCC, and no unequivocal cholangiocarcinomas were found. It is likely that a longer period of FB<sub>1</sub> treatment is required to cause HCC and cholangiocarcinomas in these animals. In this regard, Gelderblom *et al.* (7) showed previously that treatment of BD IX rats with FB<sub>1</sub> (50 mg/kg diet) for 26 months resulted in the development of HCCs (in 60% of rats) and cholangiocarcinomas. However, the basal diet used in the study was deficient in multiple vitamins and lipotropes, and such dietary deficiencies could modulate FB<sub>1</sub>-induced hepatocarcinogenesis (26). More recently, Howard *et al.* (27) found that male F344/N rats fed diets containing FB<sub>1</sub> (up to 150 mg/kg diet) for 2 years developed renal tubular neoplasms but no liver tumors, and there was no evidence of carcinogenic activity of FB<sub>1</sub> in females. The reason for the lack of hepatocarcinogenic effects of FB<sub>1</sub> in this long-term feeding study is unclear.

To date, *in vivo* studies on cellular carcinogenesis by FB<sub>1</sub> have focused on the role of initiated hepatocytes and the foci-nodule-carcinoma sequence (9), according to the resistant hepatocyte model of Solt and Farber (28,29). Here we provide evidence of a potential role for oval cells in FB<sub>1</sub>-induced hepatocarcinogenesis. Prolonged treatment of rats with FB<sub>1</sub> caused both hepatocellular and cholangiocellular tumors (frequently in the same animal). This propensity of FB<sub>1</sub> to cause both hepatocellular and bile duct tumors is shared by only a few other experimental carcinogens, e.g. 2-AAF (30) and furan (31), and suggests that both the hepatocellular and cholangiocellular tumors induced by FB<sub>1</sub> may originate from a common bipotential progenitor cell capable of differentiating along both the hepatocytic and biliary lineages. OV-6 is a mouse monoclonal antibody directed at cytokeratin 14/19 (32), which normally stains oval cells and also may stain some bile duct cells, adenomatous hepatocytes (within HAs) and HCCs in rat liver (33,34). We noted an intimate spatial relationship between proliferating OV-6<sup>+</sup> oval cells and ductules, GSTP<sup>+</sup> hepatocellular lesions (foci and adenomas) and bile duct lesions (CFs). Furthermore, several cell types besides oval cells and ductular cells were found to express OV-6 antigen, including some adenomatous hepatocytes and also cells within HAs that resembled hepatocytes, but which lined curious cystic structures or appeared to form ductules. While these observations do not provide direct proof that oval cells are the source of the hepatocellular and cholangiocellular tumors in the FB<sub>1</sub>-fed rat, they add to the growing weight of evidence from other carcinogenesis models of such a concept (15,22).

From the biopsy and post-mortem liver data, it appears that 2-AAF and prolonged FB<sub>1</sub> treatment have transient effects only on oval cell proliferation, but they exert sustained and enhancing effects on the growth of GSTP<sup>+</sup> hepatocellular lesions. Although not specifically monitored in this study, it is unlikely that the liver biopsy procedures themselves resulted in any significant enhancing effects on the development of liver lesions. The enhancing effects of 2-AAF and FB<sub>1</sub> on GSTP<sup>+</sup> lesions were particularly evident in post-mortem liver specimens, especially when using the raw data for percentage area occupied by GSTP<sup>+</sup> lesions rather than the GSTP<sup>+</sup> score. Examination of whole liver specimens avoids the potential problem of sampling error, which is inherent in liver biopsy specimens, and is thus the most accurate method for assessing focal liver lesions in the rat. Although there appeared to be discordance in the kinetics of oval cell proliferation and GSTP<sup>+</sup> lesion growth during the course of the study, it is quite possible that some oval cells may have differentiated into adenomatous hepatocytes at an early time point. These

findings thus do not militate against the possibility of an oval cell origin for the hepatocellular tumors caused by FB<sub>1</sub>. Interestingly, 2-AAF (but not prolonged FB<sub>1</sub> treatment) had a marked enhancing effect on the incidence of CFs in post-mortem livers. Although not specifically measured in this study, 2-AAF is known for marked inhibition of proliferation of normal hepatocytes (19,20), and this property was thought to underlie its potent promoting effects on FB<sub>1</sub>-induced GSTP<sup>+</sup> lesions and CFs, possibly through resultant activation of a facultative stem cell compartment (oval cells) in the liver.

In conclusion, this study supports the involvement of dietary FB<sub>1</sub> in liver carcinogenesis in male F344 rats. Oval cells may be the source of both the hepatocellular and cholangiocellular tumors induced by prolonged treatment with FB<sub>1</sub>. 2-AAF appears to have an enhancing effect on FB<sub>1</sub>-induced liver tumors, presumably due to its potent inhibitory effects on hepatocyte regeneration.

### Acknowledgements

The authors wish to thank Mrs Heather McCleod, Mr Willem Ryneveldt and Mr Gert Engelbrecht for expert technical assistance; Mrs Petra Snijsman for the purification of FB<sub>1</sub> and Ms Charon Lambrechts for humane care of the animals. We are grateful to Dr Peter Cruse for valuable discussions and to Dr Stewart Sell for the OV-6 antibody.

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Received September 11, 2003; revised February 6, 2004;  
accepted February 19, 2004





## Chemoprotective properties of rooibos (*Aspalathus linearis*), honeybush (*Cyclopia intermedia*) herbal and green and black (*Camellia sinensis*) teas against cancer promotion induced by fumonisin B<sub>1</sub> in rat liver

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### ARTICLE INFO

#### Article history:

Received 20 March 2008

Accepted 2 November 2008

#### Keywords:

Rooibos  
Honeybush  
Fumonisin B<sub>1</sub>  
Cancer promotion  
Rat liver

### ABSTRACT

The chemoprotective properties of unfermented and fermented rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*) herbal teas, and green and black teas (*Camellia sinensis*) were investigated against fumonisin B<sub>1</sub> (FB<sub>1</sub>) promotion in rat liver utilizing diethylnitrosamine (DEN) as cancer initiator. The various teas differently affected the clinical chemical parameters associated with liver and kidney damage associated with FB<sub>1</sub> suggesting specific FB<sub>1</sub>/iron/polyphenolic interactions. Green tea enhanced ( $P < 0.05$ ) the FB<sub>1</sub>-induced reduction of the oxygen radical absorbance capacity, while fermented herbal teas and unfermented honeybush significantly ( $P < 0.05$ ) decreased FB<sub>1</sub>-induced lipid peroxidation in the liver. The teas exhibited varying effects on FB<sub>1</sub>-induced changes in the activities of catalase, glutathione peroxidase (GPx) glutathione reductase (GR) as well as the glutathione (GSH) status. Unfermented rooibos and honeybush significantly ( $P < 0.05$ ) to marginally ( $P < 0.1$ ) reduced the total number of foci ( $>10 \mu\text{m}$ ), respectively, while all the teas reduced the relative amount of the larger foci. Fermentation seems to reduce the protective effect of the herbal teas. Differences in the major polyphenolic components and certain FB<sub>1</sub>/polyphenolic/tissue interactions may explain the varying effects of the different teas on the oxidative parameters, hepatotoxic effects and cancer promotion in rat liver.

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### 1. Introduction

The popularity of the two South African indigenous herbal teas, rooibos (*Aspalathus linearis*) and honeybush (*Cyclopia intermedia*), is increasing as health beverages worldwide. This is mainly due to the absence of caffeine (Morton, 1983), as well as antioxidant (Von Gadow et al., 1997; Yoshikawa et al., 1990; Hubbe and Joubert, 2000a,b; Joubert et al., 2004, 2008), anticlastogenic (Sasaki et al., 1993; Shimoi et al., 1994) and antimutagenic properties (Marnewick et al., 2000; Van der Merwe et al., 2006; Snijman et al., 2007). Although the antioxidant and antimutagenic proper-

ties of rooibos and honeybush herbal teas have been investigated, little information is available about their potential cancer modulating properties *in vivo*.

A recent study indicated that the consumption of rooibos and honeybush herbal teas enhanced the activity of phase II detoxifying enzymes, as well as altering the oxidative status in the liver of rats (Marnewick et al., 2003). Cytosolic liver fractions of the herbal tea treated rats protected against the mutagenesis of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) and 2-acetylaminofluorene (2-AAF). The microsomal bioactivation of AFB<sub>1</sub> was reduced, indicating the potential of the herbal tea components to modulate their metabolic fate *ex vivo* (Marnewick et al., 2004). These findings suggest that aqueous extracts of rooibos and honeybush are likely to alter the carcinogenic potency of hepatocarcinogens *in vivo*. A study in mouse skin showed that extracts of the herbal teas disrupt cancer promotion subsequently reduces the development of papillomas (Marnewick et al., 2005).

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Based on studies with green and black teas (*Camellia sinensis*), polyphenolic components have been considered as potential chemopreventive agents (Fujiki et al., 2002; Yang et al., 2000a). Tea polyphenols are known to modulate the metabolic fate of carcinogens in several ways to render them less active, thus protecting the target tissue against their adverse effects (Yang et al., 2000b). Several studies in animals showed that black and green teas modulate cancer development *in vivo* (Mukhtar et al., 1994; Steele et al., 1999). The consumption of tea polyphenols and tea pigments, comprising of the oxidized flavanol products, theaflavins and thearubigins, significantly reduced the number as well as the average area of glutathione-S-transferase placental form positive (GSTP<sup>+</sup>) foci in the liver (Gong et al., 2000; Jia et al., 2002). An aqueous extract of green tea inhibited both cancer initiation and promotion of AFB<sub>1</sub>- and carbon tetrachloride-induced hepatocarcinogenesis in male Fischer rats (Qin et al., 2000).

Whereas the major polyphenolic constituents of green and black tea are flavanols and oxidation products of flavanols, the main monomeric phenolic constituents of rooibos are the flavonoid aspalathin, a dihydrochalcone, and its flavone analogues (Joubert, 1996; Bramati et al., 2002). The major monomeric phenolic constituents of honeybush are the xanthone, mangiferin and the flavanone, hesperidin (Ferreira et al., 1998; Joubert et al., 2003; Kamara et al., 2003). Differences in the polyphenolic constituents could explain variations in the biological properties, e.g. when comparing the antioxidant and antimutagenic properties of the different teas (Van der Merwe et al., 2006; Joubert et al., 2008).

The fumonisin B (FB) mycotoxins, which are natural contaminants produced by *Fusarium verticillioides* on maize, are potent liver cancer promoters (Gelderblom et al., 1988) and were recently shown to synergistically interact with AFB<sub>1</sub> in the induction of liver nodules in a short-term carcinogenesis model (Gelderblom et al., 2002). As these mycotoxins co-occur naturally, the modulation of their carcinogenic characteristics is of importance (Zhang et al., 1997; Ali et al., 1998). Numerous studies reported on the modulation of the carcinogenicity of AFB<sub>1</sub> by natural occurring dietary constituents (Qin et al., 1997; Maxuitenko et al., 1998) whereas the modulation of the carcinogenic properties of the fumonisins has not been investigated. Carcinogenesis by FB<sub>1</sub>, the main fumonisin produced, is associated with a chronic hepatotoxic effect and the induction of oxidative damage (Abel and Gelderblom, 1998; Sahu et al., 1998; Lemmer et al., 1999). The role of reactive oxygen species in carcinogenesis is well established as it regulates critical events regarding cell proliferation and apoptosis (Klaunig and Kamendulis, 2004). The modulating role of antioxidants in the ROS-induced effects on the different cell survival parameters is therefore of interest with respect to fumonisin-induced hepatocarcinogenesis. The present study investigated the modulating properties of aqueous extracts of fermented and unfermented South African herbal teas, as well as green and black teas, against different oxidative parameters and cancer promoting activity induced by FB<sub>1</sub> in rat liver.

## 2. Materials and methods

### 2.1. Chemicals and diets

Diethylnitrosamine (DEN) was purchased from Sigma (Sigma-Aldrich, Cape Town, South Africa) and prepared in dimethylsulfoxide (DMSO). FB<sub>1</sub> was purified according to the method of Cawood et al. (1991) at the PROMEC Unit, Medical Research Council, Tygerberg, South Africa to a purity of 93–95%. The FB<sub>1</sub>-containing diet for the rats was prepared by evaporating FB<sub>1</sub>, dissolved in methanol (Gelderblom et al., 1994), on a sub sample (200 g) of Epol (Epol Ltd., Johannesburg, South Africa) rat mash diet and mixing it with the control diet to obtain the desired dietary level of 250 mg/kg diet. The diet was stored under nitrogen at 4 °C. Glutathione-S-transferase placental form (GSTP) antibody was obtained from Novacastra, Newcastle, UK. Reduced (GSH) and oxidized (GSSG) glutathione

were purchased from Roche (Cape Town, South Africa). Glutathione reductase, perchloric acid (PCA), trichloro acetic acid (TCA), 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate (M2VP), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), NADPH, EDTA, gallic acid, (+)-catechin, quercetin, mangiferin and rutin were purchased from Sigma (Sigma-Aldrich). Phycoerythrin (B-PE) was purchased from ProZyme and 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), Trolox, hesperidin and hesperetin were obtained from Aldrich Chemical Company (Cape Town, South Africa). Aspalathin and nothofagin, with >95% purity were isolated at the PROMEC Unit, while isovitexin, vitexin, isoquercitrin, orientin, iso-orientin were purchased from Extrasynthese (Genay, France). All other chemicals used were of analytical grade.

### 2.1.1. Plant material and tea preparation

Black tea (*C. sinensis* var *assamica*), a blend of locally produced African and Sri Lankan teas, was bought from a retail outlet in Cape Town, while green tea (*C. sinensis* var *sinensis*), imported from China, was a gift from Vital Health Foods (Kuilsriver, South Africa). Fermented ("oxidized") and unfermented ("green" or "unoxidized") rooibos (*A. linearis*) and honeybush (*C. intermedia*) herbal teas were obtained from Rooibos Ltd. (Clanwilliam, South Africa) and the Agriculture Research Council, Infruitec-Nietvoorbij (Stellenbosch, South Africa), respectively. Green and black tea and rooibos herbal tea were prepared at concentrations customarily used for tea making purposes, while for honeybush a higher ratio of plant material to water was used, to compensate for its relatively high stem content that lowers its extractable polyphenol content. Aqueous extracts of honeybush is known to exhibit lower antioxidant and antimutagenic properties than the other teas (Van der Merwe et al., 2006; Joubert et al., 2008). Freshly boiled tap water was added to the plant material at concentrations of 2 g/100 ml for fermented and unfermented rooibos herbal, green and black teas and at 4 g/100 ml for fermented and unfermented honeybush herbal tea. The aqueous extracts were allowed to cool to room temperature and fed to rats *ad libitum*. Fresh tea was prepared every second day.

### 2.2. HPLC quantification of the major flavonoids in herbal tea aqueous extracts

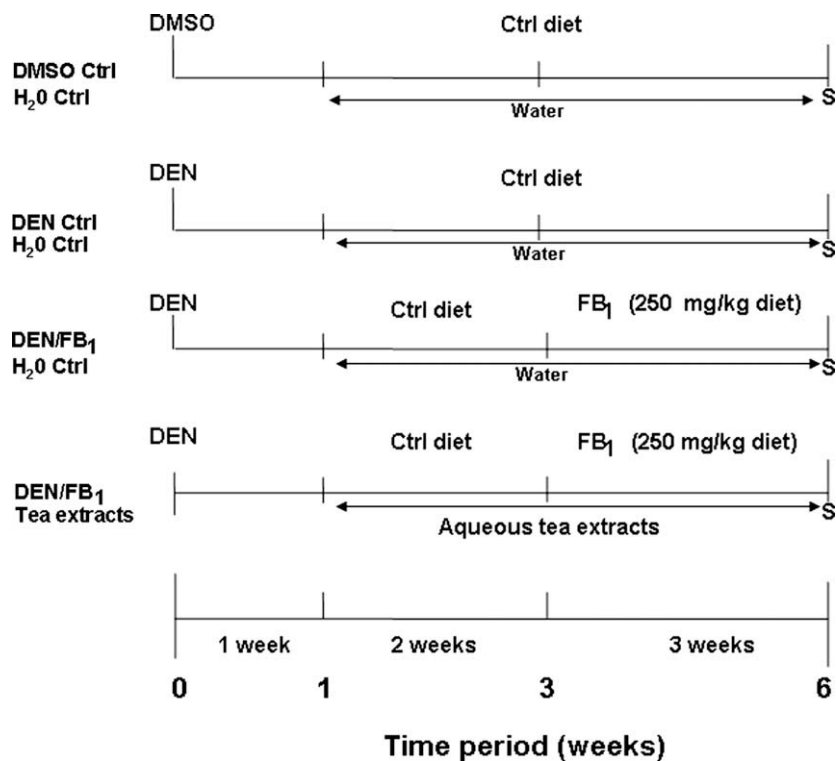
Freeze-dried samples of rooibos and honeybush aqueous extracts were reconstituted in purified water (Modulab Water Purification System from Continental Water System Corporation), filtered (Magna Nylon, 0.45 µm) and separated on a LiChrospher 100 RP-18 (5 µm, 250 × 4.6 mm) column (Joubert, 1996) and a Phenomenex Synergy Max-RP C12 column with TMS end capping (4 µm, 150 × 4.6 mm) (Joubert et al., 2003), respectively. A C<sub>18</sub> guard column was used in both cases and the HPLC system consisted of a Merck/Hitachi LaChrom system comprising of both a L-7400 UV and a L-7450 DAD detector, L-7100 pump, L-7200 autosampler, D-7000 HPLC system manager and interface module. The UV detector output was used for quantification, while the DAD detector was used to confirm peak identity, based on the spectra of the standards. Column temperature was maintained at 30 °C. The polyphenols were detected at 280 nm and quantified using calibration curves constructed for each flavonoid. Rutin co-eluted with isoquercitrin and the peak area was quantified in terms of quercetin equivalents.

### 2.2.1. Treatment of animals

Ninety male Fischer rats (150–170 g), obtained from the Primate Unit, MRC (Tygerberg, South Africa), had free access to Epol rat mash. They were housed in wired top and bottom cages, fitted with Perspex<sup>™</sup> houses and kept in a controlled environment of 23–24 °C, 50% humidity and a 12 h light/dark cycle. Rats were randomly divided into nine groups of ten rats each and caged individually. Initiation was effected by a single dose of diethylnitrosamine (DEN; 200 mg/kg body weight, *i.p.*). Tea or herbal teas were offered as the sole source of drinking fluid and commenced one week after initiation until the end of the experiment, while promotion commenced three weeks after initiation by giving the FB<sub>1</sub>-diet (250 mg/kg) for 21 days (Fig. 1). The DEN-initiated positive control group received the FB<sub>1</sub> promotion treatment (FB<sub>1</sub>) and tap water. The negative control groups either received DEN initiation or DMSO (carrier solvent) with the mash diet and tap water. All the rats were averaged fed during the FB<sub>1</sub> promotion treatment period, *i.e.*, all the FB<sub>1</sub> treated rats received an equal amount of feed than the rats of the DEN-FB<sub>1</sub> positive control group. Their body weights were monitored on a weekly basis. The FB<sub>1</sub> and tea intake profiles over the 3 week period were calculated as a function of the body weight and expressed as mg FB<sub>1</sub> or ml tea per 100 g body weight. At termination, fasted (16 h) animals were euthanized by *i.p.* injection of sodium pentobarbital (0.15 mL/100 g bw) and blood collected from the abdominal aorta. Livers were excised, weighed and sections processed in buffered formalin for histological examination. The remaining liver tissue were immediately frozen in liquid nitrogen and stored at –80 °C for biochemical analyses.

### 2.2.2. Clinical chemistry

The clinical biochemical parameters including serum creatinine, total cholesterol, total iron, aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) were measured on a Technicon RA 1000 automated analyzer.



**Fig. 1.** Schematic diagram illustrating the experimental regimens utilized to investigate the modulating properties of the different teas on cancer promotion by FB<sub>1</sub> in rat liver. DEN = diethylnitrosamine (200 mg/kg body weight; i.p., single dose); FB<sub>1</sub> (250 mg/kg) = rat mash containing FB<sub>1</sub> (250 mg/kg diet for 3 weeks) as promotion regimen; s = sacrifice animals; ctrl = control.

### 2.2.3. Oxidative parameters

**2.2.3.1. Lipid peroxidation.** The thiobarbituric acid reacting substances (TBARS) were determined according to a modified method described by Esterbauer and Cheeseman (1990). Sub samples of the excised livers were homogenized on ice in 19 volumes of 0.01 M phosphate buffer (pH 7.4) and incubated with 15  $\mu$ M FeSO<sub>4</sub> for one hour at 37 °C. The incubated homogenate (1 mL) was mixed with 2 mL of cold TCA reagent (10% TCA, 0.01% BHT). The samples were centrifuged (3000 rpm) and 2 mL of the resultant supernatant was combined with 2 mL of 0.67% TBA solution and incubated at 90 °C for 20 min. The mixture was allowed to cool to room temperature and the absorbance was measured at 532 nm. Lipid peroxidation was expressed as nmole malondialdehyde (MDA) per mg protein using a mmolar extinction coefficient of 156 (Esterbauer and Cheeseman (1990)). Non specific lipid peroxidation was prevented by the incorporation of EDTA in the phosphate buffer and BHT in the reaction solution for the TBARS assay.

**2.2.3.2. Oxygen radical absorbance capacity (ORAC).** Sub samples of the stored livers were homogenized in 4 volumes of sodium phosphate buffer (75 mM, pH 7.0) in a Thomas homogenizer (10 strokes) and centrifuged at 12,000g for 10 min at 4 °C. The supernatant was deproteinised using 0.25 M perchloric acid (PCA), centrifuged at 16,000g for 15 min and the resulting supernatants stored at –80 °C prior to analysis. The ORAC assay was conducted according to the method of Cao and Prior, 1998 on a 96 well microtiter plate using a BioTEK Fluorescence plate reader (FL-600). The reaction consisted of 10  $\mu$ l of diluted sample (1:1) and fluorescein (56 nM), which was used as a target for free radical attack. The reaction was initiated by the addition of 20  $\mu$ l AAPH (240 mM) and the fluorescence (emission 590 nm, excitation 530 nm) recorded every 5 min until the reading had declined to less than 5% of the initial reading. The ORAC values were calculated and expressed as  $\mu$ M Trolox equivalents/g wet liver weight.

**2.2.3.3. Glutathione analysis.** The total glutathione (GSH and GSSG) was measured according to a modified method of Tietze (1969). Liver samples were homogenized (1:10) in 15% (w/v) TCA containing 1 mM EDTA for GSH determination and in 6% (v/v) PCA containing freshly prepared 3 mM M2VP and 1 mM EDTA for GSSG determination on ice. After centrifugation at 10,000g for 10 min, 50  $\mu$ l of supernatant was added to glutathione reductase (1U) and 75  $\mu$ M DTNB. The reaction was initiated by addition of 0.25 mM NADPH to a final volume of 200  $\mu$ l. The change in absorbance was monitored at 410 nm for 5 min and levels calculated using pure GSH and GSSG as standards.

**2.2.3.4. Activity of antioxidant enzymes.** Liver homogenates (10% m/v) were prepared in a phosphate buffer, centrifuged for 10 min at 15,000g at 4 °C and the supernatant preserved for enzyme analyses. Catalase (CAT) activity was determined spectrophotometrically at 240 nm by monitoring the decomposition of H<sub>2</sub>O<sub>2</sub> and expressed as  $\mu$ moles H<sub>2</sub>O<sub>2</sub>/min/ $\mu$ g protein while superoxide dismutase (SOD) activity was determined by the method of Ellerby and Bredesden (2000), modified for a microplate reader and expressed as the amount of protein (ng) required to produce a 50% inhibition of auto-oxidation of 6-hydroxydopamine. Glutathione peroxidase (GPx) activity was measured spectrophotometrically (340 nm) by the method of Flohe and Gunzler (1984), expressing activity as nmoles NADPH/min/ $\mu$ g protein using the mmolar extinction coefficient of 6.22. Glutathione reductase (GR) activity was measured spectrophotometrically (340 nm) by the method of Ellerby and Bredesden (2000), expressing activity as nmoles of NADPH utilised per min per  $\mu$ g protein using the mmolar extinction coefficient of 6.22.

### 2.2.4. GST-Pi immunohistochemical assay

Histochemical staining for the placental form of glutathione-S-transferase (GSTP) was conducted on dewaxed liver sections using a three-stage indirect streptavidin–biotin technique to identify GSTP<sup>+</sup> stained hepatocytes (Ogawa et al., 1980). The enzyme altered foci were quantified microscopically (10 $\times$  magnification), according to their number and size (internal diameter) and categorized according to the following sizes, 5–10 (mini foci), 11–20, 21–30, >30  $\mu$ m. Foci were expressed as number of foci/cm<sup>2</sup> of the liver section, the area of which was determined by image analyses. The relative amount of each focal size category was expressed as a % of the total GSTP<sup>+</sup> foci ( $\geq$  5  $\mu$ m). The total GSTP<sup>+</sup> foci ( $\geq$  10  $\mu$ m) excluding the mini foci were also determined.

### 2.2.5. Statistical analysis

Data were tested for normality using the Kolmogorov–Smirnov Test and Levene's Test for Equality of variances. One-way ANOVA's (two-tailed) was used to test for significant group differences followed by the Tukey–Cramér multiple comparison tests in order to establish which groups differed significantly. The Kruskal–Wallis Test, a non-parametric analogue to the one-way ANOVA, was used to test for group differences when the data was not normally distributed. Statistical significance was at the 5% level ( $P < 0.05$ ). Statistical comparisons were conducted between (1) the DEN–FB<sub>1</sub> treated rats with the DEN and DMSO control as well as the tea treated groups and (2) among the different tea treated groups in so-called inter tea comparisons.

### 3. Results

#### 3.1. Intake of selected herbal tea flavonoids

The dihydrochalcone, aspalathin, was the major flavonoid consumed by the rats that received unfermented rooibos. Substantially lower quantities of its structural flavone analogues, orientin and iso-orientin, the flavonol glycosides, rutin/isoquercitrin and the dihydrochalcone, nothofagin and its flavone analogues, vitexin and isovitexin, were consumed (Table 1). In the aqueous extract of fermented rooibos, aspalathin and nothofagin were present at much lower concentrations, resulting in an increased intake of iso-orientin, orientin, vitexin and isovitexin relatively to aspalathin and nothofagin. The xanthone mangiferin and the flavanone hesperidin were the major monomeric polyphenols in both the unfermented and fermented honeybush. The intake of mangiferin and hesperidin was reduced substantially when consuming fermented honeybush. Only trace amounts of hesperetin, the aglycone of hesperidin were detected.

#### 3.2. FB<sub>1</sub> and tea intake profiles

The average tea intake and feed intake were significantly ( $P < 0.05$ ) reduced during the second week due to FB<sub>1</sub>-induced toxicity. After the third week the tea intake profiles returned back to the levels monitored during the first three weeks (data not shown). During the second week, the black tea intake was significantly decreased (6.2 ml/100 g bw) when compared to the other teas which ranged between 8.6 and 10.2 ml/100 g bw. As a result the averaged black tea consumption was significantly ( $P < 0.05$ ) lowered during the FB<sub>1</sub> treatment (Table 2). The average intake of fermented honeybush was significantly higher when compared to the other teas. There was no significant difference between the FB<sub>1</sub> intake profiles between the different groups.

#### 3.3. Effect of FB<sub>1</sub> and tea treatments on body weight parameters

The total body weight gain (BWG) during the experimental period was significantly ( $P < 0.05$ ) reduced in the FB<sub>1</sub> treated rats compared to the DMSO and DEN control groups consuming water, while the different tea treatments showed no additional effect (Table 2). However, inter tea comparisons indicated that unfermented rooibos significantly ( $P < 0.05$ ) lowered the total BWG compared to fermented rooibos and green tea. Compared to the DMSO control treated rats, DEN and the DEN-FB<sub>1</sub> treatments significantly ( $P < 0.05$ ) increased and decreased the relative liver weight, respectively. Fermented honeybush counteracted ( $P < 0.05$ ) the FB<sub>1</sub>-induced reduction, while unfermented rooibos marginally ( $P < 0.1$ ) further enhanced the reduction in the relative liver weight. Inter tea comparisons showed that fermented and unfermented rooibos significantly ( $P < 0.05$ ) reduced the relative liver weights compared to the other teas.

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#### 3.4. Clinical chemical parameters

Serum levels of AST, ALT, ALP, creatinine and cholesterol were significantly ( $P < 0.05$ ) increased by FB<sub>1</sub> as compared to the DMSO control group (Table 3). The DEN treatment significantly increased the ALT and ALP serum parameters as compared to the DMSO control. Unfermented rooibos significantly ( $P < 0.05$ ) enhanced the FB<sub>1</sub>-induced increase of serum AST and ALT levels while unfermented honeybush significantly ( $P < 0.05$ ) increased the ALT level. No significant reduction of the FB<sub>1</sub>-induced increase in serum ALP levels was observed for any of the teas. Inter tea comparisons showed that both rooibos herbal teas significantly ( $P < 0.05$ ) increased AST compared to black and green teas. Unfermented rooibos significantly ( $P < 0.05$ ) increased ALT when compared to fermented honeybush, black and green teas. Fermented honeybush significantly ( $P < 0.05$ ) increased the ALP level compared to black tea.

Black tea and fermented honeybush significantly ( $P < 0.05$ ) and unfermented honeybush marginally ( $P < 0.1$ ) counteracted the FB<sub>1</sub>-induced increase in serum creatinine levels. A similar trend was noticed with the inter tea group comparison. With respect to FB<sub>1</sub>-induced increase of serum cholesterol both fermented and unfermented honeybush as well as green tea significantly ( $P < 0.05$ ) decreased the level. A marginal ( $P < 0.1$ ) reduction was also noticed with black tea. A similar trend was noticed for the inter tea comparison. The total iron levels were not significantly altered by the FB<sub>1</sub> treatment while the teas appeared not to have any additional effect. However, inter tea comparisons showed that fermented and unfermented honeybush and green tea significantly ( $P < 0.05$ ) increased the total iron levels compared to the rooibos herbal and black teas.

**Table 1**

Quantification of the major flavonoids in aqueous extracts of rooibos and honeybush teas consumed by rats during FB<sub>1</sub> promotion.

| Soluble solids (mg/ml) <sup>a</sup> | Fermented                |  | Unfermented               |                            |
|-------------------------------------|--------------------------|--|---------------------------|----------------------------|
|                                     | 2.59 ± 0.44              |  | 5.36 ± 0.81               |                            |
| Phenolic compounds                  | (%) of soluble solids    | Daily intake (mg/100 g BW) <sup>**</sup> | (%) of soluble solids (%) | Daily intake (mg/100 g BW) |
| <i>Rooibos tea</i>                  |                          |  |                           |                            |
| Aspalathin                          | 0.53 ± 0.02              | 0.15 ± 0.01                              | 8.4 ± 1.38                | 4.96 ± 0.41                |
| Nothofagin                          | 0.05 ± 0.02              | 0.02 ± 0.01                              | 0.36 ± 0.13               | 0.21 ± 0.02                |
| Orientin                            | 0.46 ± 0.01              | 0.13 ± 0.01                              | 0.65 ± 0.18               | 0.38 ± 0.03                |
| Iso-orientin                        | 0.66 ± 0.04              | 0.19 ± 0.01                              | 0.80 ± 0.18               | 0.47 ± 0.04                |
| Vitexin                             | 0.19 ± 0.01              | 0.05 ± 0.01                              | 0.13 ± 0.02               | 0.08 ± 0.01                |
| Isovitexin                          | 0.18 ± 0.03              | 0.05 ± 0.01                              | 0.29 ± 0.07               | 0.14 ± 0.01                |
| Rutin/iso-quercitrin                | 0.45 ± 0.03              | 0.13 ± 0.01                              | 0.60 ± 0.10               | 0.35 ± 0.03                |
|                                     | 5.96 ± 0.78 <sup>b</sup> |  | 11.78 ± 1.18 <sup>c</sup> |                            |
|                                     | (%) of soluble solids    | Daily intake (mg/100 g BW) <sup>**</sup> | (%) of soluble solids     | Daily intake (mg/100 g BW) |
| <i>Honeybush tea</i>                |                          |  |                           |                            |
| Mangiferin                          | 1.11 ± 0.15              | 0.82 ± 0.06                              | 6.68 ± 0.84               | 8.90 ± 0.79                |
| Hesperidin                          | 0.37 ± 0.08              | 0.27 ± 0.02                              | 2.63 ± 0.16               | 3.49 ± 0.31                |
| Hesperetin                          | Trace                    |  | Trace                     |                            |

All samples used were aqueous extracts prepared as described in the methods. The values in columns represent the mean ± standard deviation of 2–3 repeats of each sample.

<sup>a</sup> Data from Marnewick et al., 2003. BW = body weight.

<sup>\*\*</sup> Mean daily tea intake during the 3 week FB<sub>1</sub> period (Table 2) was used.

**Table 2**  
Effect of DEN-FB<sub>1</sub> and various tea treatments on the rat body weight parameters.

| Groups                  | Ave tea intake (ml/100 g BW/day) | Ave FB <sub>1</sub> intake (mg/100 g BW/day) | Body weight gain (g) <sup>B</sup> | Relative liver weight (%) <sup>A</sup> |
|-------------------------|----------------------------------|--|-----------------------------------|--|
| DMSO                    | –                                | –  | 86.9 ± 8.2 <sup>a</sup>           | 2.9 ± 0.35 <sup>a</sup>                |
| DEN                     | –                                | –  | 77.7 ± 25.1 <sup>a</sup>          | 3.51 ± 0.27 <sup>c</sup>               |
| DEN-FB <sub>1</sub>     | –                                | 1.5 ± 0.3 <sup>a</sup>                       | 39.8 ± 13.8 <sup>b</sup>          | 2.50 ± 0.16 <sup>b</sup>               |
| DEN-FB <sub>1</sub> -Rf | 11.0 ± 0.9 <sup>a</sup>          | 1.6 ± 0.3 <sup>a</sup>                       | 44.0 ± 6.8 <sup>bc</sup>          | 2.36 ± 0.12 <sup>(b)d</sup>            |
| DEN-FB <sub>1</sub> -Ru | 10.8 ± 0.7 <sup>a</sup>          | 1.5 ± 0.4 <sup>a</sup>                       | 29.6 ± 9.6 <sup>bd</sup>          | 2.44 ± 0.09 <sup>bd</sup>              |
| DEN-FB <sub>1</sub> -Hf | 12.4 ± 0.8 <sup>b</sup>          | 1.3 ± 0.3 <sup>a</sup>                       | 37.9 ± 12.7 <sup>b</sup>          | 2.79 ± 0.05 <sup>ae</sup>              |
| DEN-FB <sub>1</sub> -Hu | 11.3 ± 1.0 <sup>a</sup>          | 1.5 ± 0.3 <sup>a</sup>                       | 36.1 ± 16.4 <sup>b</sup>          | 2.58 ± 0.19 <sup>be</sup>              |
| DEN-FB <sub>1</sub> -Bl | 9.0 ± 0.9 <sup>c</sup>           | 1.5 ± 0.3 <sup>a</sup>                       | 40.2 ± 19.8 <sup>b</sup>          | 2.58 ± 0.13 <sup>be</sup>              |

Values in columns represent the mean ± STD. Abbreviations: Rp = fermented rooibos, Rg = unfermented/“green” rooibos, Hp = fermented honeybush and Hg = unfermented/“green” honeybush tea extracts, Bl = black and Gr = green tea. Values followed by the same letters do not differ significantly. When letters differ then  $P < 0.05$ . Letters in parenthesis then  $P < 0.1$ . Inter tea comparison are indicated in bold.

<sup>A</sup> Relative liver weights equal liver weight/body weight × 100.

<sup>B</sup> Including DEN, Pre-FB<sub>1</sub> tea treatment and FB<sub>1</sub> treatment regimens.  $N = 7$ –10 rats per group.

**Table 3**  
Effect of DEN-FB<sub>1</sub> and various tea treatments on the serum clinical chemistry.

| Groups                  | AST (U/L)                  | ALT (U/L)                  | ALP (U/L)                  | Creatinine (umol/L)        | Cholesterol (mmol/L)      | Total iron (μmol/L)      |
|-------------------------|----------------------------|----------------------------|----------------------------|----------------------------|---------------------------|--------------------------|
| DMSO                    | 88.1 ± 16.8 <sup>a</sup>   | 53.7 ± 7.3 <sup>a</sup>    | 168.9 ± 32.8 <sup>a</sup>  | 69.0 ± 6.0 <sup>a</sup>    | 1.6 ± 0.2 <sup>a</sup>    | 23.1 ± 5.8 <sup>a</sup>  |
| DEN                     | 102.8 ± 15.4 <sup>a</sup>  | 96.7 ± 11.5 <sup>c</sup>   | 272.4 ± 69.1 <sup>c</sup>  | 67.3 ± 3.7 <sup>a</sup>    | 1.4 ± 0.3 <sup>a</sup>    | 25.9 ± 2.6 <sup>a</sup>  |
| DEN-FB <sub>1</sub>     | 258.7 ± 60.2 <sup>b</sup>  | 211.6 ± 48.0 <sup>b</sup>  | 282.4 ± 82.0 <sup>b</sup>  | 85.8 ± 4.7 <sup>b</sup>    | 3.5 ± 0.4 <sup>b</sup>    | 24.4 ± 7.4 <sup>a</sup>  |
| DEN-FB <sub>1</sub> -Rf | 296.8 ± 52.3 <sup>bd</sup> | 256.62 ± 68.9 <sup>b</sup> | 283.4 ± 61.3 <sup>b</sup>  | 85.3 ± 4.4 <sup>bd</sup>   | 3.3 ± 0.4 <sup>bc</sup>   | 21.9 ± 2.3 <sup>ab</sup> |
| DEN-FB <sub>1</sub> -Ru | 341.1 ± 50.0 <sup>cd</sup> | 321.3 ± 75.4 <sup>de</sup> | 325.3 ± 86.8 <sup>b</sup>  | 83.5 ± 5.8 <sup>bd</sup>   | 2.9 ± 1.0 <sup>bc</sup>   | 22.6 ± 4.4 <sup>ab</sup> |
| DEN-FB <sub>1</sub> -Hf | 242.9 ± 54.3 <sup>b</sup>  | 236.3 ± 63.9 <sup>bf</sup> | 355.3 ± 77.8 <sup>bd</sup> | 77.2 ± 3.8 <sup>ce</sup>   | 2.3 ± 0.8 <sup>ad</sup>   | 27.5 ± 2.2 <sup>ac</sup> |
| DEN-FB <sub>1</sub> -Hu | 283.2 ± 52.1 <sup>b</sup>  | 280.9 ± 71.7 <sup>d</sup>  | 314.3 ± 57.9 <sup>b</sup>  | 80.8 ± 5.8 <sup>(b)d</sup> | 2.1 ± 0.3 <sup>ad</sup>   | 28.3 ± 4.9 <sup>ac</sup> |
| DEN-FB <sub>1</sub> -Bl | 207.1 ± 56.3 <sup>be</sup> | 216.0 ± 90.5 <sup>bf</sup> | 245.0 ± 58.4 <sup>be</sup> | 78.4 ± 2.2 <sup>(c)d</sup> | 3.0 ± 0.6 <sup>(b)c</sup> | 24.1 ± 6.3 <sup>ab</sup> |
| DEN-FB <sub>1</sub> -Gr | 226.4 ± 22.5 <sup>be</sup> | 229.3 ± 41.4 <sup>bf</sup> | 296.8 ± 62.1 <sup>bd</sup> | 83.6 ± 3.0 <sup>bd</sup>   | 2.4 ± 0.5 <sup>ad</sup>   | 27.1 ± 4.4 <sup>ac</sup> |

Values in columns represent the mean ± STD. Abbreviations: Rp = fermented rooibos, Rg = unfermented/“green” rooibos, Hp = fermented honeybush and Hg = unfermented/“green” honeybush tea extracts, Bl = black and Gr = green tea. Values followed by the same letters do not differ significantly. When letters differ then  $P < 0.05$ . Letters in parenthesis then  $P < 0.1$ . Inter tea comparison are indicated in bold.  $n = 7$ –10 rats per group.

### 3.5. Effect on hepatic oxidative status (Table 4)

#### 3.5.1. Oxygen radical absorbance capacity (ORAC)

The DEN treatment significantly ( $P < 0.05$ ) lowered the hepatic ORAC status compared to the DMSO control group. Of the teas, only green tea further decreased ( $P < 0.05$ ) the hepatic ORAC values while unfermented rooibos marginally ( $P < 0.1$ ) increased the ORAC status when compared to the DEN treatment group.

#### 3.5.2. Hepatic glutathione

Rats receiving the FB<sub>1</sub>-dietary treatment showed significantly ( $P < 0.05$ ) elevated GSH levels in the liver when compared to the DMSO and DEN treated control groups. Consumption of the rooibos herbal teas resulted in a significant ( $P < 0.05$ ) reduction in hepatic GSH concentration when compared to the DEN-FB<sub>1</sub> treatment group. A similar effect on the GSH concentration was noticed for the inter tea comparison. The GSSG levels were not affected by FB<sub>1</sub> resulting in a significantly ( $P < 0.05$ ) increased GSH:GSSG ratio in the liver when compared to the DMSO and DEN treated control groups. The GSSG level was significantly ( $P < 0.05$ ) elevated with the consumption of green, black, unfermented rooibos and honeybush herbal teas, resulting in a significantly ( $P < 0.05$ ) decreased GSH:GSSG ratio, while a marginal ( $P < 0.1$ ) decrease was noticed with fermented rooibos.

#### 3.5.3. Antioxidant enzyme activity

FB<sub>1</sub> treatment significantly ( $P < 0.05$ ) increased the activity of GPx (Table 3) while CAT activity was significantly ( $P < 0.05$ ) reduced. No effect was observed on the activity of SOD. Treatment with green, black and herbal teas did not alter the FB<sub>1</sub>-induced ef-

fect on GPx. Treatment with both honeybush herbal, green and black teas resulted in a significant ( $P < 0.05$ ) recovery in the FB<sub>1</sub>-induced decrease in the activity of CAT. FB<sub>1</sub> significantly increased the GR activity while none of the teas showed any modulating effect.

### 3.6. Lipid peroxidation

The TBARS levels were significantly ( $P < 0.05$ ) increased in the liver of the FB<sub>1</sub> treated rats (Table 4) when compared to the DMSO and DEN treated groups. The rats consuming fermented rooibos, and fermented and unfermented honeybush showed a significant ( $P < 0.05$ ) decrease in the TBARS levels. Consumption of unfermented rooibos, black and green teas showed no effect on the FB<sub>1</sub>-induced lipid peroxidation. Among the different teas, fermented rooibos and honeybush significantly ( $P < 0.05$ ) decreased lipid peroxidation compared to their unfermented counterparts, and unfermented honeybush significantly ( $P < 0.05$ ) reduced lipid peroxidation compared to black tea.

### 3.7. Effect of various tea treatments on FB<sub>1</sub>-induced promotion

FB<sub>1</sub> significantly ( $P < 0.05$ ) promoted the growth of GSTP<sup>+</sup> foci by increasing the number and relative number [(expressed as a % of the total foci (>5 μm))] of GSTP<sup>+</sup> foci in all size categories as well as the total number (>10 μm) when compared to the DEN treated control rats (Table 5). No GSTP<sup>+</sup> foci were detected in the liver of the DMSO control rats. When considering the positive control treatment (DEN-FB<sub>1</sub>), all the tea preparations significantly ( $P < 0.05$ ) to marginally ( $P < 0.1$ ) increased the number of

**Table 4**

Effect of unfermented and fermented herbal, green and black teas on reduced glutathione (GSH), oxidized glutathione (GSSG), the ratio GSH:GSSG, oxidative enzyme parameters and lipid peroxidation in livers of rats.

| Groups                  | ORAC<br>(umole Trolox<br>eq/g wet liver) | GSH<br>(umole/g wet liver) | GSSG         | GSH: GSSG<br>Ratio | GR<br>(nmoleNADPH/<br>min/ $\mu$ g protein) | GPx<br>(nmoleNADPH/<br>min/ $\mu$ g protein) | CAT ( $\mu$ mole H <sub>2</sub> O <sub>2</sub> /<br>min/ $\mu$ g protein) | SOD*          | TBARS<br>(nmol MDA /mg<br>protein) |
|-------------------------|--|----------------------------|--------------|--------------------|---|--|---|---------------|------------------------------------|
| DMSO                    | 15.5 ± 2.4a                              | 5.3 ± 1.3a                 | 0.41 ± 0.06a | 12.8 ± 1.5a        | 0.025 ± 0.001a                              | 0.024 ± 0.001a                               | 0.21 ± 0.04a  | 413.6 ± 30.9a | 0.08 ± 0.01a                       |
| DEN                     | 12.8 ± 2.2b                              | 5.3 ± 1.1a                 | 0.50 ± 0.12a | 11.1 ± 2.9a        | Nd  | Nd   | Nd  | Nd            | 0.07 ± 0.01a                       |
| DEN-FB <sub>1</sub>     | 13.6 ± 0.7b                              | 8.8 ± 1.5b                 | 0.41 ± 0.05a | 21.6 ± 3.1b        | 0.032 ± 0.002b                              | 0.032 ± 0.003b                               | 0.14 ± 0.02b  | 450.2 ± 89.5a | 0.32 ± 0.08b                       |
| DENFB <sub>1</sub> -Rf  | 14.5 ± 1.5b                              | 6.0 ± 1.8a                 | 0.40 ± 0.11a | 15.9 ± 4.7(b)      | 0.031 ± 0.001b                              | 0.033 ± 0.002b                               | 0.17 ± 0.03b  | 454.0 ± 65.6a | 0.21 ± 0.07a                       |
| DEN-FB <sub>1</sub> -Ru | 14.7 ± 1.3(b)                            | 5.2 ± 1.3a                 | 0.54 ± 0.08b | 9.9 ± 2.8a         | 0.032 ± 0.003b                              | 0.035 ± 0.003b                               | 0.15 ± 0.03b  | 418.5 ± 53.2a | 0.30 ± 0.04b                       |
| DEN-FB <sub>1</sub> -Hf | 14.4 ± 1.1b                              | 8.5 ± 0.7b                 | 0.46 ± 0.14a | 20.5 ± 8.5b        | 0.029 ± 0.002b                              | 0.033 ± 0.003b                               | 0.20 ± 0.02a  | 381.4 ± 59.9a | 0.16 ± 0.04ac                      |
| DEN-FB <sub>1</sub> -Hu | 13.2 ± 1.1b                              | 7.0 ± 0.9b                 | 0.57 ± 0.13b | 12.7 ± 2.4a        | 0.030 ± 0.002b                              | 0.034 ± 0.004b                               | 0.22 ± 0.03a  | 425.3 ± 67.3a | 0.26 ± 0.06ad                      |
| DEN-FB <sub>1</sub> -Bl | 13.6 ± 1.1b                              | 7.8 ± 2.8b                 | 0.6 ± 0.12b  | 13.1 ± 4.6a        | 0.030 ± 0.003b                              | 0.036 ± 0.004b                               | 0.22 ± 0.03a  | 400.3 ± 69.4a | 0.35 ± 0.07be                      |
| DEN-FB <sub>1</sub> -Gr | 10.0 ± 0.88c                             | 7.4 ± 2.3b                 | 0.71 ± 0.17b | 11.1 ± 4.9a        | 0.030 ± 0.003b                              | 0.038 ± 0.006b                               | 0.20 ± 0.04a  | 420.9 ± 39.5a | 0.31 ± 0.09bd                      |

Values in columns represent average of 5–8 values per group ± STD. Means followed by the same letter do not differ significantly, when letters differ then  $P < 0.05$ . Letters in parenthesis indicated  $P < 0.1$ . Abbreviations: Rf = fermented rooibos, Ru = unfermented/"green" rooibos, Hp = fermented honeybush and Hg = unfermented/"green" honeybush tea extracts, Bl = black and Gr = green tea. Amount of protein (ng) required to produce a 50% inhibition of auto-oxidation of 6-hydroxydopamine. GR = glutathione reductase; GPx = glutathione peroxidase; CAT = catalase; SOD = superoxide dismutase.

**Table 5**

Effect of various tea treatments on the induction of GSTP<sup>+</sup> foci by combined treatment of DEN and FB<sub>1</sub>.

| Treatment groups        | GSTP <sup>+</sup> Liver Foci              |                              |  |                              |  |                              |   |                              |                                   |  |
|-------------------------|---|------------------------------|--|------------------------------|--|------------------------------|---|------------------------------|-----------------------------------|--|
|                         | No foci/cm <sup>2</sup><br>(5–10 $\mu$ m) | % of the total<br>no of foci | No foci/cm <sup>2</sup><br>(11–20 $\mu$ m) | % of the total<br>no of foci | No foci/cm <sup>2</sup><br>(21–30 $\mu$ m) | % of the total<br>no of foci | No foci/cm <sup>2</sup><br>(>30 $\mu$ m/cm <sup>2</sup> ) | % of the total<br>no of foci | Total no of foci<br>(>10 $\mu$ m) |  |
| DMSO                    | Nd  | nd                           | nd   | nd                           | nd   | nd                           | nd  | nd                           | nd                                |  |
| DEN                     | 4.7 ± 1.7a                                | 58.7 ± 13.4a                 | 1.9 ± 0.8a                                 | 32.9 ± 7.6a                  | 0.5 ± 0.8a                                 | 6.5 ± 8.7a                   | 0.14 ± 0.34a  | 2.0 ± 4.0a                   | 2.6 ± 1.8a                        |  |
| DEN-FB <sub>1</sub>     | 8.0 ± 2.8b                                | 23.7 ± 7.5b                  | 18.2 ± 5.6b                                | 50.2 ± 8.4b                  | 6.5 ± 1.4b                                 | 19.6 ± 5.0b                  | 2.3 ± 1.4b  | 6.5 ± 3.9b                   | 27.0 ± 11.1b                      |  |
| DEN-FB <sub>1</sub> -Rf | 15.9 ± 8.7(b)                             | 39.2 ± 14.3c                 | 15.5 ± 5.7b                                | 39.1 ± 4.3a                  | 6.5 ± 2.9b                                 | 16.1 ± 3.6b                  | 2.3 ± 1.1b  | 6.0 ± 3.2b                   | 24.2 ± 8.8b                       |  |
| DEN-FB <sub>1</sub> -Ru | 12.6 ± 4.9c                               | 53.9 ± 17.1a                 | 10.4 ± 5.3c                                | 36.0 ± 9.0a                  | 3.1 ± 2.4c                                 | 10.3 ± 6.6a                  | 0.7 ± 1.1a  | 1.9 ± 2.8a                   | 14.2 ± 7.8c                       |  |
| DEN-FB <sub>1</sub> -Hf | 17.9 ± 5.4c                               | 42.3 ± 7.9a                  | 15.1 ± 6.3b                                | 33.9 ± 4.6a                  | 7.6 ± 4.8b                                 | 16.4 ± 7.0b                  | 3.5 ± 2.7b  | 7.5 ± 4.1b                   | 26.3 ± 12.3b                      |  |
| DEN-FB <sub>1</sub> -Hu | 19.7 ± 8.5c                               | 52.7 ± 14.7a                 | 11.9 ± 5.9(b)                              | 31.8 ± 6.2a                  | 4.0 ± 2.7c                                 | 10.6 ± 5.1a                  | 0.9 ± 1.7(b)  | 2.3 ± 4.1(b)                 | 17.1 ± 14.2(b)                    |  |
| DEN-FB <sub>1</sub> -Bl | 30.0 ± 3.5c                               | 54.0 ± 12.3a                 | 17.3 ± 9.9b                                | 27.9 ± 15.8a                 | 7.7 ± 3.0b                                 | 13.8 ± 6.1(b)                | 2.5 ± 1.4b  | 4.3 ± 2.1b                   | 27.5 ± 10.9b                      |  |
| DEN-FB <sub>1</sub> -Gr | 23.9 ± 11.3c                              | 37.8 ± 13.3a                 | 18.2 ± 6.2bd                               | 33.3 ± 4.6a                  | 10.4 ± 6.6b                                | 17.5 ± 8.8b                  | 5.9 ± 5.2b  | 9.7 ± 7.2b                   | 33.7 ± 4.7b                       |  |

Values represent the average of ten rats per group ± STD. Means (column) followed by the same letter do not differ significantly. When letters differ then  $P < 0.05$ . Letters in parenthesis indicated  $P < 0.1$ . Abbreviations: Rf = fermented rooibos, Ru = unfermented rooibos, Hf = fermented honeybush and Hu = unfermented honeybush tea extracts, Bl = black and Gr = green tea, nd = not detected. Shaded areas; foci number expressed as a % of the total GSTP<sup>+</sup> foci (5  $\mu$ m).

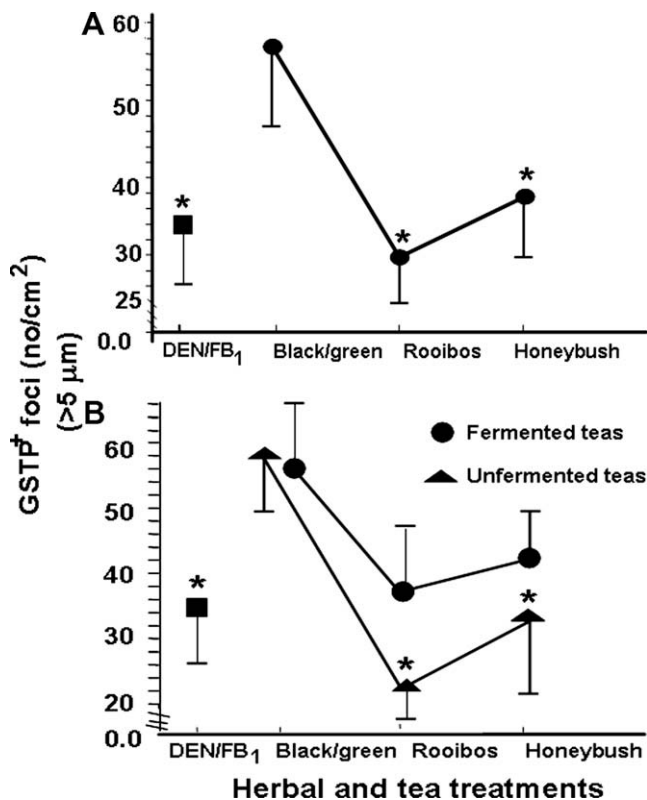
mini foci (5–10  $\mu$ m) as well as the relative number. A similar effect was noticed when considering the relative amount of larger foci (11–20  $\mu$ m) constituting 50% of the number of foci. The number of foci was significantly decreased by unfermented rooibos ( $P < 0.05$ ) and marginally by honeybush ( $P < 0.1$ ). The unfermented herbal teas significantly ( $P < 0.05$ ) decreased the number of foci (size 21–30  $\mu$ m) constituting approximately 20% of the total number of foci in the positive control group. The unfermented herbal teas and black tea significantly ( $P < 0.05$ ) and marginally ( $P < 0.1$ ) decreased the relative amount of foci in this size category, respectively. When considering the focal size >30  $\mu$ m, which constituted approximately 6% of the total amount of foci unfermented rooibos significantly ( $P < 0.05$ ) and unfermented honeybush marginally ( $P < 0.1$ ) decreased the number and relative amount. The total number of foci (>10  $\mu$ m), excluding the mini foci, was significantly ( $P < 0.05$ ) and marginally ( $P < 0.1$ ) reduced by unfermented rooibos and honeybush, respectively.

Interactive plots showed that the herbal teas (combined effect) significantly ( $P < 0.05$ ) decreased the total amount of foci (>5  $\mu$ m) as compared to the black and green teas (Fig. 2A). The separate effects of the different teas indicated that the unfermented rooibos and honeybush significantly ( $P < 0.05$ ) decreased the total number of foci when compared to the green and black teas (Fig. 2B). A similar effect was noticed with the fermented herbal teas, although differences were not significant, implying that fermentation reduced the protective effects of the herbal teas.

#### 4. Discussion

FB<sub>1</sub> was characterized as a non-genotoxic liver cancer promoter (Gelderblom et al., 1996) and shown to be hepato- and nephrocarcinogenic in rats (Gelderblom et al., 1991; Howard et al., 2001). In mice, FB<sub>1</sub> significantly increased the incidence of adenomas and carcinomas that developed spontaneously in the liver (Howard et al., 2001). The mechanism of cancer induction is not known at present but studies in rats indicate that FB<sub>1</sub> could effect both cancer initiation and promotion in the liver (Gelderblom et al., 1992, 1994, 1996, 2008). The disruption of growth-stimulatory responses in normal and genetically altered initiated cells is suggested to be important in establishing a growth differential whereby the preneoplastic cell populations are clonally expanded, subsequently leading to carcinogenesis by FB<sub>1</sub> in the liver. At a cellular level, FB<sub>1</sub> disrupts sphingolipid, phospholipid and fatty acid metabolism, which have been suggested to be the underlying mediators responsible for cancer promotion in the liver (Riley et al., 2001; Gelderblom et al., 2001; Burger et al., 2007).

The present study confirmed the FB<sub>1</sub>-induced hepatotoxic and nephrotoxic effects (Gelderblom et al., 1991; Voss et al., 1995), which were manifested by a significant ( $P < 0.05$ ) increase in the clinical chemical parameters associated with liver and kidney function, decreased body weight gain and relative liver weight. Black tea and fermented honeybush counteracted the reduction in serum creatinine suggesting a protective effect against FB<sub>1</sub>-induced nephrotoxicity. Honeybush and green tea also protected against FB<sub>1</sub>-induced accumulation of serum cholesterol. Both hes-



**Fig. 2.** (A) Interactive plots illustrating the combined effects ( $n = 20$ ) of the various tea treatments on the induction of total number of hepatic GSTP<sup>+</sup> foci  $>5 \mu\text{m}$  by FB<sub>1</sub> and (B) separate effects ( $n = 10$ ) illustrating the affect of tea processing on the induction of GSTP<sup>+</sup> foci. Values are the means  $\pm$  STD. \*Significantly different ( $P < 0.05$ ) from black and green tea.

peridin and mangiferin, present in large quantities in unfermented honeybush, is of interest as the aglycone of hesperidin, hesperetin and mangiferin reduce the level of rat plasma cholesterol (Kim et al., 2003; Nair and Devi, 2006). FB<sub>1</sub> and the different tea treatments did not alter the total serum iron levels when compared to the control. However, fermented and unfermented rooibos and black teas significantly ( $P < 0.05$ ) decreased the levels when compared to green tea and fermented and unfermented honeybush. As rooibos did not affect serum iron after a 10 week feeding study (Marnewick et al., 2003) specific iron/polyphenolic interactions, the decreased feed intake profiles and associated hepatotoxic effects during the 3 week FB<sub>1</sub> feeding regimen seems to be involved in the reduction of serum iron levels as compared to the other teas.

Unfermented rooibos further enhanced the FB<sub>1</sub>-induced hepatotoxic effect when considering the increased liver function enzymes, reduced body weight gain and relative liver weight. Unfermented honeybush also increased the ALT levels but the body and liver weight parameters were not altered. Specific interactions between the tea polyphenolic constituents and FB<sub>1</sub>-induced toxic effects are likely to be involved as the tea consumption profiles, except for the lower black tea intake, and total FB<sub>1</sub> intake profiles were similar. One possibility could be the induction of a pro-oxidative effect by the unfermented herbal tea polyphenols in the presence of iron. Aspalathin-enriched extracts of unfermented rooibos exhibit pro-oxidative effects in a Fenton-type reaction *in vitro*, a property that was significantly reduced by fermentation (Joubert et al., 2005). It has been suggested that high levels of mangiferin, the major antioxidant of unfermented honeybush, could also result in pro-oxidant activity (Joubert et al., 2008). The rat intake profiles

of mangiferin given unfermented honeybush were almost double that of aspalathin receiving the unfermented rooibos, suggesting that under these conditions mangiferin could act as a pro-oxidant. However, studies regarding the bioavailability of these polyphenolic constituents in the herbal extracts that are currently under investigation will provide more information on their *in vivo* antioxidant potencies. FB<sub>1</sub>-induced hepatotoxicity was associated with an increased oxidative damage (Abel and Gelderblom, 1998) and the mobilization of iron (Lemmer et al., 1999) that create an ideal environment for the induction of pro-oxidant effects by aspalathin and mangiferin. In this regard, the antioxidant potency of specific tea polyphenols is likely to determine whether antioxidant and/or pro-oxidant effects prevail. Fermented rooibos and honeybush, exhibiting weaker antioxidant properties (Von Gadow et al., 1997; Hubbe and Joubert, 2000a,b) and containing far lower levels of aspalathin and mangiferin, reduced the FB<sub>1</sub>-induced lipid peroxidation. Similar to the major unfermented herbal tea polyphenols, the potent green and black tea antioxidants, epigallocatechingallate (EGCG) and theaflavins, are known to act as pro-oxidants in the presence of copper and/or iron (Azam et al., 2004; Schuck et al., 2008). It would appear that under the current conditions, the tea flavonoids with the highest antioxidant potency are likely to enhance free radical production via a Fenton-type reaction thereby potentiating the FB<sub>1</sub>-induced hepatotoxicity effects. The reason why green and black teas did not affect the FB<sub>1</sub>-induced hepatotoxicity could be related to the iron binding capacity of the tannins (Andrade et al., 2006) as well as the differential induction of antioxidant enzymes discussed below.

Protection against lipid peroxidation, proposed to be a secondary event to FB<sub>1</sub>-induced hepatotoxicity (Lemmer et al., 1999), will not necessarily reduce FB<sub>1</sub>-induced hepatotoxicity. The suggested pro-oxidative effect of unfermented rooibos and the potentiating effects on FB<sub>1</sub>-induced hepatotoxic effects vs the protection against the resultant lipid peroxidation appear to be two separate events. The FB<sub>1</sub>/polyphenolic interactions are further complicated when considering different oxidative parameters in the liver. FB<sub>1</sub> significantly ( $P < 0.05$ ) reduced the ORAC as a result of the increased oxidative stress which is in agreement with a recent study in glioblastoma cells showing that the FB<sub>1</sub>-induced lipid peroxidation and oxidative stress which was associated with a reduced GSH level (Stockmann-Juvala et al., 2008). In the present study, the herbal and black teas did not alter the reduction in ORAC, while green tea significantly ( $P < 0.05$ ) further reduced the level, implying that the antioxidant capacity is further depleted, which may lead to a depletion of GSH. This is in agreement with the findings of a previous study where green tea reduces ORAC in the liver although the GSH level was not altered (Marnewick et al., 2003). In contrast, FB<sub>1</sub> significantly increased the GSH level which was associated with the development of preneoplastic lesions known to stain positively for gamma glutamyl transpeptidase positive (GGT<sup>+</sup>) foci (Gelderblom et al., 1991). This is in agreement with the findings that these preneoplastic lesions had an increased level of GSH (Marinho et al., 1997; Abel and Gelderblom, 1998). The increased levels of GSH in preneoplastic lesions provide these lesions with a selective growth advantage when compared to the surrounding tissue (Hanigan and Pitot, 1985) and will therefore be more resistant to the oxidative stress induced by FB<sub>1</sub>. The reduction of the GSH level by unfermented rooibos and to some extent unfermented honeybush was associated with a decrease in the number of the preneoplastic foci. When considering the GSH:GSSG ratio unfermented rooibos, honeybush, black and green teas significantly ( $P < 0.05$ ) decreased the ratio mainly due to an increased GSSG level while GSH was reduced as a result of the decreased number and size of the GSTP<sup>+</sup> foci. It is known that, under stressed conditions, the GSH:GSSG ratio decreases either due to increased GSSG or decreased GSH

levels (Dickinson and Forman, 2002). The teas exhibiting the highest protective effect against FB<sub>1</sub>-induced lipid peroxidation, fermented rooibos and honeybush did not alter the GSSG level and the GSH:GSSG ratio although fermented rooibos marginally ( $P < 0.1$ ) reduced the ratio due to the reduction in the GSH level. The development of the preneoplastic lesion therefore complicated interpretations regarding the effect of the herbal teas on the GSH level and hence the oxidative status in the liver. However, unfermented rooibos and honeybush have been reported to significantly increase the GSH level in the liver after a 10 week feeding study (Marnewick et al., 2003), an aspect that is masked in the present study by the development of preneoplastic lesions.

None of the teas affected the FB<sub>1</sub>-induced activity of GR, implying that the increased GSH levels in the liver, as stated above, are more related to the induction of GSTP<sup>+</sup> foci. FB<sub>1</sub> increased and decreased the activities of GPx and CAT, respectively, implying that the production of hydrogen peroxide is involved in the FB<sub>1</sub>-induced oxidative stress. None of the teas affected the increased activity of the GPx while honeybush, green and black teas effectively counteracted the FB<sub>1</sub>-induced decrease in CAT. This could explain the above arguments that green tea, black tea and fermented honeybush did not enhance the FB<sub>1</sub>-induced hepatotoxic effects. The activity of SOD was not affected suggesting that the formation of superoxide is not a prominent feature during FB<sub>1</sub>-induced hepatotoxic effects as was suggested previously (Sahu et al., 1998).

Herbal tea consumption significantly ( $P < 0.05$ ) arrested the proliferation of GSTP<sup>+</sup> altered cells in the presence of the cancer promoter FB<sub>1</sub> as the relative number of foci (11–20 μm), constituting approximately 50% of the total amount of foci, were significantly ( $P < 0.05$ ) decreased, while the relative number of mini foci (5–10 μm) was significantly ( $P < 0.05$ ) increased. The reduction of the total number of foci (>10 μm) by unfermented rooibos and to a certain extent with unfermented honeybush, could be related to the increased oxidative stress, which could further enhance apoptosis known to be effected by FB<sub>1</sub> in the liver (Lemmer et al., 1999). It was reported that apoptosis, induced by FB<sub>1</sub>, delayed cancer induction by removing initiated cells from the liver (Gelderblom et al., 1992, 1994). Similar to the herbal teas, green and black teas significantly decreased and increased the relative number of foci (11–20 μm) and minifoci (5–10 μm), respectively. Inhibition of cell proliferation by green tea polyphenols during cancer promotion has been associated with a reduction of the number and size of enzyme altered foci (Gong et al., 2000). Green and black teas significantly enhanced the total number of foci (>5 μm) as compared to the herbal teas and the DEN-FB<sub>1</sub> treatments, mainly due to an increase in the number of mini foci, which could be related to the reduction in liver ORAC, in the case of green tea, and GSH:GSSG ratio suggestive of an increased oxidative stress. It is known that a dual role for oxidative stress exists that could either stimulate cell proliferation or inhibit cell proliferation thereby increasing cell depletion via the induction of apoptosis (Klaunig and Kamendulis, 2004). In this regard the green tea flavanol, (–)-epigallocatechin-3-gallate was shown to inhibit the proliferation of cancer cells through the induction of apoptosis (Yang et al., 1998; Kuo and Lin, 2003). However, the present study indicated that the polyphenolic constituents that differ between the teas selectively modulate oxidative stress and the cancer promoting potency of FB<sub>1</sub> in rat liver.

Fermentation of the herbal teas tended to reduce the inhibition of cancer promotion effected by FB<sub>1</sub>, implying that the chemopreventive properties of the unfermented herbal teas should be further evaluated. Fermentation significantly reduced the soluble solid content which was associated with a decrease in the major antioxidants constituents resulting in a reduction in the rat antioxidants intake profiles. It is known that the antioxidant potency of plant polyphenolic components may play a determining role in

maintaining the balance between cell growth and cell death in preneoplastic lesions (Lee et al., 2002). In the present study the involvement of antioxidant properties of the different teas modulates the oxidative stress during FB<sub>1</sub>-induced cancer promotion by selectively affecting the ORAC and GSH levels, antioxidant enzymes and the level of lipid peroxidation presumably due to differences in their polyphenolic constituents. The reduced protective effect of honeybush against FB<sub>1</sub>-induced cancer promotion further emphasized the role of antioxidants as rooibos extracts exhibit higher antioxidant properties than honeybush (Joubert et al., 2008). It would appear that at similar concentrations, honeybush would have exhibited a far weaker protection. However, the protection against FB<sub>1</sub>-induced cancer promotion by the different teas may not be entirely related to their antioxidative activities and other features, e.g. their pro-oxidant activity due to specific polyphenol/FB<sub>1</sub>/iron interactions, affecting cell regulatory processes related to cell survival need to be further investigated.

### Conflict of interest statement

The authors declare that there are no conflicts of interest.

### Acknowledgements

The authors gratefully acknowledge the technical support provided by the Primate Unit of the MRC for maintenance of the experimental animals and also the Subprogramme Microbiology of PROMEC for the preparation of the culture material needed for FB<sub>1</sub> extraction and purification. The authors also thank D de Beer and CJ Malherbe from ARC Infruitec-Nietvoorbij, Stellenbosch, for HPLC analyses. This study was jointly supported by grants to WG and EJ from the Cancer Association of South Africa, Medical Research Council of South Africa, Rooibos Tea Council, National Research Foundation (Indigenous Knowledge Systems, GUN 2050353) and the Department of Trade and Industry (Technology and Human Resources for Industry Programme, THRIP 2424).

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## CHAPTER IV

### FUMONISIN-INDUCED HEPTOCARCINOGENESIS: MECHANISMS OF CANCER INITIATION AND PROMOTION

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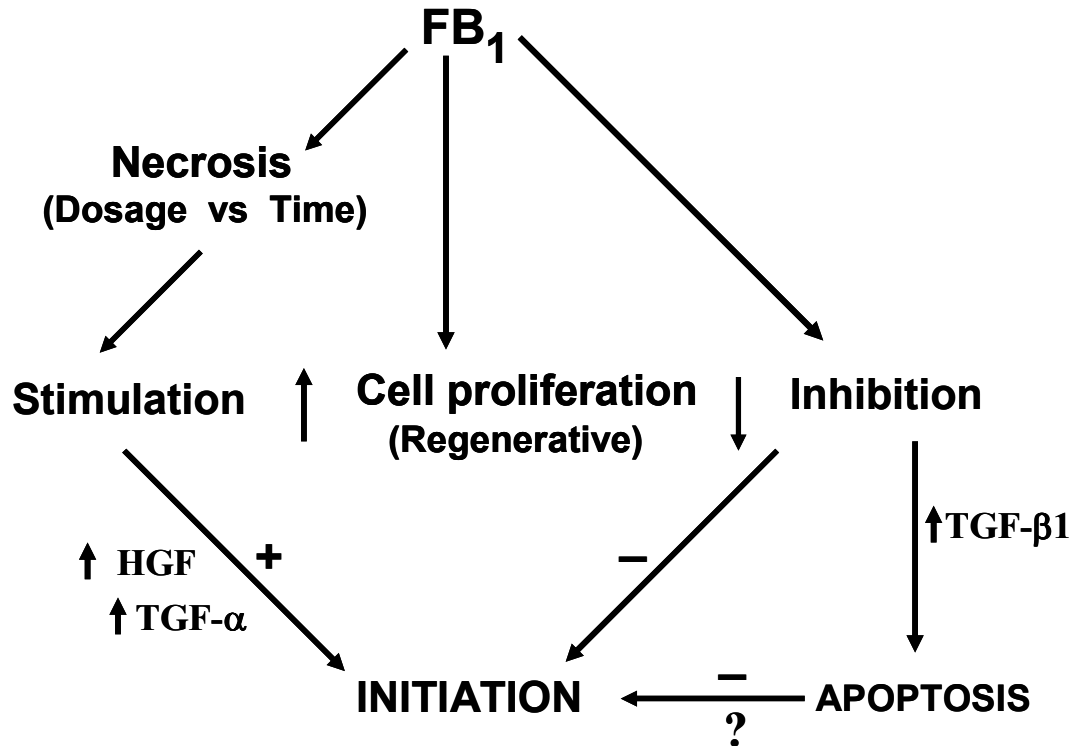
## FUMONISIN-INDUCED HEPATOCARCINOGENESIS: MECHANISMS OF CANCER INITIATION AND PROMOTION

### 4.1 *Cancer initiation by fumonisin B<sub>1</sub>*

#### 4.1.1 Studies in rat liver

Several studies have been carried out to investigate the mechanism of cancer initiation by FB<sub>1</sub> in an *in vivo* rat liver cancer initiation/promotion model. The cancer initiating potential of the apparent non-genotoxic FB<sub>1</sub> was therefore of interest as it is normally accepted that this stage of cancer induction is associated with a mutation-like event (Farber, 1984). At present very little is known about the nature of the initiating step of either genotoxic or non-genotoxic carcinogens although many other cell parameters such as protein and RNA can be altered independently from changes to the DNA. Apoptosis is also known to play a determining role in the outcome of the initiating event, as initiated cells are more prone to undergo apoptosis (Bursch *et al.*, 1992). It is known that FB<sub>1</sub> induces necrosis as well as apoptosis in the liver that could determine the outcome of cancer initiation by the mycotoxin (Lemmer *et al.*, 1999a; Gelderblom *et al.*, 2008). A biphasic effect of FB<sub>1</sub> on cell proliferation is proposed in the liver that results in the genesis of altered hepatic lesions ultimately leading to cancer (Figure 1). FB<sub>1</sub> either stimulates compensatory/regenerative cell proliferation as a result of cell death induced in the liver or inhibits cell proliferation, which will result in the induction of apoptosis. These processes are controlled by the induction of specific growth regulatory factors. The balance between these processes will determine the level of cell proliferation that prevails and the extent of cancer initiation achieved which will depend on the dose and duration of exposure.

When utilising the classical model for cancer initiation generally used for genotoxic carcinogens, i.e. a single dosage of a carcinogen 18-20 hr after partial hepatectomy, the fumonisins failed to effect initiation after a single or multiple dosages (Gelderblom *et al.*, 1992). One possible explanation for the lack of cancer initiation was ascribed to the inhibitory effect of the fumonisins on cell proliferation. Cancer initiation by FB<sub>1</sub> was only effected after prolonged feeding in the diet, and depending on the dosage, could be induced over a 14-day period (Gelderblom *et al.*, 1994).



**Fig 1.** The effect of the biphasic response of  $FB_1$  on cell proliferation in relation to cancer initiation. The overexpression of several growth stimulating and inhibitory factors are effected which are likely to play a determining role in the induction of the altered resistant hepatocytes (Gelderblom *et al.*, 1994; Lemmer *et al.*, 1999b).

The “Effective Dosage Level” for cancer initiation in male Fisher rats fed the AIN 76 diet was determined and interesting aspects regarding exposure levels and initiation were noticed. In this regard a total dosage of 30 mg  $FB_1$ /100 g body weight failed to initiate cancer over a 7-day period whilst the same dosage effected initiation over a period of 21 days. A critical balance between compensatory or regenerative cell proliferation resulted from the hepatotoxicity and the inhibitory effect on cell proliferation thereof seems to exist, which will eventually determine initiation by  $FB_1$  (Fig 1). A dose dependent effect on  $FB_1$ -induced cancer initiation was also observed during a gavage dosage regimen over a period of 14 days, although it was more effective than the dietary treatment.

A gavage  $FB_1$  treatment protocol was utilized to investigate the effect of cell proliferation on the cancer initiating properties of  $FB_1$  over 14 days (Gelderblom *et al.*, 2001a). The role of regenerative cell proliferation and mitogen-induced hyperplasia,

induced by partial hepatectomy and lead nitrate, respectively, were monitored as a single insult after 7 days. Similar to genotoxic carcinogens, lead nitrate failed to, while partial hepatectomy supported cancer initiation when using the induction of placental glutathione S-transferase positive (PGST<sup>+</sup>) foci as the endpoint. This study supports the initial studies utilizing FB<sub>1</sub> that a non-toxic threshold exists for cancer initiation and that regenerative cell proliferation plays an important role which in turn will depend on the FB<sub>1</sub> dose and duration of exposure. In this regard a low dose exposure for an extended period of time will initiate cancer in the absence of a hepatotoxic effect (Gelderblom *et al.*, 2001b), which provides additional information regarding threshold effects and the complex nature of the cancer initiating step.

Additional studies of the cancer initiating potential of fumonisin B<sub>1</sub> (FB<sub>1</sub>) were conducted in rat liver to (i) monitor the effect of another cancer promoter, such as phenobarbital (PB) and (ii) to evaluate the involvement of spontaneously initiated cells (Gelderblom *et al.*, 2008). The PB promoting regimen was found to stimulate the outgrowth of FB<sub>1</sub>-induced PGST<sup>+</sup> positive initiated hepatocytes. A specific role for spontaneously initiated cells and promotion by FB<sub>1</sub> into the development of eosinophilic clear cell foci could not be established. The ability of different stimuli to selectively promote the outgrowth of FB<sub>1</sub>-induced initiated cells and the lack of selectively promoting eosinophilic clear cell subtype foci in older rats, excluding the role of spontaneous initiated cells, verifying the cancer initiating potency of this apparent non-genotoxic mycotoxin.

#### 4.1.2 Genotoxicity

FB<sub>1</sub> has been shown to be non-genotoxic in *in vitro* mutagenic and genotoxic assays and in *in vivo* unscheduled DNA repair assays in rat liver. Fumonisin B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub> lack mutagenicity in the *Salmonella* mutagenicity test against TA97a, TA98, TA 100 and TA 102, both in the absence and presence of metabolic activation (Gelderblom & Snyman, 1991). In contrast fusarin C exhibited a mutagenic response against TA 97a, TA 98 and TA 100 in the presence of metabolic activation. The lack of mutagenicity by FB<sub>1</sub> was confirmed against TA 98 and TA 100 while it was also negative when conducting the SOS chromotest assay with E coli PQ37 and the induction of reparable DNA damage with E coli K-12 strains (Knasmuller *et al.*, 1997). *In vitro* and *in vivo* unscheduled DNA repair assays in primary hepatocytes related to

genotoxic cell damage showed FB<sub>1</sub> to lack genotoxic effects (Gelderblom *et al.*, 1992). Clastogenic effects, however, were noticed when utilizing primary cultures of hepatocytes. The induction of micronuclei were less clear as FB<sub>1</sub> only induces significant effects at certain doses although no clear dose response effects could be established. Induction of chromosomal aberrations was dose dependent with a 7-fold increase above the background (Knasmuller *et al.*, 1997).

#### 4.1.3 Role of oxidative damage.

As cancer initiation by FB<sub>1</sub> is associated with the induction of a hepatotoxic effect a role of oxidative damage in the induction of the altered genetic events has been suggested. Oxidative damage was closely associated to FB<sub>1</sub>-induced cancer initiation *in vivo* while the plasma and microsomal membranes and to some extent the mitochondria and the nuclei, appear to be significantly affected by lipid peroxidation (Abel & Gelderblom, 1998). *In vitro* studies in primary hepatocytes revealed a similar association between cytotoxicity and lipid peroxidation, measured as the induction of thiobarbituric acid reactive substances (TBARS). FB<sub>1</sub>-induced lipid peroxidation in primary hepatocytes was potentiated in the presence of cumol hydroperoxide, a potent oxidising agent, while it was reduced by the addition of vitamin E. The protection of vitamin E was only partly effective suggesting that the induction of lipid peroxidation is a result and not necessarily a cause of the FB<sub>1</sub>-induced hepatotoxic effects. Many cell processes, including cellular membranes, proteins, DNA and carbohydrates could be altered as a result of oxidative damage and these processes appear to occur as a result of cell injury induced by FB<sub>1</sub>. These changes could result from the over production of free radicals and/or reduced cellular defense mechanisms which could eventually effect cellular damage and altered biological responses within the cell. As it is known that oxidative damage could effect cancer initiation (Rushmore *et al.*, 1984), the slow cancer initiating potency of FB<sub>1</sub> as well as the close association of hepatotoxicity and oxidative damage suggests that it is likely to be an important determinant during cancer induction by this non-genotoxic mycotoxin. Potentiation of FB<sub>1</sub>-induced lipid peroxidation was also effected by dietary iron (Lemmer *et al.*, 1999a). As dietary iron caused lipid peroxidation in the absence of hepatic injury while FB<sub>1</sub> only induces mild lipid peroxidation as a result of the hepatotoxicity further provide evidence that lipid peroxidation is secondary to FB<sub>1</sub>-induced hepatic injury. Although Fe potentiates lipid peroxidation the effect on cancer

initiation could not be determined, as iron overload tends to protect against cancer promotion due to the stimulatory effect on cell proliferation.

#### 4.2 *Structure activity relationships*

The toxicological and/or biochemical effects of structural related compounds provide important information about the underlying mechanism involved as well as possible targets for strategising deactivation procedures. The occurrence of different structurally related fumonisins, eg the N-acetyl derivatives, the hydrolysed products (aminopolyols) and the mono methyl esters, which are purification artifacts, provided a unique opportunity to conduct comparative cytotoxicity and *in vivo* cancer induction assays (Gelderblom *et al.*, 1993; Van der Westhuizen *et al.*, 1998).

##### 4.2.1 Studies using primary hepatocyte cultures

Comparative cytotoxicity studies showed that FB<sub>2</sub> exhibited the highest cytotoxic effect followed by FB<sub>3</sub> and FB<sub>1</sub>. The respective hydrolysed products of FB<sub>1</sub> and FB<sub>2</sub> showed a higher cytotoxic effect than the parent molecules, with AP<sub>2</sub> exhibiting a significantly higher effect than AP<sub>1</sub>. It became evident that the polarity of the specific analogue is of importance as the less polar compound exhibiting a higher cytotoxicity. FA<sub>1</sub> and FA<sub>2</sub> exhibited significant weaker cytotoxic effects, emphasizing the role of the free amino group in the biological properties of the fumonisins (Van der Westhuizen *et al.*, 1998).

The disruption of sphingolipid biosynthesis was also monitored in primary hepatocytes using the different structural analogues as well as the *Alternaria alternata* toxins, TA and TB (Van der Westhuizen *et al.*, 1998). FB<sub>1</sub> (1 uM) maximally disrupt sphingolipid biosynthesis after 24 hrs in an irreversible manner (section 4.4.1). FB<sub>1</sub> was shown to be more cytotoxic than TA and TB, although the latter two toxins were more effective in disrupting sphingolipid biosynthesis in the order TB>TA>FB<sub>1</sub>. FA<sub>1</sub> was more, and AP<sub>1</sub> was far less effective in disrupting sphingolipid metabolism than FB<sub>1</sub>. It was suggested that the structural requirements for the cytotoxic effect differs from that required for ceramide synthase inhibition, especially when considering the activities of the hydrolysed structural analogues vs the parent fumonisin compounds. It would appear that additional effects, apart from the



disruption of sphingolipid biosynthesis, are required to explain the cytotoxic effects in primary hepatocytes.

#### 4.2.2 *In vivo* studies in rat liver

The cancer initiating properties of FB<sub>1</sub>, FB<sub>2</sub>, FB<sub>3</sub> and the structural analogues, FA<sub>1</sub>, AP<sub>1</sub>, AP<sub>2</sub>, the momomethylesters of FB<sub>1</sub> (MME) and tricarballic acid moiety of the fumonisins (TCA) were monitored in a 21-day feeding study in two separate experiments (Gelderblom *et al.*, 1993). The cancer promoting regimen 2-AAF/PH was used two weeks following the initiating treatment to select the outgrowth of initiated cells with the induction of GGT positive foci as the endpoint for cancer initiation. The FB series were fed at dietary levels of 0.1% for 7 days and 0.05% for 14 days in the first experiment and 0.05% for 21 days during the second experiment. FA<sub>1</sub> was fed at a dietary level of 0.1% for 21 days in both experiments. MME was only used in one experiment and fed at a dietary level of 0.1% for 1 week followed by 0.05% for two weeks. AP<sub>1</sub> and AP<sub>2</sub> were fed at 0.05% for three weeks in the first experiment while AP<sub>1</sub> was fed at a dietary level of 0.1 for three weeks. Only the compounds with an intact fumonisin structure and a free amino group initiated cancer, which was associated with a significant reduction in body weight gain over the 21 days feeding period. It was of interest that AP<sub>1</sub>, which exhibited a higher cytotoxic effect than FB<sub>1</sub> in primary hepatocytes, lacks any cancer initiating properties even when fed at double the concentration.

#### 4.3 *Mechanisms related to cancer promotion.*

The purification of FB<sub>1</sub> was based on the cancer promoting characteristics of maize culture material of *F. verticillioides* in a short-term liver cancer initiating promoting model using diethylnitrosamine as cancer initiator. Although two mechanisms for cancer promotion exist, FB<sub>1</sub> seems to select the outgrowth of initiated cells by the process of differential inhibition. During this selection process the growth of normal hepatocytes is inhibited while the initiated cells, resistant to the growth inhibitory effects of FB<sub>1</sub>, proliferate. This model of cancer promotion was based on the resistant hepatocyte model originally developed in rat liver by Solt *et al.*, (1977). Additional models for cancer induction by FB<sub>1</sub> have been proposed in rat kidney (Riley *et al.*, 2001; Dragan *et al.*, 2001).

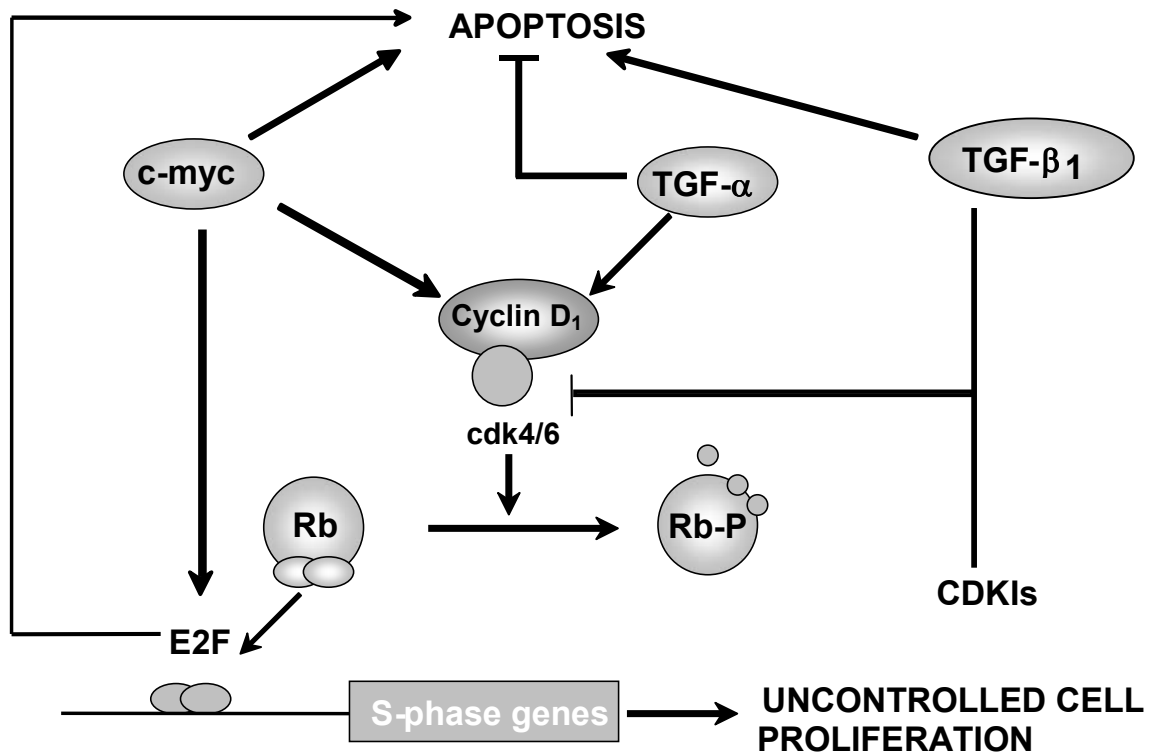
#### 4.3.1 Studies in rat liver

The inhibitory effect of FB<sub>1</sub> on hepatocyte cell proliferation formed the basis of the bioassay during the isolation of the compound from the culture material of the fungus (Gelderblom *et al.*, 1988). It would appear that, with respect to the inhibition of cell proliferation, the fumonisins exhibit a dual response on cancer induction, i.e. on the one hand it delays cancer initiation via the induction of apoptosis while on the other hand it promotes the growth of genetically altered cells resistant to the adverse biological properties (Fig 1). Consequently fumonisin B<sub>1</sub> is a far better cancer promoter than cancer initiator, an aspect that should be considered when establishing risk assessment parameters (see section V).

The first evidence that FB<sub>1</sub> altered growth responses in the liver came from the initial study on the isolation of FB<sub>1</sub> and FB<sub>2</sub> indicating that the fumonisins caused numerous toxic lesions leading to a toxic hepatitis (Gelderblom *et al.*, 1988a). Short-term studies in rats showed that FB<sub>1</sub> significantly inhibited hepatocyte cell proliferation induced by partial hepatectomy, either by a single gavage dosage or by feeding in a diet over a period of 21 days (Gelderblom *et al.*, 1994). Hepatocyte proliferation was effectively delayed and 3 days after removal of FB<sub>1</sub> from the diet, the FB<sub>1</sub>-treated rats exhibited a higher level of cell proliferation than the controls suggesting that the inhibitory effect of FB<sub>1</sub> is reversible. Dose-response studies indicated that a dietary level up to 50 mg FB<sub>1</sub>/kg significantly reduced hepatocyte cell proliferation induced by partial hepatectomy (Gelderblom *et al.*, 1996b). Of interest was the fact that this inhibitory effect was closely associated with the cancer promoting activity of diethylnitrosamine (DEN)-induced GSTP positive foci. Cancer promotion by FB<sub>1</sub> was induced in the absence of any adverse hepatotoxic effects at dietary levels that lack cancer initiation activity over a 21-day feeding period. It would appear that in contrast to cancer initiation, cancer promotion is effected in the absence of hepatotoxicity, which was originally thought not to be the case (Gelderblom *et al.*, 1988a; 1988b; 1994). Although other mechanisms also exist to explain cancer promotion the inhibitory effect of the fumonisins on hepatocyte proliferation and several cell culture systems strongly suggest that cancer promotion in the liver results from the induction of differential growth inhibition of normal hepatocytes allowing the “resistant” initiated hepatocytes to proliferate.

In order to correlate the histopathological alterations with changes in gene expression in rat liver during short-term treatment with FB<sub>1</sub>, male Fisher rats were fed either a diet containing FB<sub>1</sub> at 250 mg/kg or a control diet for 5 weeks (Lemmer *et al.*, 1999b). FB<sub>1</sub> caused a predominantly zone 3 'toxic' liver injury, with hepatocyte death due to necrosis and apoptosis. Hepatocyte injury and death were mirrored by desmin-positive hepatic stellate cell proliferation and marked fibrosis, with progressive disturbance of architecture and formation of regenerative nodules. Despite the inhibition of hepatocyte regenerative activity, OV-6 positive oval cell proliferation was noted from week 2, PGST<sup>+</sup> hepatic foci and nodules developed and, at later time points, oval cells were noted inside some of the 'atypical' nodules. Northern blot (mRNA) analysis of liver specimens from weeks 3 to 5 showed a progressive increase in gene expression for  $\alpha$ -fetoprotein (AFP), hepatocyte growth factor (HGF), TGF- $\alpha$  and especially TGF- $\beta$ 1 and *c-myc*. Immunostaining with LC (1-30) antibody demonstrated a progressive increase in expression of mature TGF- $\beta$ 1 protein by zone 1 and 2 hepatocytes over the 5 week feeding period. FB<sub>1</sub> thus appears to be a unique liver carcinogen, which causes the rapid induction of oval cell proliferation and preneoplastic lesions despite marked apoptosis. The overexpression of TGF- $\beta$ 1 by hepatocytes may be causally related to the prominent apoptosis and fibrosis seen with liver injury due to FB<sub>1</sub>. Increased expression of *c-myc* and TGF- $\beta$ 1 may cooperate in the promotion of liver tumour development in the FB<sub>1</sub>-fed rat, possibly by providing an environment that stimulates the outgrowth of TGF- $\beta$ 1-resistant transformed hepatocytes and/or oval cells via the regulation of cyclin D<sub>1</sub>, cyclin D kinases, retinoblastoma protein (Rb) phosphorylation and E<sub>2</sub>F (Fig 2). The interaction of cyclin D<sub>1</sub> and *c-myc* expression with the subsequent inactivation of Rb may alter the expression of E<sub>2</sub>F and the deregulation of cell cycle control.

A study in male Fischer 344 and BD IX rats showed that FB<sub>1</sub> increased the levels of cyclin D<sub>1</sub> in preneoplastic and neoplastic liver lesions (Ramljak *et al.*, 2000). FB<sub>1</sub> was shown to activate protein kinase B (Akt), which stimulates the phosphorylation of cyclin D<sub>1</sub> via glucocorticoid synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), thereby inhibiting its proteosomal breakdown. Subsequent events include an increase in the cyclin dependent kinase 4 (Cdk4) complex with cyclin D<sub>1</sub> resulting in the elevation of Cdk4

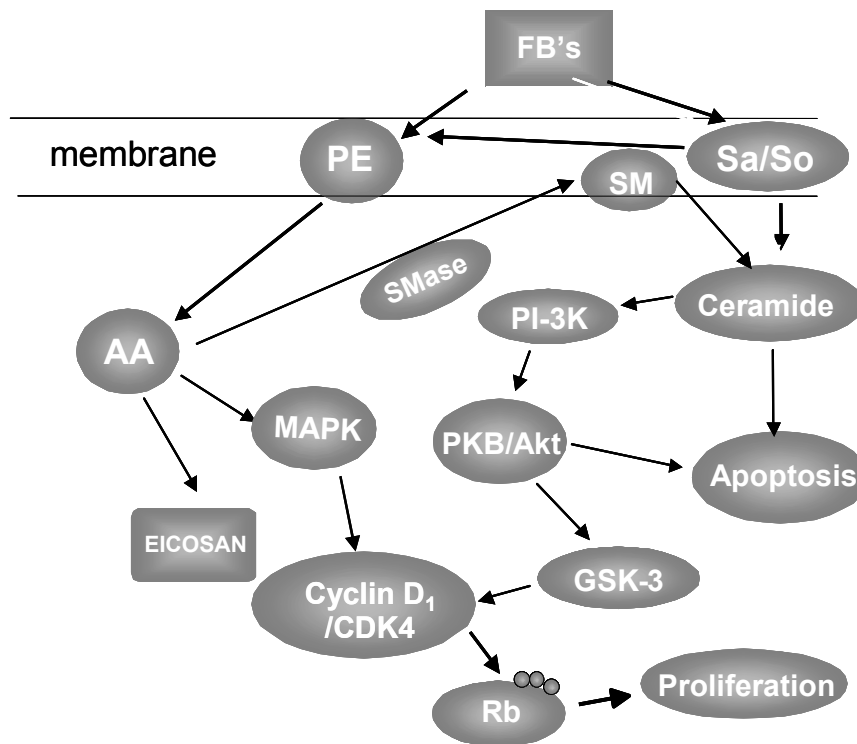


**Fig 2.** Alteration of cell cycle control genes in regulating cell proliferation and apoptosis. Differential effects of FB<sub>1</sub> on c-myc, TGF-α and TGF-β<sub>1</sub> in normal and altered hepatocytes forms the basis of cancer promotion via the regulation of cyclin D<sub>1</sub> and E2F (Lemmer et al., 1999b).

activity and phosphorylation of retinoblastoma, which will provide a selective growth advantage to the altered preneoplastic and neoplastic liver cells. Akt is also known to inhibit apoptosis in the resistant preneoplastic cells resulting from cancer initiation while normal cells will undergo apoptosis in the absence of cyclin D<sub>1</sub> stabilisation. The disruption of sphingolipid, phospholipid and fatty acid metabolism and the subsequent effects on cyclin D<sub>1</sub> stabilisation are illustrated in below (Fig 3).

Upstream membranal events are likely to be involved in the FB<sub>1</sub>-induced effects on lipid biosynthesis, especially the regulatory roles of arachidonic acid (AA; C20:4n-6) and ceramide on cell cycle parameters, such as MAPK and PI3K pathways determining cell survival. The differential effects of these lipid-associated changes on normal and preneoplastic lesions in the liver could provide a plausible explanation for the cancer promoting properties of the fumonisins. The disruption of sphingolipid

metabolism in hepatocyte nodules, induced by either FB<sub>1</sub> or DEN, followed by 2-acetylaminofluorene/partial hepatectomy (AAF/PH) promotion was investigated by monitoring the effect of a secondary FB<sub>1</sub> treatment on the levels of sphinganine (Sa) and sphingosine (So) in the nodules and surrounding tissue (Van der Westhuizen *et al.*, 2004). The initiating treatment of FB<sub>1</sub> (250 mg FB<sub>1</sub>/kg diet for 21 days) significantly increased the Sa, So and Sa/So ratio in the liver which returned to baseline levels after the 2-AAF/PH promoting treatment except for So in the nodules.



**Fig 3.** Membrane effects of FB<sub>1</sub> disrupting lipid biosynthesis at different levels, affecting membrane integrity and key cell survival regulatory molecules related to cell proliferation and apoptosis (Ramljak *et al.*, 2000; Gelderblom *et al.*, 2001, 2008)

The secondary treatment increased the Sa levels to a similar extent in the nodules and surrounding tissue while So was further increased in the FB<sub>1</sub>- and DEN-induced hepatocyte nodules. DEN initiation sensitized the nodules and surrounding tissue to the accumulation of Sa when compared to FB<sub>1</sub> initiation, resulting in a higher Sa/So ratio. The nodules resulted from FB<sub>1</sub>-initiation are sensitized to accumulate So which could selectively support proliferation, presumably via the production of So-1-P.

#### 4.3.2 Studies in primary hepatocytes

A common property of many cancer promoters is their mitoinhibitory effect in primary rat hepatocytes when using epidermal growth factor (EGF) as a mitogen. In a comparative study 2-AAF, a known cancer promoter, exhibited the highest inhibitory effect followed by FB<sub>1</sub> and phenobarbital (PB), on the EGF-induced mitogenic response (Gelderblom *et al.*, 1996b). PB is generally regarded as a mitogen that selectively stimulates the development of preneoplastic lesions in the liver via the disruption of apoptosis (Schulte-Herman *et al.*, 1990). From these studies it would appear that the inhibition of cell proliferation by FB<sub>1</sub> is a key event in creating the differential growth response in the liver during cancer promotion. At present very little is known about the mechanisms involved in the mitoinhibitory effect and/or the inhibition of cell proliferation by FB<sub>1</sub>. Studies in primary hepatocytes indicated that mitoinhibition was reversible and that the pretreatment of cells has very little effect on the EGF response (Gelderblom *et al.*, 1995). It would appear that hepatocytes have very little "memory" and that the continued presence of FB<sub>1</sub> is required for maximal inhibition. The mitoinhibition by FB<sub>1</sub> was not associated with cytotoxicity as it was effected well below (10x) the cytotoxic levels. When compared to cancer promotion *in vivo* similarities exist with respect to the reversibility and the lack of an association with hepatotoxicity. Due to these similarities the mitoinhibitory effect in primary hepatocytes was used as a tool to investigate the possible mechanism(s) involved.

The EGF response in primary hepatocytes was associated with changes in the n-6 fatty acid (FA) metabolism as C20:4n-6 and C18:2n-6 was significantly decreased and increased, respectively (Gelderblom *et al.*, 1999). Addition of C20:4n-6 increased the EGF response while C20:5n-3 decreased not only the levels of C20:4n-6, but also the EGF mitogenic response. The inhibition of the EGF mitogenic response by C20:5n-3 was associated with a decrease in the total n-6 FA, PUFA and an increase in the saturated (SATS) and mono unsaturated FA (MONO-UNSATS) resulting in a decrease in the polyunsaturated/saturated (P/S) FA ratio. The underlying mechanisms are not known, but the inhibition of the EGF response was associated with changes in the membrane environment, specifically the FA content of PC. The stimulation of the EGF response was directly associated with the increased level of C20:4n-6 in the membranal PC phospholipid fraction. The role of C20:4n-6 was further emphasized as the addition of PGE<sub>2</sub>, derived from C20:4n-6, stimulated, while

ibuprofen, a non steroidal anti-inflammatory drug known to inhibit PGE<sub>2</sub> formation, decreased the EGF mitogenic response, respectively. PGE<sub>2</sub> also counteracted the FB<sub>1</sub>-induced mitoinhibitory effect suggesting that this biosynthetic pathway is disrupted in primary hepatocytes. The disruption of C20:4n-6 levels and subsequent prostanoid metabolism are likely to be one of the underlying mechanisms responsible for the modulation of the growth regulatory effects in primary hepatocytes and rat liver *in vivo*.

#### 4.4 *Metabolism and disruption of lipid biosynthesis*

##### 4.4.1 *In vitro* studies using primary hepatocytes

FB<sub>1</sub> and FB<sub>2</sub> were found not to be highly toxic to primary hepatocytes when compared to AFB<sub>1</sub> (Gelderblom *et al.*, 1993). Of the fumonisins, FB<sub>2</sub> exhibited the highest cytotoxic effects with FB<sub>1</sub> and FB<sub>3</sub> having similar potencies. In comparative studies using <sup>14</sup>C radiolabelled fumonisins a higher specific binding was noticed with FB<sub>2</sub>, presumably due to a lower polarity (Cawood *et al.*, 1994). The effective dose level, i.e. the lowest levels to elicit a cytotoxic effect, was similar. About 10% of the fumonisins are bound irreversibly to the hepatocytes indicating that the mycotoxins are associated with both the soluble and insoluble (membranal) compartments of the cell. Some of the radiolabelled fumonisins were tightly bound to the microsomes and plasma membranes. FB<sub>1</sub> was not metabolized by the primary hepatocytes as no radiolabelled metabolites could be detected after the fractionation of the culture media. Enzymatic incubation assays also provide evidence that FB<sub>1</sub> was not metabolised by cytochrome P450, the hepatic esterases, the tryglyceride hepatic endothelial lipase or the porcine pancreatic lipase. The study provides evidence that the intact molecule is responsible for the toxicological effects in the liver and hydrolisation to the aminopolyols does not occur.

A study using [<sup>14</sup>C] leucine provided evidence that FB<sub>1</sub> did not affect the incorporation in primary hepatocytes at both cytotoxic and non-cytotoxic doses (Gelderblom *et al.*, 1996a). In contrast the incorporation of [<sup>14</sup>C] labeled palmitic acid was significantly reduced in triacylglycerol (TAG) and sphingomyelin (SM) while it was increased in phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The increased accumulation in the phospholipids closely mimics the increased concentration of the

respective phospholipids in the treated cells. The relative levels of C18:2n-6 and C20:4n6 was significantly increased in the hepatocytes resulting in an increase in the total PUFA. Cholesterol, free cholesterol and SM levels were significantly lowered while sphinganine was increased. The increased PC/free cholesterol ratio was indicative of a more rigid membrane structure while the decreased C20:3n-6/C18:2n-6 ratio implies the impairment of the delta-6 desaturase, the rate limiting enzyme in fatty acid metabolism. Changes in the conversion of C18:2n-6 to C20:4n-6 also regulates TAG synthesis.

The kinetics of sphingolipid metabolism inhibition was investigated in primary hepatocytes (Van der Westhuizen *et al.*, 1998). The accumulation of sphinganine (Sa) and sphingosine (So) was maximally increased by FB<sub>1</sub> (1µM) after 40 hr resulting in a significant increase in the Sa/So ratio in the absence of any cytotoxic effects. Removal of FB<sub>1</sub> after 12 or 24 hrs did not effect the accumulation of Sa although the level of So was significant reduced. This would imply that the accumulation of Sa is irreversible in primary hepatocytes and the accumulation of Sa was not associated with a cytotoxic effect.

#### 4.4.2 In vivo studies in rat liver

Lipid alterations in the livers of male Fischer and BD IX rats were monitored in a short-term, high dose and a long-term, low dose feeding treatment regimens, respectively (Gelderblom *et al.*, 1997). Phospholipid changes noticed involved an increase in PE and decrease in SM at the highest dose (250 mg FB<sub>1</sub>/kg) while PE was markedly increased at the 50 and 100mg FB<sub>1</sub>/kg diet groups. Total serum and liver cholesterol was significantly increased at the highest dose level. Sa was markedly to significantly increased at all the dietary treatments while the Sa/So ratio was significantly increased at 100 mg FB<sub>1</sub> dietary level. When considering the relative fatty acid (FA) levels, C18:2n-6 and C22:5n-6 were significantly increased and decreased respectively, in PE and markedly in PC suggesting an impairment of the delta 6-desaturase enzyme. This resulted in a decrease in the total n-6 FA and PUFA at the highest dose. In the long-term study PE was significantly increased in the liver at 1, 10 and 25 mg FB<sub>1</sub>/kg diet, while Sa and So were markedly increased at the highest dose level. Total FA analyses showed that C18:2n-6 was significantly increased at all the dose levels while C22:5n-6 was markedly decreased again



suggesting an impaired delta 6-desaturase. In the long-term experiment the n-6/n-3 ratio was significantly decreased in PC due to an increase in the total n-3 FA. Retrospective analyses of the C20:4n-6 PC/PE ratio in both the short-term and long-term experiments showed that FB<sub>1</sub> decreased the ratio implying that the level was increased in PE relatively to PC (See section 4.5 for additional comments).

Table 1. The modulating effect of FB<sub>1</sub> on the arachidonic acid (C20:4n-6) distribution in phosphatidylcholine (PC) and phosphatidyl-ethanolamine (PE) in rat liver of short-term and long-term feedings studies.

| Dietary FB <sub>1</sub><br>(21 days)* | C20:4n-6<br>(PC/PE ratio) | Dietary FB <sub>1</sub><br>(2 yr)** | C20:4n-6<br>(PC/PE ratio) |
|---------------------------------------|---------------------------|-------------------------------------|---------------------------|
| Ctrl                                  | 1.15                      | Ctrl                                | 1.05                      |
| 50                                    | 1.17                      | 1                                   | 0.91                      |
| 100                                   | 1.10                      | 10                                  | 0.93                      |
| 250                                   | 1.02                      | 25                                  | 0.76                      |

\*Male Fischer 344 rats; \*\*Male BD IX rats (Data derived from Gelderblom *et al.*, 1997)

A study in liver microsomal preparations of rats receiving different dietary levels of FB<sub>1</sub> (10, 50, 100 and 250 mg/kg) over 21 days showed a dose dependent increase in PC, PE, PI and cholesterol (Chol) resulting in changes in the membrane lipid parameters (Gelderblom *et al.*, 2002). This resulted in a decrease in the PC/PE and an increase in the total phospholipid/Chol ratios suggesting an increase in the rigidity of the microsomal membrane. The absolute FA levels, including SATS, MONO-UNSATs and polyunsaturated (PUFA), increased as a function of the elevated phospholipid levels and were already altered at the lowest FB<sub>1</sub> dietary level. The relative levels of the monounsaturated (C16:1n-7, C18:1n-9) as well as C18:2n-6 increased in the liver of the rats receiving the 100 mg FB<sub>1</sub>/kg/kg diet and higher. Similarly, the relative levels of the long chained PUFA's, C20:4n-6 and C22:5n-6 decreased implying an impairment of the delta 6-desaturase enzyme. Enzyme analyses showed that FB<sub>1</sub> inhibited the activity of the enzyme in a dose dependent manner and coincided with the changes of the relative FA levels. The significant increase in the C18:3n-6/20:4n-6 and the C20:3n6/C20:4n-6 ratios also imply the impairment of the delta 5-desaturase enzyme. The mechanism of inhibition is not known at present, although the disruption of the integrity of the microsomal membrane is likely to affect the activity of the enzyme, especially at higher dietary

levels. The increase of PE relative to PC implies that certain FA, especially the C20:4n-6 PC/PE ratio is enhanced (Table 2) which has been suggested to be an important growth stimulus in hepatocyte nodules (Abel *et al.*, 2001). A specific role of C20:4n-6 has been proposed for the development of liver cancer induced by FB<sub>1</sub> (see section 4.5).

Table 2. The modulating effect of FB<sub>1</sub> on the arachidonic acid (C20:4n-6) distribution in phosphatidylcholine (PC) and phosphatidyl-ethanolamine (PE) in rat liver after a 21 day feedings study.

| Dietary FB <sub>1</sub><br>(21 days)* | Ctrl | 10  | 50  | 100 | 250 |
|---------------------------------------|------|-----|-----|-----|-----|
| C20:4n6<br>(PC/PE ratio)              | 2.7  | 2.4 | 2.6 | 1.8 | 1.9 |

\* male Fischer rats (Adapted from Gelderblom *et al.*, 2002)

Changes in the lipid composition by FB<sub>1</sub> was further expanded by including different subcellular fractions of the liver of rats exposed to the highest dietary level of 250 mg FB<sub>1</sub>/kg for 21 days (Burger *et al.*, 2007). This dietary level effects both cancer initiation and promotion (Gelderblom *et al.*, 1994, 1996b). Similar changes related to cholesterol, phospholipid and FA metabolism as described above were noticed in the subcellular fraction although it differs between the subcellular membrane fractions. PE was increased in the microsomal, mitochondrial and plasma membrane fractions while PC was increased in the microsomes but decreased in the mitochondria. SM was decreased and increased in the mitochondria and nuclear fractions, respectively. Cholesterol was increased in both the microsomal and nuclear fractions. It would appear that most of the changes in these lipid parameters are located in the microsomes, mitochondria and plasma membrane. SATS increased in PE in the microsomes and mitochondria due to an increase in both C16:0 and C18:0 and decreased in the mitochondrial PC due to a decrease in C18:0. The MONO-UNSATs were increased in PC and PE in the microsomes and mitochondria mainly due to an increase in C18:1n-9. The PUFA were decreased in PE in the microsomes, PC in the mitochondria and plasma membrane mainly due to a decrease in the long chain PUFAs (C20:4n-6, C22:4n-6 and C22:5n-6). The reduction in the levels of PUFA and increased in the MONO-UNSATs, C16:1n-7 and C18:1n-9, were indicative of an

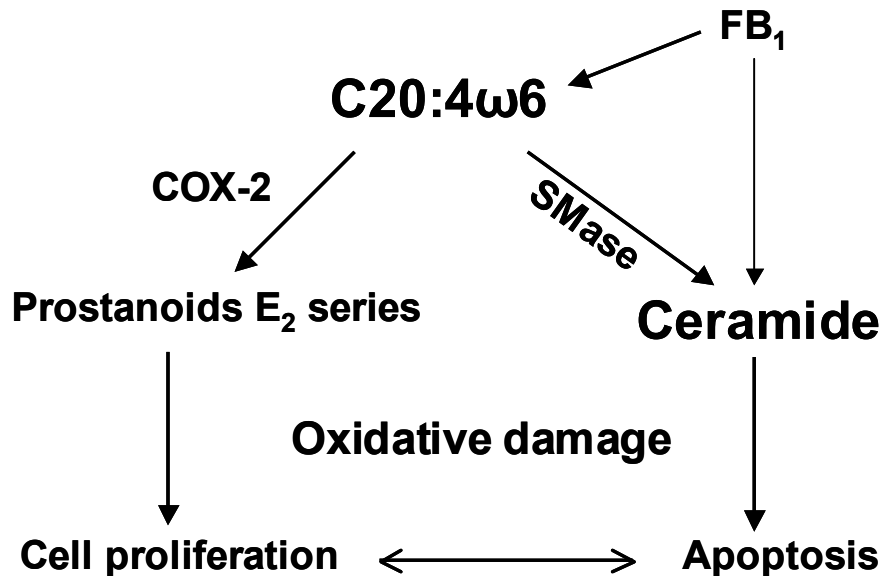
impaired FA desaturase enzymes. Changes in the different lipid parameters in the various cellular compartments regarding the PC/PE and P/S ratios suggest a more rigid membrane structure. Changes in the n-6/n-3 ratio and the decreased C20:4n-6 PC/PE ratio due to a relative increase of C20:4n-6 in PE, suggest a shift in the prostanoid synthesis of the E<sub>2</sub> series. Changes in the PE and C20:4n-6 in the plasma membrane could also impact on the key growth regulatory cell receptors specifically those located in lipid rafts known to be altered by FB<sub>1</sub>. An interaction between C20:4n-6 and ceramide was suggested to play a critical role in the mitochondria to regulate the balance between cell proliferation and apoptosis which could be important during cancer promotion of the resistant hepatocytes induced in the liver (Fig 4). Changes in the level of oxidative stress in the normal and the preneoplastic lesions (section 4.1.3) will also contribute to the balance that exists between the proliferative and apoptotic status prevailing in the liver.

#### 4.5 Perspectives

The genotoxicity of the fumonisins has been debated although it is generally accepted that it lacks DNA interactive reactivity in different short-term genotoxicity assays utilising bacteria (Knasmuller *et al.*, 1997; Aranda *et al.*, 2000; Ehrlich *et al.*, 2002) and *in vivo* and *in vitro* DNA repair assays in primary hepatocytes (Gelderblom and Snyman, 1991). No direct interaction of FB<sub>1</sub> with oligonucleotides could be detected (Pocsfalvi *et al.*, 2000). However, recent studies showed that FB<sub>1</sub> exhibited clastogenic and apoptotic effects in different human cells including human fibroblasts (Galvano *et al.*, 2002), HepG<sub>2</sub> cells (Ehrlich *et al.*, 2002), lymphocytes (Lerda *et al.*, 2005; De Lorenzi *et al.*, 2005), monkey and, rabbit kidney cells (Abado-Becognee *et al.*, 1998; Rumora *et al.*, 2002). DNA damage of the apoptotic type was induced in the fibroblast in the absence of cytotoxic effects. A study in C6 gliomal cells with normal p-53 status underwent apoptosis after incubation with FB<sub>1</sub> associated with a dose-related increased in oxidative DNA adduct formation via the formation of 8-hydroxy deoxyguanine (8-OH-dG) and fragmentation (Mobio *et al.*, 2003).

The loss in the suppression of cell cycle progression and absence of apoptosis in p-53-null mouse embryonic fibroblasts cells suggest that p53 gene governed DNA lesions induced by FB<sub>1</sub> further supported DNA reactivity. In the HepG2 cells

chromosomal aberrations and DNA migration patterns as monitored by the comet assay were detected at very low concentrations in the absence of any cytotoxic effects.



**Fig 4.** Interactive pathways of FB<sub>1</sub>-induced lipid and oxidative changes in the regulation of cell proliferation and apoptosis in the liver (Burger *et al.*, 2007; Gelderblom *et al.*, 2008)

Oxidative DNA damage appears to be one of the major mechanisms proposed for disrupting membrane structures associated with an increased lipid peroxidation (Yin *et al.*, 1998; Sahu *et al.*, 1998; Atroshi *et al.*, 1999), which could explain the genotoxic effects of the fumonisins via the formation of hydroxyl and peroxy radicals. An *in vivo* study in rats suggested that the disruption of sphingolipid biosynthesis played an important role in DNA damage in the kidney caused by FB<sub>1</sub> as it occurs in the absence of different markers associated with oxidative damage (Domijan *et al.*, 2007). In the liver, however, oxidative markers and changes to the sphingolipid levels coincided with DNA damage. The study confirms the notion that oxidative damage appears to be a secondary event following the FB<sub>1</sub>-induced cytotoxic effects, which was associated with DNA alterations in the liver.

The *in vitro* and *in vivo* studies confirmed that FB<sub>1</sub>-induced DNA damage, presumably by indirect means associated with cell toxicity and resultant oxidative damage. The implications of these genetic alterations in cancer development have been evaluated by utilising the rat liver cancer initiating/promotion model. These studies provided sufficient evidence that FB<sub>1</sub> possesses cancer initiating activity as defined below:

- Studies using male BD IX, Fischer 344 and Sprague Dawley rats showed that FB<sub>1</sub> exhibits cancer-initiating properties (Gelderblom *et al.*, 1988a, 1992, 1994, 2001a, 2008; Metha *et al.*, 1998).
- The induction of a hepatotoxic effect, as discussed for the induction of hepatocarcinogenesis, is a prerequisite for cancer initiation. An apparent no-effect threshold related to hepatotoxicity and hepatocyte regeneration exists for cancer initiation, which in turn is determined by the dosage and the duration of exposure. In this regard a low non-toxic dose effect initiation in the absence of a hepatotoxic effect (Gelderblom *et al.*, 2001b). The inhibition of hepatocyte proliferation and the induction of apoptosis seem to play a critical role in the genesis and/or the threshold of the initiating event (Gelderblom *et al.*, 1994; Lemmer *et al.*, 1999b).
- FB<sub>1</sub> initiates cancer in rat liver similarly to the genotoxic carcinogens, by inducing “resistant” hepatocytes with a dependence on cell proliferation, although the kinetics differ. Phenotypically these initiated cells develop into eosinophilic clear cell foci and nodules that stained positively PGST and  $\gamma$ -glutamyl-transferase (GGT). The absolute level of events (DNA mutations, etc), resulting in initiation, occurs at a far slower rate when compared to genotoxic carcinogens (Gelderblom *et al.*, 1991; 1992, 1994).
- Similarly to genotoxins, cell proliferation associated with hyperplasia failed to enhance, while stimulation of regenerative cell proliferation enhanced the cancer initiating potency of FB<sub>1</sub> (Gelderblom *et al.*, 2001a).

- Irreversibility of cancer initiation by FB<sub>1</sub> was demonstrated as a five-week exposure resulted in liver adenomas after 1 year in male Fischer 344 rats (Lemmer *et al.*, 2004).
- Different cancer promoting stimuli, 2-AAF/PH, 2-AAF/CCl<sub>4</sub> and PB promote the development of altered preneoplastic hepatic lesions (Gelderblom *et al.*, 1991, 2008).
- FB<sub>1</sub> lacks peroxisome proliferative activity (Gelderblom *et al.*, 1996b) while the role of promoting spontaneous initiated cells in older rats (Gelderblom *et al.*, 2008) seems not to play a role in FB<sub>1</sub>-induced carcinogenesis.

The intact fumonisin structure is a prerequisite for the toxicological effects in the liver while the hydrolysis to the aminopolyols does not occur in the liver. Studies in vervet monkeys indicated the presence of the aminopentol and -tetrol and partial hydrolysed forms of FB<sub>1</sub> and FB<sub>2</sub> in the faeces is only a minor metabolic pathway, presumably related to the breakdown by the gut microflora (Shephard *et al.*, 1995, 1999). However, the aminopentol (AP<sub>1</sub> of HFB<sub>1</sub>) has been implicated in the toxicological effects in rats (Hendrich *et al.*, 1993; Voss *et al.*, 1996). These studies showed that nixtamalised culture material containing HFB<sub>1</sub>, but no FB<sub>1</sub>, exhibited cancer promoting and toxic effects in the liver and nephrotoxic effects in kidneys of rats. It was suggested that HFB<sub>1</sub> still retains hepatotoxic and nephrotoxic effects in rats when fed at dietary levels between 7-10 and 39 mg/kg diet, although it was less potent than the parent FB<sub>1</sub>. Culture material containing 58 mg HFB<sub>1</sub>/kg diet and fed to rats for 4 weeks significantly increased the So level and Sa/So ratio and toxic effects in the liver and kidneys (Voss *et al.*, 1998). Studies in rats using purified HFB<sub>1</sub> (AP<sub>1</sub>) and HFB<sub>2</sub> (AP<sub>2</sub>), however, showed contrasting results in that levels up to 500 and 1000 mg/kg over a period of 21 days lack any toxic and/or cancer initiating potency (Gelderblom *et al.*, 1993). A study in mice also indicated that HFB<sub>1</sub>, fed at a dietary level of up to 140 ppm for 27 days lacks any histopathological effects and changes in sphingolipid biosynthesis (Howard *et al.*, 2002) suggesting that the hydrolysed products lack any biological activity *in vivo*. These studies showed that the free amino group is a prerequisite for biological activity *in vivo*.

Studies utilising primary hepatocytes showed that HFB<sub>1</sub> and HFB<sub>2</sub> were less effective in disrupting sphingolipid metabolism while it was more cytotoxic than the parent molecule (Gelderblom *et al.*, 1993; Van der Westhuizen *et al.*, 1998). Cytotoxic effects of FA<sub>1</sub> and FA<sub>2</sub> were similar to those of FB<sub>1</sub> and FB<sub>2</sub>, respectively although it tended to be less toxic at higher dose levels (Gelderblom *et al.*, 1993). At a low dose (1 µM) FA<sub>1</sub> was more effective in disruption of Sa and So levels in primary hepatocytes than FB<sub>1</sub>. However, studies with liver slices showed that purified FA<sub>1</sub> lacks any inhibition of ceramide synthase (Norred *et al.*, 2001). It was reported that FA<sub>1</sub> is converted to 3-O-acetyl and 5-acetyl FB<sub>1</sub> thereby liberating the free amino group in solution, especially under acidic conditions, which could explain the lack of effect on sphingolipid biosynthesis. Irrespective whether FA<sub>1</sub> underwent rearrangement in the study of Van der Westhuizen *et al.* (1998) the conversion under neutral conditions in saline at 4 °C is not known and highly unlikely to be complete yielding the free amino derivatives as suggested by Norred *et al.*, (2001). This must be considered against the background that a high conversion is noticed under acid conditions. Norred *et al.* (2001) also directly linked the disruption of sphingolipid metabolism in liver slices in which no cytotoxic assessment was conducted to the study in primary hepatocytes where the disruption of sphingolipid metabolism was associated with cytotoxic effects. It would appear that both the hydrolysed FB's and the N-acetyl derivatives lack activity *in vivo* while they either exhibited similar or increased effects on sphingolipid metabolism and cytotoxicity in primary hepatocytes. However, the role of the hydrolysed FB's in the toxicological effects of the fumonisins *in vivo* is therefore controversial and should be re-evaluated.

In contrast to the cancer initiating properties of FB<sub>1</sub> several studies have been conducted to investigate the cancer promoting properties and including *in vitro* studies in primary hepatocytes and cancer cell lines as well as in rats and mice *in vivo*. Studies in rat liver have been described in detail above (Section 4.3) which was based on the resistant hepatocyte model (Solt *et al.*, 1977; Farber, 1990). An additional hypothesis has been developed in the liver and kidney of rats, which was based on a compensatory cell proliferative model for non genotoxic carcinogens (Dragan *et al.*, 2001; Howard *et al.*, 2001a; Riley *et al.*, 2001). Introducing concepts such as oncotic necrosis and apoptotic necrosis further developed the model, both leading to a sustained cell proliferation which will lead to an increased incidence of

tumors in the target tissue. This theoretical model was based on the cell death and compensatory regeneration hypothesis formulated for a variety of non-genotoxic chemical carcinogens in different tissues (Cohen, 1998; Cohen and Ellwein, 1990; Butterworth and Goldsworthy, 1991). The development of a different subset of cells, resistant to the apoptotic necrosis has also been considered to be involved in the genesis of renal tumors induced by FB<sub>1</sub> (Riley *et al.*, 2001). The induction of renal tumors in rats by FB<sub>1</sub> was argued to be an example of apoptotic necrosis regenerative cell proliferation carcinogenesis model. In the liver, however, apoptotic necrosis and oncotic necrosis occur at the same time, which complicates the apoptotic cancer model. However, the high incidence of nephropathy in the kidneys and the resultant formation of many proliferative lesions including atypical tubule hyperplasia (Hard *et al.*, 2004) suggest a possible FB<sub>1</sub>/high protein diet interaction (Chapter III). The study on the carcinogenic effects of FB<sub>1</sub> in female B6C3F1 mice was also complicated by the spontaneous development of hepatocellular adenomas and carcinomas (Howard *et al.*, 2001b). Although no carcinomas developed in the mice due to feed restriction it is not clear whether the increased development of adenomas and carcinomas could be ascribed to the cancer promoting properties of FB<sub>1</sub> rather than being considered as a carcinogen.

The role of sphingolipids in the development of kidney cancer has been reviewed in detail (Merrill *et al.*, 2001; Dragan *et al.*, 2001; Riley *et al.*, 2001) and will not form part of the current discussions. The role of cell proliferation in the development of cancer has also been debated in the past after the questioning of the role of cell proliferation as a risk factor *per se* in the long process of cancer development (Perera *et al.*, 1991; Farber, 1995). During a follow up debate (Letters to Editor) by Butterworth, Cohen, Ames and Ellwein regarding the role of cell proliferation in carcinogenesis it was concluded that, due to the complexity of cancer development, there is no scientific evidence that a sustained proliferation is a risk factor for cancer (Ward *et al.*, 1993; Tomatis, 1993; Weistein, 1993; Farber 1996). As carcinogenesis in rat kidney by FB<sub>1</sub> has not been well characterised regarding the different phases as compared to the liver, the oncotic apoptotic cell proliferative model is debatable, especially against the background of the numerous proliferative tubular lesions due to the development of CPN (Chapter III). This is of particular importance when the



underlying principles of such a model impact on setting risk assessment parameters for the fumonisins, an aspect that will be further debated in Chapter V.

The role of the disruption of lipid metabolism by FB<sub>1</sub>, involving phospholipid and fatty acids in rat liver cancer promotion, has been reviewed extensively (Gelderblom *et al.*, 1996; 2001a, b; 2008). The model was based on studies regarding the role of lipid metabolism in liver cancer development (Abel *et al.*, 2001; 2004 – see addenda 1, 2) during which different lipid parameters were monitored in hepatocyte nodules over a period of 9 months. In short the decrease in the PC/PE phospholipid ratio, impairment of the delta 6-desaturase and a decrease in the oxidative status in hepatocyte nodules were identified as the key components responsible for the altered growth characteristics in these lesions. These changes closely mimicked regenerating liver, which further provides proof that they are associated with cell proliferation. Persistent changes in the nodules include an increase in cholesterol and PE which were associated with an increase in C18:1n-9 and C18:2n-6 while C20:4n-6 decreased in PC and increased in PE resulting in an increase C20:4n-6 PC/PE ratio. The end products of both the n-6 and n-3 FA pathways, C20:5n-6 and C22:6n-3 also decreased in PC resulting in a reduction in the total PUFA and long chain PUFA levels in the nodules. The specific role of these altered FA parameters in the growth of nodules can be summarised as follow:

- The increased and decreased levels of C18:1n-9 and long chained PUFA levels, respectively, render the nodules less susceptible to oxidative stress and hence to undergo apoptosis. C18:1n-9 has been proposed to act as a membranal antioxidant known to be enhanced in many cancer cells (Horrobin, 1994). These lesions were shown to exhibit a low level of lipid peroxidation, the latter, which was increased due to an increased level of PUFA using a diet high in long chain (LC) n-3 PUFA (Abel *et al.*, 2004). The increased level of lipid peroxidation was associated with a decrease in the incidence and sizes of hepatocyte nodules, presumably due to increases their susceptibility to undergo apoptosis.
- The increased C20:4n-6 PE/PC ratio has been implicated in the altered proliferation of hepatocyte nodules due to the role of C20:4n-6 and the prostaglandin E<sub>2</sub> series products via the activation of protein kinase C and the

mitogen activation protein kinases. It has been proposed to be involved in the development of preneoplastic foci via its effect on different growth factors and the deregulation of *c-myc* involving ceramide (Jayadev, *et al.*, 1994).

FB<sub>1</sub>-induced effects in the liver closely mimic the lipid changes in hepatocyte nodules and are therefore likely to selectively stimulate the growth in hepatocyte nodules, suggesting that similar changes in the normal surrounding tissue resulted in an adverse effect on cell viability. Altered lipid changes induced by FB<sub>1</sub>:

- Impaired delta 6-desaturase, leading to the accumulation of C18:1n-6, C18:2n-6 and a reduction in LC PUFA levels in the cell (Gelderblom *et al.*, 2002).
- Increase PE resulting in an increased C20:4n-6 PE/PC ratio. Inhibition of ceramide synthase with accumulation of sphinganine and decrease in sphingomyelin. Changes in these parameters differ in the subcellular fractions with the increased PE noticed in the plasma membrane, mitochondria and microsomes while SM was decreased in mitochondria. The C20:4n-6 PE/PC ratio was altered in all the membranal fractions (Burger *et al.*, 2007).
- Differential effects of these lipid associated changes on the viability of normal and preneoplastic cells provide the basis for cancer promotion. The association of the lipid changes and the selective induction of growth factors and signaling molecules are likely to inhibit cell proliferation and induce apoptosis in normal cells whilst inducing cell proliferation in preneoplastic cells (Lemmer *et al.*, 1999a; Ramljak 2000). This inhibitory effect on cell proliferation was demonstrated by FB<sub>1</sub> in proliferating hepatocytes in normal liver following partial hepatectomy (Gelderblom *et al.*, 1994).
- Effect of FB<sub>1</sub> on cellular oxidative status played an important differential role in the regulation of apoptosis and cell proliferation between normal and neoplastic cells. A recent study showed that FB<sub>1</sub> modulated the cellular redox balance in the liver by altering oxidative enzymes and redox sensitive signaling molecules including the GSH levels, MAPK activation, heat shock proteins, ERK and p38 (Rumora *et al.*, 2007; Marnewick *et al.*, 2009)

Interaction of the fumonisins with cellular membranes has become a major focus point of research to explain the toxicological and carcinogenic effects in experimental animals. A study in model membranes showed that FB<sub>1</sub> interacted with the polar head group region effecting the membrane organization and dynamics, which will impact on the permeability and integral proteins such as the ion channels and lyolytic enzymes (Theumer *et al.*, 2008). Subsequent disruption of sphingolipid, phospholipid and fatty acid metabolism and their underlying interaction are likely to play a determining role in the selection of altered hepatocytes and the development of hepatocellular carcinoma in rat liver. These interactions may occur at several levels that could affect the growth signaling and apoptotic pathways determining cell survival and hence cancer promotion in the liver.

*In vitro* studies utilising oesophageal cancer cells (WHC03) showed that FB<sub>1</sub> interfered with the effects of C20:4n-6 on cell cycle progression, apoptosis and tyrosine- and cyclin D dependent kinase 2 (CDC2-kinase) activity (Seegers *et al.*, 2000). FB<sub>1</sub> inhibited apoptosis induction by C20:4n-6 and PGE<sub>2</sub>, which was associated with a reduction in tyrosine kinase, increase in CDC2-kinase and a reduction in the p53 levels. C20:4n-6-induced apoptosis has been shown to be effected via the modulation of the ceramide levels by increasing the sphingomyelinase activity (Jayadev *et al.*, 1994). Ceramide-induced apoptosis is also dependent on an increased tyrosine kinase activity (Stewart *et al.*, 1995) which was markedly enhanced by C20:4n-6. The latter also enhanced the p53 level, which in turn reduced the level of cyclin B<sub>1</sub> and the activity of CDC2-kinase via the expression of p21. The disruption of ceramide synthase by FB<sub>1</sub> will therefore abrogate the anti-mitotic and apoptotic-inducing effects of C20:4n-6. Additional evidence for the role of C20:4n-6 in the FB<sub>1</sub>-induced effects was obtained from a study in human bronchial epithelial cells (Pinelli *et al.*, 1999). FB<sub>1</sub> increased protein kinase C translocation and mitogen-activated protein kinase (MAPK) resulting in the down regulation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and C20:4n-6 metabolism. Activation of PLA<sub>2</sub> was related to the phosphorylation by MAPK and the increase in sphingosine due to the disruption of ceramide synthase. The activation of MAPK by FB<sub>1</sub> was also demonstrated in Swiss 3T3 fibroblasts, which were related to the stimulation of mitogenesis in these cells (Wattenberg *et al.*, 1996). Apart from the PUFA, the levels of SATS and

MONO-UNSATs, both disrupted by the fumonisins, also plays a determining role in cell survival. Of interest is the study in breast cancer cells indicating that C16:0 and C18:1n-9, increased and decreased phosphatidylinositol 3-kinase (PI3-K), an important modulator of cell proliferation and apoptosis (Hardy *et al.*, 2000). As reported previously, FB<sub>1</sub> activated AKt the activity of which is also regulated by PI3-K (Ramljak *et al.*, 2000). The disruption of these cell cycle dependent pathways and bioactive fatty acids and sphingolipids in cells could play an important role in the selective outgrowth of pre-neoplastic cells *in vivo* and during cancer promotion. A study in Sprague-Dawley rats showed that menhaden oil, high in n-3 fatty acids (C20:5n-3, C22:6n-3), inhibited cancer promotion by FB<sub>1</sub> following DEN initiation (Lee *et al.*, 2000). The protection against cancer promotion was also associated with a decrease in hepatic PGE<sub>2</sub> and PGF<sub>2</sub>α providing further proof for the involvement of these lipid parameters.

The FA/sphingolipid interactive pathways (Figs 3, 4) represent alterations related to membranal changes affecting subsequent resultant signal transduction pathways involved in cell survival. However, these pathways are proposed to exist in the liver, the outcome of which will differ when considering the differential effects on normal and pre-neoplastic cells.

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