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

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Oxidative damage and fumonisin B_1-induced toxicity in primary rat hepatocytes and rat liver in vivo

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Abstract

Dietary fumonisin B_1 (FB_1) levels of 250 and 500 mg FB_1/kg increased the level of thiobarbituric acid reactive substances (TBARS) significantly ($P < 0.05$) in the liver of rats fed FB_1 over 21 days. Levels of 10, 50 and 100 mg FB_1/kg also markedly (not significantly) increased the level of TBARS in the liver homogenate. Subcellular fractionation of the liver of the rats fed the 250 mg FB_1/kg diet, showed a marginally significant increase of TBARS in the plasma membranes ($0.05 < P < 0.1$) and a significant increase in the microsomes ($P < 0.05$). In vitro investigations in primary rat hepatocytes indicated that the level of TBARS was increased in a dose dependent manner associated with an increase in cytotoxicity. Addition of the antioxidant, α-tocopherol, significantly decreased the cytotoxicity whereas the level of TBARS was decreased to basal levels, suggesting that lipid peroxidation is likely to contribute to the cytotoxic effect of FB_1. Addition of cumene hydroperoxide (CMHP) to primary hepatocytes exposed to FB_1 for 44 h, enhanced the CMHP-induced TBARS release suggesting that the hepatocytes exposed to FB_1 are more susceptible to chemically-induced oxidative stress. Free radical production could result in excessive cellular damage and/or metabolic abnormalities that are likely to be involved in FB_1-induced altered growth responses and cell death in primary hepatocytes. The hepatotoxic effects and resultant oxidative damage induced by FB_1 may be important during cancer induction in rat liver by this apparently non-genotoxic compound.

Keywords: Fumonisin B_1; Oxidative damage; Lipid peroxidation; Malondialdehyde; Rat hepatocytes; Rat liver

Abbreviations: AA, arachidonic acid; AIN, American Institute of Nutrition; ANOVA, analysis of variance; BHT, butylated hydroxytoluene; CMHP, cumene hydroperoxide; EGF, epidermal growth factor; FA, fatty acid/s; FB_1, fumonisin B_1; LA, linoleic acid; LDH, lactate dehydrogenase; MDA, malondialdehyde; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid/s; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances.

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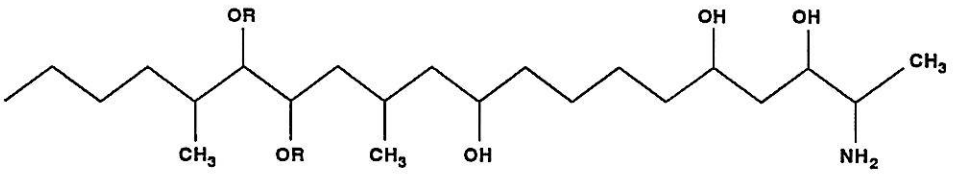
1. Introduction

Fumonisin B_1 (FB_1; Fig. 1), a mycotoxin produced by the fungus *Fusarium moniliforme*, is known to be the causative agent of several diseases in animals (Marasas, 1996). Cancer induction in rat liver is associated with a cytotoxic/proliferative response, as studies have indicated that FB_1-induced hepatotoxicity is a prerequisite for cancer initiation (Gelderblom et al., 1994), while liver cancer develops against a background of a chronic toxic hepatitis (Gelderblom et al., 1991). The changes associated with FB_1-induced hepatotoxicity could, therefore, indirectly lead to events that are involved in the process of cancer induction. At present very little is known about the mechanisms involved in the hepatotoxicity of fumonisins in rats.

Reactive oxygen species (ROS), such as the superoxide anion (Halliwell, 1994) and nitric oxide (Ohshima and Bartsch, 1994), produced within the cell or by activated phagocytes present during inflammation, have been proposed to play an important role in the multistage carcinogenesis process, presumably by damaging and altering target cells. The induction of lipid peroxidation has been implicated as the primary cause of cell death caused by several halogenated hydrocarbons (Halliwell et al., 1992) while it is also associated with cancer induction by the choline deficient (CD) diet in rat liver (Ghoshal and Farber, 1993). In this regard, nuclear lipid peroxidation and subsequent changes to DNA has been proposed to be important during the cancer initiation stage by the CD diet. Apart from genetic damage due to oxidative damage, changes involving the lipid composition of cellular membranes could alter the responsiveness of cells to growth related processes and subsequently contribute to the induction of carcinogenesis (Spector and Burns, 1987). Fumonisins have been shown to disrupt lipid biosynthesis in vitro and in vivo in rat liver with changes occurring in the major cellular membrane phospholipid fractions and their fatty acid (FA) content (Gelderblom et al., 1996a, 1997). Depending on the dietary level, in vivo changes include the accumulation of the membrane phospholipid, phosphatidylethanolamine (PE), and increased levels of free cholesterol. The sphingolipid, sphingomyelin (SM), decreases while the sphingoid base, sphinganine, increases as a result of the FB_1-induced disruption of sphingolipid biosynthesis (Gelderblom et al., 1996b, 1997). FA analyses of PE and PC (phosphatidylcholine) indicated that the total $\omega 6$ FA and the PUFA were significantly reduced. In vitro studies in primary rat hepatocytes (Gelderblom et al., 1996a), showed an increase in the level of linoleic acid (LA) and arachidonic acid (AA) in the major phospholipids and the neutral lipid, triacylglycerol (TAG). In contrast to the in vivo studies, the PUFA increased and the cholesterol level decreased in vitro. The PE concentration also increased, as in the in vivo study. The FB_1-induced changes in the phospholipids and FA biosynthesis could play an important role in the cytotoxic effects of the fumonisins by disruption of the integrity and function of hepatocyte membranes. As the toxic effects of FB_1 seems to be important during cancer induction the aim of the present study was to



R = CO-CH$_2$-CH(COOH)-CH$_2$-COOH

Fig. 1. Structure of fumonisin B_1.

investigate whether FB_1 could effect oxidative damage in the rat liver in vivo and in primary rat hepatocytes in vitro.

2. Materials and methods

2.1. Chemicals and animal treatment

α-Tocopherol (95%; CAS number: 10191-41-0) was obtained from Sigma Chemical Company (St Louis, MI, USA). For the in vitro study FB_1 (98% pure) (Cawood et al., 1991) was dissolved in saline according to the desired concentration and 50 μl quantities added to the hepatocyte culture dishes. The FB_1-containing diets for the in vivo study were prepared by evaporating FB_1 (90–95%) (Cawood et al., 1991), dissolved in methanol, on a sub sample of the AIN-76A diet (200 g). After drying in a fume-cupboard for 4 h, the sub samples were mixed with the control diets to obtain the desired FB_1 levels and stored under nitrogen at 4°C. The FB_1 preparations were purified at PROMEC/MRC, Tygerberg, South Africa.

2.2. Preparation of hepatocyte cultures

Rat hepatocytes were isolated from male Fischer-344 rats (150–200 g body weight) according to the collagenase perfusion technique of Hayes et al. (1984). Cells (viability of > 90%) were plated at a density of 6×10^5 viable cells/culture dish (6 cm) and incubated for 3 h in 6 ml modified Williams E (WE) medium, containing fetal bovine serum (10%), insulin (20 U/l), L-glutamine (2 mM), HEPES (10 mM) and penicillin (100 U/ml). The cells were washed with Hanks buffer solution and finally incubated in 3 ml serum-free modified WE medium containing L-proline (2 mM) and sodium pyruvate (10 mM) in addition to the components described above.

2.3. In vivo experiment in rats

Rats were caged individually in a controlled environment (23–25°C) with a 12 h light–dark cycle, had free access to feed and water and were weighed three times per week. The rats (three animals per group) were fed FB_1 dietary levels of 10, 50, 100, 250 and 500 mg/kg diet for 21 days parallel with a control group fed the basal diet. The 250 mg FB_1/kg dietary and a control group (five rats/group) were repeated in a separate experiment for the preparation of the subcellular fractions. To monitor the effect of feed refusal (Gelderblom et al., 1994) on lipid peroxidation, control rats were pair-fed with the 250 mg FB_1/kg dietary group for 21 days. All the rats were sacrificed after 21 days, the livers were removed, weighed and stored in saline at −80°C for biochemical analyses. The livers from the pair-fed rats were immediately stored in liquid nitrogen after removal.

2.4. Preparation of liver homogenate, membraneous and sub-cellular fractions

Liver homogenates (three rats/group) were prepared by homogenising tissue in 1.15% KCl containing 3 mM EDTA, pH 7.4 to obtain a 10% solution. The subcellular fractions, i.e. plasma membranes, nuclei (Loten and Redshaw-Loten, 1986), mitochondria and microsomes (Shen et al., 1994), were isolated from the liver homogenates of rats (five rats/group) fed the basal and the FB_1 diet (250 mg/kg) and stored at −80°C in 1.15% KCl containing 3 mM EDTA, pH 7.4 until analysed. The EDTA was added to the samples to prevent further occurrence of lipid peroxidation during the isolation and fractionation method (Recknagel and Ghoshal, 1966; Esterbauer and Cheeseman, 1990). Aliquots of the whole liver homogenate and the various cell fractions were subjected to protein determination according to the method of Kaushal and Barnes (1986), using bovine serum albumin as standard.

2.5. In vitro FB_1-induced effects on TBARS generation

Three different experiments were carried out in primary hepatocyte cultures:

(a) Primary hepatocytes were treated with 75, 150, 250 and 500 μM FB_1 for 44 h after which the growth medium was removed and the cells

washed (three times) with ice cold saline. The cells were harvested into 3 ml saline and an aliquot (100 μl) removed for protein determination (Kaushal and Barnes, 1986) while the remainder was subjected to TBARS analyses (see below). Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH) according to the method of Hayes et al. (1984).

(b) The effect of α-tocopherol on FB_1-induced TBARS formation and cytotoxicity in primary hepatocytes was similarly monitored. The hepatocytes were treated with different concentrations (75, 150 and 250 μM) of FB_1 in the presence and absence of 10 μM α-tocopherol for 44 h and the cytotoxicity and the level of TBARS formation determined.

(c) The effect of cumene hydroperoxide (CMHP)-induced TBARS production in hepatocytes incubated with and without FB_1 was also monitored. Hepatocytes were incubated with 75 and 150 μM FB_1 for 44 h after which 10 μl of 0.1 mM CMHP (0.11 μmol/1 ml incubation medium) was added to both the FB_1 treated and untreated hepatocytes for 5 min and the TBARS determined.

Eight culture dishes were used per treatment for each of the three above mentioned experiments and, for the analyses, two of the eight culture dishes were combined to give four samples per treatment.

2.6. Determination of TBARS

A mixture of 2 ml of the hepatocyte suspension (in saline) and 2 ml of the thiobarbituric acid (TBA) reagent consisting of 20% trichloroacetic acid (TCA), 0.67% TBA and 0.01% butylated hydroxytoluene (BHT) was incubated at 90°C for 20 min in a water bath (Kinchington et al., 1993). For the in vivo experiments, 1 ml of the liver homogenate and 1 ml from the various subcellular fractions (0.1–2.0 mg protein/ml) were mixed with 2 ml of a cold 10% TCA solution containing 0.01% BHT, respectively. The samples were centrifuged (3000 rpm), 2 ml of the supernatant was combined with 2 ml of a 0.67% TBA solution and heated for 10 min in boiling water. The mixture was allowed to cool and the absorbance measured at 532 nm (Esterbauer and Cheeseman, 1990). Lipid peroxidation was expressed as nmole MDA equivalents per mg protein using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ at 532 nm for MDA (Buege and Aust, 1978). The determination of MDA as described above is known not to be specific for MDA as various substances such as sucrose, metal ions and whole tissue homogenates may also react with TBA or influence the assay procedure, therefore the term thiobarbituric acid reacting substances (TBARS) is used to describe the reaction product in the assay (Esterbauer and Cheeseman, 1990). In the present study whole tissue homogenates were used but subsequent experiments were performed on membrane and sub-cellular fractions to confirm MDA formation. Non-specific lipid peroxidation was prevented by the incorporation of EDTA in the buffers and BHT in the reaction solutions for the TBARS assay.

2.7. Statistical analyses

All statistical analyses (ANOVA) were performed with the statistical analysis system (SAS) program. The Tukey *T*-test was used to determine whether the means of the treatment groups differed significantly. The Wilcoxon rank sum test was used to determine whether α-tocopherol significantly affected the cytotoxicity and level of TBARS in FB_1 treated and control hepatocytes (Fig. 3). The same procedure was used to determine whether the TBARS levels in the sub-cellular and membraneous fractions in FB_1-treated (250 mg/kg diet; Fig. 6) rats differed significantly from the relevant controls. The data used for statistical analyses were the means of three separate experiments for the in vitro studies and two separate experiments for the in vivo studies.

3. Results

3.1. Effect of FB_1 on body weight gain, relative liver weight and feed intake

The effect of FB_1 feeding on body weight gain, relative liver weight and feed intake has been

described in detail elsewhere (Gelderblom et al., 1994). The body weight gain and relative liver weight decreased significantly in the rats fed the 250 mg FB_1/kg diet for 21 days compared to the control group fed the basal diet. The reduction in body weight gain was accompanied by a reduced feed and FB_1 intake. No significant effects on the body weight gain, relative liver weight and feed intake of the rats fed 10, 50 and 100 mg FB_1/kg diet were observed. The relative liver weight of the pair-fed rats receiving 250 mg FB_1/kg diet was significantly ($P < 0.05$) reduced, while the body weight gain was also markedly reduced (data not shown).

3.2. In vitro studies in primary hepatocytes

3.2.1. FB_1-induced LDH release and TBARS generation

FB_1 induced a cytotoxic dose response effect in the primary hepatocytes as determined by LDH release in the incubation medium. A significant ($P < 0.01$) increase in LDH release above the control cultures occurred at all the FB_1 concentration levels tested (Fig. 2A). The TBARS levels were significantly increased in a dose dependent manner in the hepatocytes exposed to 75 μM ($P < 0.05$) and 150, 250 and 500 μM FB_1 ($P < 0.01$; Fig. 2B).

3.2.2. Effect of α-tocopherol on LDH release and level of TBARS

α-Tocopherol (10 μM) significantly decreased the LDH release in the hepatocytes treated with FB_1 (75 μM: $P < 0.05$; 150 μM: $0.05 < P < 0.1$; 250 μM: $P < 0.05$; Fig. 3A). The same effect was obtained with the TBARS levels in the hepatocytes treated with FB_1 (Fig. 3B; FB_1 75 μM: $P < 0.01$; FB_1 150 μM: $P < 0.05$ and FB_1 250 μM: $P < 0.01$). No significant differences were observed in the TBARS levels between the α-tocopherol treated and untreated control cultures. The magnitude of inhibition of LDH release by α-tocopherol was not reflected to the same extent when compared to the TBARS production.

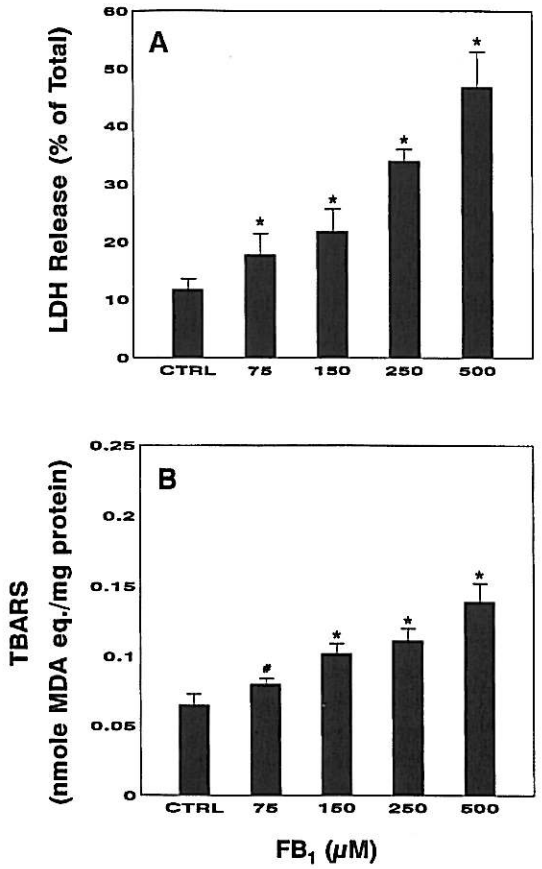


Fig. 2. Dose-response of LDH release (A) and TBARS generation (B) in vitro as a function of FB_1 concentration. Hepatocytes were incubated with various concentrations of FB_1 for 44 h. Data are presented as the means and S.D. Significant differences from the control are shown by * $P < 0.01$ and # $P < 0.05$.

3.2.3. Effect of cumene hydroperoxide

The TBARS level in the hepatocyte cultures treated with CMHP (0.11 μmole/ml) in the absence and presence of FB_1, were significantly ($P < 0.01$) increased as compared to the hepatocytes not treated with CMHP (Fig. 4). The addition of CMHP to the FB_1 treated hepatocytes (75 and 150 μM), significantly ($P < 0.01$) increased the TBARS level above the control treated only with CMHP. The difference between the controls ± CMHP (Δ^1) and 75 μM FB_1 treated hepatocytes ± CMHP (Δ^2) was statistically significant ($P < 0.05$; Fig. 4 insert). The effect obtained with 150 μM FB_1 ± CMHP (Δ^3) was only slightly (not

significant) higher than Δ^1, as FB_1 also significantly enhanced the production of TBARS and therefore reduced the lipid peroxidative capacity of the hepatocytes to CMHP exposure which was only added 44 h after the FB_1.

3.3. *In vivo studies in rats*

3.3.1. *Liver homogenates*

The TBARS level was significantly increased ($P < 0.05$) in the liver of the rats fed the FB_1 dietary level of 250 and 500 mg FB_1/kg diet (Fig. 5). No significant increase was observed with the 10, 50 and 100 mg FB_1/kg diets, although the TBARS levels were raised. Comparison of the TBARS levels of the liver homogenates of pair-fed rats fed the basal diet and 250 mg FB_1/kg for 21 days also showed a significant increase ($P < 0.01$) in the FB_1 fed rats (data not shown). This experiment was performed to monitor the effect of feed refusal induced by FB_1 on lipid peroxidation as it has been shown that feed restriction reduces the level of lipid peroxidation in the liver (Hart and Turturro, 1997). Comparative TBARS analyses of the liver samples frozen in saline as well as frozen in liquid nitrogen did not show up any significant differences between the two storage methods.

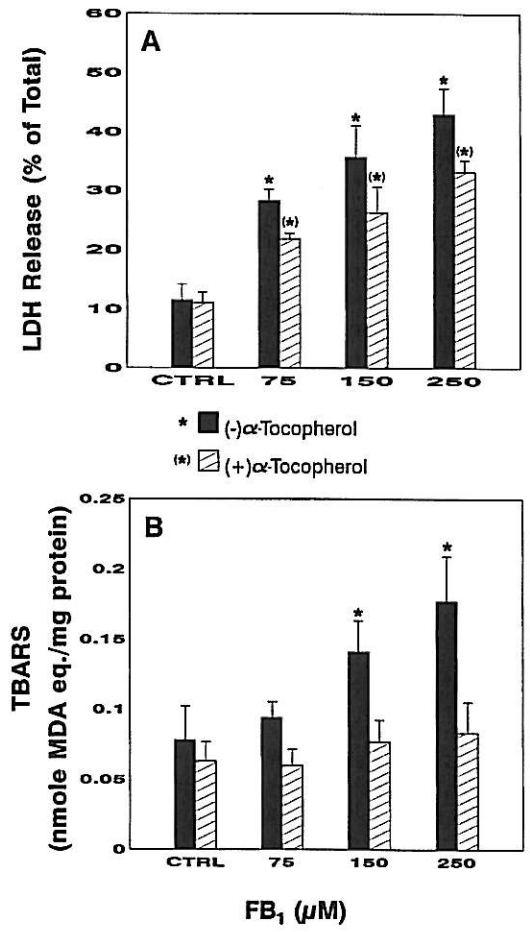


Fig. 3. Dose-response of LDH release (A) and TBARS generation (B) in vitro as a function of FB_1 concentration with and without the addition of α-tocopherol. Hepatocytes were incubated with various concentrations of FB_1 and 10 μM α-tocopherol for 44 h. Data are presented as the means and SD. Significant differences (Tukey *T*-test) from the control are shown by * $P < 0.01$.

3.3.2. *Membraneous and sub-cellular fractions*

Significant to marginally significant increases in the TBARS level occurred in the microsomal ($P < 0.05$) and plasma membranal ($P < 0.10$) fractions of the livers of the rats fed the 250 mg FB_1/kg diet for 21 days (Fig. 6). Although increased TBARS levels were observed in the nuclei and mitochondria, it was not significant ($P > 0.10$).

3.3.3. *Histopathological changes*

The pathological changes induced by FB_1 have been described in detail by Gelderblom et al. (1991). In short, morphologically altered hepatocyte foci and early signs of fibrosis occurred in the liver of the rats fed the 250 and 500 mg FB_1/kg diets, while early signs of nodular regeneration were seen with the 500 mg FB_1/kg dose. In the 100 mg FB_1/kg dose group, mild changes were observed such as single cell necrosis and duct epithelial cell proliferation. Only a few necrotic cells were observed in the 50 mg FB_1/kg group. No specific lesions were detected in the liver of the rats that received the 10 mg FB_1/kg diet (Gelderblom et al., 1991).

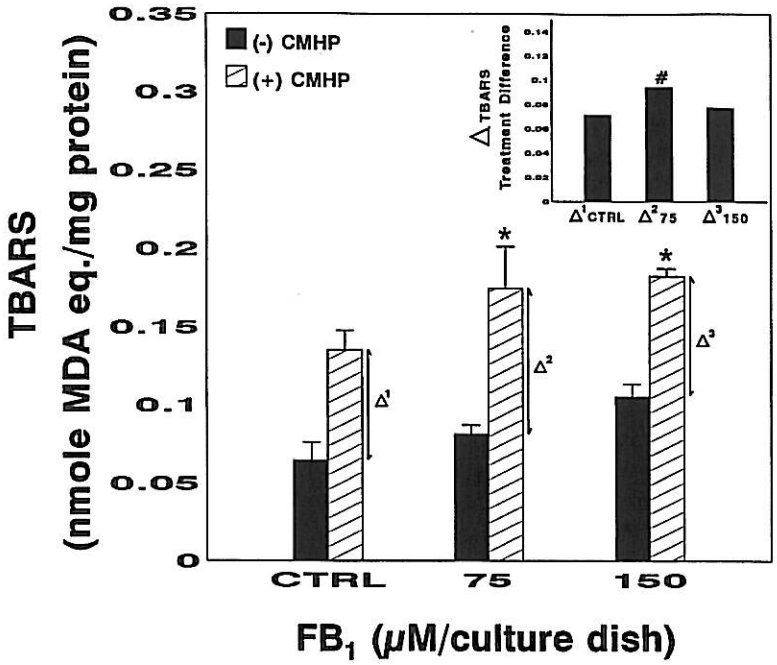


Fig. 4. Generation of TBARS in vitro in FB_1 (75 and 150 μM) incubated (44 h) hepatocytes treated with CMHP (0.11 μmole/ml) for 5 min. Data are presented as the means and SD. Significant differences (Tukey *T*-test) from the relevant control are shown by * $P < 0.01$. Insert: The delta (Δ) significant difference (Tukey *T*-test) from the control is shown by # $P < 0.05$.

4. Discussion

Free radicals, more specifically ROS, are continuously produced within the cell as a result of normal physiological processes and many of them are required for certain biological reactions (Halliwell, 1994; Martínez-Cayuela, 1995). An overproduction of these free radicals and/or the deactivation of the cellular defense mechanisms involved in free radical protection, will result in an overall rise in their concentrations and eventually give rise to cellular damage. Many factors, including toxins, have the ability to interfere with free radical related processes, thereby inducing oxidative stress in the target cells which could lead to metabolic and cellular disturbances mainly due to the damage of membrane lipids and proteins, DNA, and carbohydrates (Martínez-Cayuela, 1995). MDA, commonly used as an indicator for lipid peroxidation, is known to crosslink and polymerize membrane components and react with DNA nitrogenated bases forming MDA–DNA adducts (Vaca and Harms-Ringdahl, 1989; Chaudhary et al., 1994). Except for the halogenated hydrocarbons such as carbon tetrachloride and bromobenzene that probably cause cell injury by the induction of lipid peroxidation, oxidative damage and lipid peroxidation seem to occur as a result of cell injury induced by toxins (Halliwell et al., 1992).

The present investigation demonstrated that FB_1-induced cytotoxicity, measured as the release of LDH in the growth medium (Fig. 2A), is associated with an increase in TBARS levels in a dose dependent manner in primary hepatocytes (Fig. 2B). Addition of the antioxidant, α-tocopherol, known to interfere with the subsequent formation and breakdown of cyclic endoperoxides to MDA (Gavino et al., 1981; Halliwell, 1994; Sugihara et al., 1995), to FB_1-treated hepatocytes inhibited TBARS production, thereby preventing lipid peroxidation (Fig. 3B). However, the α-tocopherol did not completely counteract the cytotoxicity of FB_1, although LDH release was

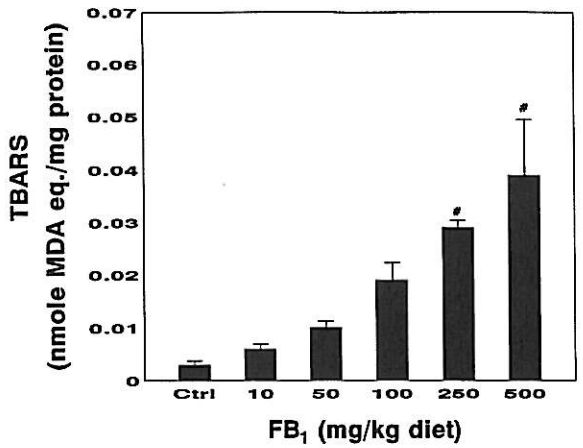


Fig. 5. Dose-response of TBARS generation in vivo from liver homogenate as a function of dietary FB_1 concentration in rats fed for 21 days. Data are presented as the means and S.D. Significant differences from the control are shown by $^{\#}$ $P <$ 0.05.

significantly ($P < 0.05$) decreased (Fig. 3A). This implies that lipid peroxidation is not solely responsible for the cytotoxic effects of FB_1 and, presumably, plays a secondary role as a result of cell injury. It is known that FB_1 is not metabolised in primary hepatocytes or by rat liver

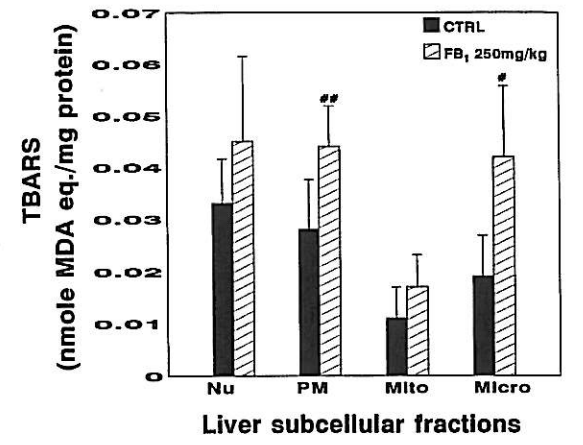


Fig. 6. TBARS generation in vivo in liver subcellular fractions (Nu, nuclei; PM, plasma membranes; Mito, mitochondria; Micro, microsomes) from the liver of rats fed 250 mg FB_1/kg dietary feed for 21 days and control rats fed the basal diet. Data are presented as the means and S.D. Significant differences from the control are shown by $^{\#}$ $P < 0.05$ and $^{\#\#}$ $P <$ 0.10.

microsomal preparations (Cawood and Gelderblom, 1994), suggesting that the compound is not converted, de novo, to an active toxic metabolite.

Lipid peroxidation causes cellular alterations and specifically alters membranal lipids, thereby affecting membrane fluidity which can lead to the loss of membrane function, impairment of membrane-bound enzymes and the destabilisation of membranal receptors (Winrow et al., 1993). A recent in vitro study showed that FB_1 affects the composition of membrane phospholipids as well as the FA profiles of phospholipids and triglycerides in primary hepatocytes treated with non- and cytotoxic FB_1 dosages (Gelderblom et al., 1996a,b). These changes suggest that the alteration of cellular membranes by FB_1 could be an important determinant in the responsiveness of hepatocytes to growth stimulatory responses. It has also been shown that FB_1 inhibits cell proliferation in many cell types (Tolleson et al., 1996) as well as the epidermal growth factor (EGF) mitogenic response in primary hepatocytes (Gelderblom et al., 1995), indicating that growth regulatory responses are altered. In addition, changes to the FA profiles of the major phospholipids also resulted in the accumulation of PUFA. As the addition of PUFA to both normal and cancer cells, has been associated with an increase in lipid peroxidation (Gavino et al., 1981; Begin et al., 1992), the accumulation of PUFA was suggested to be important in the cytotoxic effects of the fumonisins in primary hepatocytes. Long-chained unsaturated FA have also been shown to increase the susceptibility of hepatocytes to undergo lipid peroxidation thereby facilitating cell injury (Sugihara et al., 1995). With the addition of CMHP to the hepatocytes treated with 75 μM FB_1, a significant increase in the TBARS level was noticed over and above the TBARS level observed in hepatocytes treated with either CMHP or FB_1 alone (Fig. 4). This implies that FB_1-exposed hepatocytes are more susceptible to undergo lipid peroxidation and therefore cellular damage.

The in vivo data supports the in vitro findings in that lipid peroxidation occurred in a dose dependent manner in the liver of rats fed different dietary levels of FB_1. Lipid peroxidation was

closely associated with the hepatotoxicity of FB_1 as there was only a slight increase (not significant) in TBARS production with the occurrence of only mild hepatotoxic effects i.e. in the liver of the rats fed the 50 and 100 mg FB_1/kg diets (Fig. 5). In the livers of the rats fed the 250 and 500 mg FB_1/kg diets, the TBARS level increased dose dependently, presumably as a result of advanced hepatotoxic effects such as necrosis, fibrosis and nodular regeneration. The feed refusal effect of FB_1 at these dietary levels does not seem to affect the oxidative changes, although it is known that it is likely to reduce lipid peroxidation (Hart and Turturro, 1997). FA analyses of the major phospholipids in the liver of rats fed a 250 mg FB_1/kg diet for 21 days has shown that, in contrast to the in vitro experiments, PUFA levels were decreased (Gelderblom et al., 1997). The decreased PUFA in the liver of these rats and the increased TBARS level in the whole liver and subcellular fractions (plasma and microsomal membranes) are indicators that lipid peroxidation occurred in the liver. Sevanian and Hochstein (1985) reported a similar correlation between decreased PUFA levels and increased TBARS levels as indicators of lipid peroxidation.

Short-term carcinogenesis studies in rat liver have indicated that FB_1 is a slow cancer initiator and is dependent on the induction of a cytotoxic/proliferative effect. Cancer initiation only occurred in the rats fed the 250 mg FB_1/kg dietary level for 14–21 days in the presence of advanced toxic lesions (Gelderblom et al., 1994). It is therefore probable that an increase in free radicals induced by FB_1 cytotoxicity can overwhelm or deplete the antioxidant defense mechanisms thereby leading to oxidative stress. In this regard, a study in pig kidney cells indicated that FB_1 significantly reduced the levels of glutathione resulting in changes in the glutathione redox cycle status (Kang and Alexander, 1996) and therefore a reduced cytoprotective role against FB_1 toxicity. A study performed in monkey kidney cells indicated that FB_1-induced lipid peroxidation, depending on the concentration used, inhibits the synthesis of macromolecules, protein and DNA (Abado-Becognee et al., 1998). It was suggested that the oxidative damage induced by FB_1 might indirectly lead to mutagenicity and genotoxicity. The increase in lipid peroxidation in the subcellular fractions of the FB_1-treated liver in the present study (plasma membrane, microsomes and also to some extent in the nuclei and mitochondria; Fig. 6) could be an attractive hypothesis for oxidative DNA damage induced by this non-genotoxin.

Some similarities exist in rat liver when using the CD diet, as a toxic effect preceded cancer initiation which occurred after 9 weeks (Ghoshal et al., 1987). As in the case of FB_1 (Gelderblom et al., 1996b), studies have shown that a CD diet initiates hepatocellular carcinogenesis in the liver with the induction of resistant hepatocytes (Rushmore et al., 1987). The exact kinetics of lipid peroxidation and cell death in relation to FB_1-induced cancer initiation still needs to be elucidated. Recent studies have indicated that oxidative damage is also implicated in the carcinogenicity of aflatoxin B_1, as shown in in vitro experiments in primary hepatocytes and in vivo in rat liver. Aflatoxin B_1 exposure has been associated with the formation of 8-hydroxydeoxyguanosine, a DNA adduct commonly used as a biomarker for oxidative damage (Shen et al., 1995a,b). Apart from its possible role during cancer initiation, oxidative stress is also known to cause apoptosis in many cell types and a recent study indicated that a product of lipid peroxidation induces apoptosis in macrophages (Li et al., 1996). Fumonisins has been shown to induce apoptosis in vivo in the liver and in many cell types in vitro (Tolleson et al., 1996).

The present data suggests that FB_1 causes lipid peroxidation secondary to cytotoxic effects while it also increases the susceptibility of cells to undergo lipid peroxidation. The subsequent interaction between the products of lipid peroxidation and cellular constituents such as DNA, in an environment of regenerative cellular proliferation is likely to effect the processes of cancer initiation and apoptosis. Ongoing cell death, the continued production of ROS, together with the resultant compensatory cellular proliferation, may all be important events in creating conditions ideal for cancer promotion and the subsequent development of neoplasia. The mechanisms involved in cell injury, cancer initiation and apoptosis are not

known, but the present in vitro and in vivo studies indicate that oxidative stress as a result of FB_1 treatment and the subsequent lipid peroxidation could be important mediators for the induction and/or modulation of these processes in the liver.

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ORIGINAL ARTICLE

Altered Lipid Parameters in Hepatic Subcellular Membrane Fractions Induced by Fumonisin B_1

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Abstract Alteration of lipid constituents of cellular membranes has been proposed as a possible mechanism for cancer promotion by fumonisin B_1 (FB_1). To further investigate this hypothesis a dietary dosage which initiates and promotes liver cancer (250 mg FB_1/kg) was fed to male Fischer rats for 21 days and the lipid composition of plasma, microsomal, mitochondrial and nuclear subcellular fractions determined. The effect of FB_1 on the cholesterol, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), as well as sphingomyelin (SM) and the phospholipids-associated fatty acid (FA) profiles, were unique for each subcellular membrane fraction. PE was significantly increased in the microsomal, mitochondrial and plasma membrane fractions, whereas cholesterol was increased in both the microsomal and nuclear fraction. In addition SM was decreased and increased in the mitochondrial and nuclear fractions, respectively. The decreased PC/PE and polyunsaturated/saturated (P/S) FA ratio in the different membrane fractions suggest a more rigid membrane structure. The decreased levels in polyunsaturated fatty acids in PC together with a pronounced increase in C18:1ω9 and C18:2ω6 were indicative of an impaired delta-6 desaturase. The increased ω6/ω3 ratio and decreased C20:4ω6 PC/PE ratio due to an increase in C20:4ω6 in PE relatively to PC in the different subcellular fractions suggests a shift towards prostanoid synthesis of the E2 series. Changes in the PE and C20:4ω6 parameters in the plasma membrane could alter key growth regulatory and/or other cell receptors in lipid rafts known to be altered by FB_1. An interactive role between C20:4ω6 and ceramide in the mitochondria, is suggested to regulate the balance between proliferation and apoptosis in altered initiated hepatocytes resulting in their selective outgrowth during cancer promotion effected by FB_1.

Keywords Fumonisin B_1 · Microsomes · Mitochondria · Plasma membrane · Nuclei · Phospholipids · Fatty acids · Cholesterol · Sphingomyelin · Hepatocarcinogenesis

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Introduction

The structure, function and integrity of biological membranes are governed by the lipid composition of its bilayer [1–3]. Membranes show an asymmetric bilayer of aminophospholipids, such as phosphatidylethanolamine (PE) and phosphatidylserine located in the inner leaflet, whereas phosphatidylcholine (PC)

and the sphingolipid, sphingomyelin (SM), are located in the outer leaflet [4, 5]. The membrane structure and dynamics are important in maintaining cellular function that regulates signalling pathways related to cellular homeostasis [6, 7]. The role of cellular membranes during carcinogenesis has become more prominent and forms a part of subtle changes underlying epigenetic events [8, 9]. Isolated membrane fractions from tumour cells demonstrated alterations in their composition, structure and organization and thus in their functional properties [10, 11].

Fumonisin B_1 (FB_1), a natural occurring mycotoxin with cancer promoting properties produced by the fungus *Fusarium verticillioides* in maize [12]. FB_1 causes several diseases in animals and is associated with a high incidence of human oesophageal and liver cancer in certain geographical areas in the world [13, 14], and the development of neural tube defects [15]. FB_1 is hepatotoxic and hepatocarcinogenic [16, 17] when chronically fed to rats and effects both cancer initiation and promotion properties in a short-term rat liver carcinogenesis model [18, 19]. This model provides an excellent opportunity to study the mechanisms associated with cancer induction by this ''apparent'' non-genotoxic compound as it lacks genotoxicity in the *Salmonella* mutagen and DNA repair assays [20–22]. However, FB_1 induces oxidative damage [23] and exhibited clastogenic properties [24], suggesting that the compound could either directly or indirectly, induce DNA damage. Short-term studies utilizing a cancer initiating/promoting model in rat liver indicated that FB_1 closely mimics the characteristics of other genotoxic carcinogens with respect to initiation [18]. With respect to cancer promotion, evidence supports a hypothesis that FB_1 effects a growth differential, during which initiated hepatocytes proliferate in an environment where the growth of normal cells is inhibited [25]. This became evident as FB_1 inhibits the epidermal growth factor (EGF) stimulatory response in primary hepatocytes in vitro [26] and hepatocyte regeneration following partial hepatectomy in vivo [18] suggesting that FB_1 induces a growth differential similar to most cancer promoters [27, 28].

The disruption of lipid metabolism and the subsequent affect on membrane integrity and function has been proposed as a possible mechanism for cancer promotion by fumonisin B_1 [25, 29, 30]. The present study described the effect of FB_1 on the lipid profiles of different rat hepatic subcellular membrane fractions including the microsomes, mitochondria, plasma membrane and the nuclei. The possible role of these changes during cancer promotion of FB_1 in rat liver is critically evaluated.

Materials and Methods

Chemicals and Reagents

Fumonisin B_1 was extracted and purified (>90%) according to the method described by Cawood et al. [31]. Fatty acid (FA) analytical standards (C14:0 to C24:1), used for calibration and identification, were obtained from Sigma Chemical Company (St Louis, MO, USA). All the chemical solvents were of analytical grade and glass distilled prior to use.

Animals and Diets

Male Fischer rats (150 g body weight) were fed a modified AIN-76 diet after weaning [18] and housed individually in a controlled environment (23–25 °C) with a 12 h light/dark cycle with free access to feed and drinking water. FB_1 was dissolved in methanol prior to application on a subsample (200 g) of the AIN-76A diet and dried overnight. The subsample was mixed with the standard diet to obtain a concentration of 250 mg FB_1/kg, a dose that both initiates [18] and promotes [19] cancer in rat liver. The diet was stored under nitrogen at 4 °C. A control diet was prepared in the same way using an equal volume of methanol. The control and FB_1-containing diets were fed to the rats over a 21-day period. Following the feeding regimen, animals were sacrificed under sagatal anaesthesia after which the livers were harvested and stored in saline at –80 °C.

Preparation of Membrane Subcellular Fractions

Membrane subcellular fractions were isolated at 4 °C according to the method of Bartoli et al. [32] and Loten and Redshaw-Loten [33] with modifications. In short, the livers were homogenized in a buffer containing 250 mM sucrose, 10 mM Tris–HCl, 1 mM EDTA (pH 7.4) and centrifuged at 1,500*g* for 10 min. Both the supernatant (S1) and the pellet (P1) were retained for further isolation of the different subcellular membrane fractions. The supernatant (S1) was centrifuged at 18,000*g* for 10 min to obtain the mitochondrial subcellular pellet (P2) whilst the microsomal fraction remained in the supernatant (S2). The microsomal subfractions were subsequently collected from the S2 fraction by ultra centrifugation at 105,000*g* for 60 min. The mitochondrial and microsomal subcellular fractions were suspended in 10 mM Tris–HCl buffer (pH 7.4) and centrifuged at 18,000*g* for 10 min and 105,000*g* for 30 min, respectively. All the subcellular fractions were stored at –80 °C until analysed.

Liver nuclear and plasma membrane subcellular fractions were prepared by fractionating the pellet (P1) on a self-forming Percoll (Sigma Chemical Co., St Louis, MO, USA) gradient by centrifugation at 35,000*g* for 20 min. By retaining the top layer and applying another Percoll gradient (45,000*g* for 30 min), the subsequent top and bottom layers, respectively, yielded the plasma membrane and nuclear fractions. The collected fractions were centrifuged in 10 mM Tris–HCl buffer and stored at –80 °C prior to analysis. The protein concentration of the different fractions was determined according to the method of Kaushal and Barnes [34].

Lipid Analyses

The different subcellular membrane fractions were subjected to detailed lipid analysis; these included PC, PE, SM, total cholesterol and FA profiles of PC and PE. In short, the different subcellular fractions (1–2 mg protein/ml) were extracted with chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene as anti-oxidant [35, 36] and stored under nitrogen until analysed.

The sample extracts were analysed for cholesterol and subsequently for phospholipids by thin layer chromatography on 20 × 20 silica plates [37] using chloroform–methanol–petroleum ether–acetic acid–boric acid (40:20:30:10:1.8, v/v/v/v/w) as developing solvent. Plates were developed for 90 min at room temperature followed by drying under N_2 gas for 30 min, and the respective phospholipid concentrations and FA content determined.

Phospholipids and Cholesterol

The phospholipid concentrations were quantified colourimetrically with malachite green dye [38] following digestion in saturated perchloric acid at 170 °C for approximately 1 h. Total cholesterol was determined on aliquots of the original lipid extract by an enzymatic iodide method [39] with cholesterinoxidase and cholesterinesterase (Boehringer Mannheim, South Africa).

Fatty Acids

The PC and PE fractions were transmethylated with 2.5 ml methanol:18 M sulphuric acid (95:5, v/v) at 70 °C for 2 h as described by Tichelaar et al. [40]. The resultant FA methyl esters (FAME) were analysed on a Varian 3700 Gas Chromatograph equipped with 30 m fused silica megabore DB-225 columns of 0.53 mm internal diameter (J & W Scientific, Cat. No. 25-2232). The individual FAME were identified by comparison of the retention times with those of a standard mixture of free FA C14:0 to C24:1 and quantified with an internal standard (C17:0) as μg FA/mg protein.

Statistical Analyses

For comparisons to test whether two independent diet groups with normal distributions had the same mean, the *t*-test was used. The pooled method was used if variances were equal, and the Satterthwaite method for unequal variances. Otherwise the non-parametric Wilcoxon Rank Sum test was used to compare the two independent diet groups with numerical observations that were not normally distributed.

Results

Cholesterol and Phospholipid Content

The FB_1 treatment significantly increased the cholesterol content in the microsomal ($P = 0.0001$) and nuclear ($P = 0.05$) subcellular fractions, while no significant differences were observed in the mitochondrial and plasma membrane subcellular fractions. The phospholipid, PC was significantly increased in the microsomal ($P = 0.003$) and decreased in the mitochondrial ($P = 0.026$) fractions, respectively. PE was significantly increased in the microsomal ($P = 0.0001$), mitochondrial ($P = 0.026$), plasma membrane ($P = 0.048$) fractions. The SM content was significantly decreased and increased in the mitochondrial ($P = 0.013$), and nuclear ($P = 0.01$) fractions, respectively, in the FB_1-treated rats (Table 1).

Membrane Lipid Parameters

No significant change in the cholesterol/phospholipid (PC + PE) ratio was observed in any of the fractions of the FB_1-treated rats (data not shown). In contrast, a significant decrease in the PC/PE ratio was observed in the microsomal ($P = 0.005$), mitochondrial ($P = 0.005$) subcellular fractions of the FB_1-treated rats, mainly due to an increase in the phospholipid, PE (Table 1).

The polyunsaturated/saturated (P/S) FA ratio was significantly decreased in PC of the microsomal ($P = 0.039$); mitochondria ($P = 0.024$) and plasma membrane ($P = 0.015$) fraction. In PE, the P/S ratio was only decreased in mitochondrial ($P = 0.035$) and plasma membrane ($P = 0.001$) fractions.

Table 1 The effect of FB_1 treatment on the lipid and FA parameters of different membrane subcellular fractions from rat liver

Lipid and FA parameters	Control				FB_1 treated			
	Microsomes	Mitochondria	Plasma membrane	Nuclei	Microsomes	Mitochondria	Plasma membrane	Nuclei
Cholesterol (μg/mg protein)	23.53 ± 1.53	30.24 ± 3.22	34.03 ± 5.60	2.76 ± 0.82	**35.37 ± 3.48 (*P* = 0.0001)**	31.36 ± 4.26	37.99 ± 4.13	**8.31 ± 4.50 (*P* = 0.05)**
PC (μg/mg protein)	181.00 ± 34.50	276.20 ± 34.10	153.00 ± 25.90	24.89 ± 4.55	**246.20 ± 21.50 (*P* = 0.003)**	**227.40 ± 30.40 (*P* = 0.026)**	157.90 ± 38.10	22.94 ± 1.67
PE (μg/mg protein)	53.10 ± 9.40	85.10 ± 9.70	45.60 ± 12.30	9.70 ± 0.97	**122.80 ± 13.10 (*P* = 0.0001)**	**122.30 ± 9.50 (*P* = 0.026)**	**70.20 ± 21.20 (*P* = 0.048)**	10.40 ± 2.31
SM (μg/mg protein)	10.80 ± 2.50	12.90 ± 2.50	7.40 ± 3.10	3.25 ± 0.72	9.80 ± 3.00	**8.8 0 ± 2.20 (*P* = 0.013)**	8.10 ± 2.90	**7.95 ± 2.94 (*P* = 0.01)**
PC/PE	3.23 ± 0.40	3.11 ± 0.50	2.80 ± 1.69	2.77 ± 0.58	**1.92 ± 0.30 (*P* = 0.005)**	**1.76 ± 0.22 (*P* = 0.005)**	2.24 ± 0.66	3.35 ± 0.18
P/S ratio								
PC	1.25 ± 0.12	1.24 ± 0.06	1.25 ± 0.10	1.12 ± 0.10	**1.12 ± 0.12 (*P* = 0.039)**	**1.10 ± 0.11 (*P* = 0.024)**	**1.07 ± 0.09 (*P* = 0.015)**	1.03 ± 0.07
PE	1.33 ± 0.07	1.55 ± 0.17	1.55 ± 0.05	1.31 ± 0.40	1.40 ± 0.11	**1.35 ± 0.10 (*P* = 0.035)**	**1.38 ± 0.06 (*P* = 0.001)**	1.25 ± 0.08
PUFA								
PC (μg/mg protein)	55.27 ± 7.03	66.14 ± 4.00	73.96 ± 18.31	6.40 ± 0.70	55.79 ± 5.02	**49.32 ± 1.50 (*P* = 0.0001)**	**50.99 ± 10.93 (*P* = 0.05)**	**5.17 ± 0.87 (*P* = 0.04)**
PC (% of total FA)	50.81 ± 1.50	50.52 ± 0.95	50.43 ± 2.21	46.60 ± 2.95	**46.21 ± 1.79 (*P* = 0.001)**	**45.81 ± 1.50 (*P* = 0.0001)**	**45.70 ± 1.69 (*P* = 0.002)**	44.99 ± 1.50
PE (μg/mg protein)	22.56 ± 4.60	31.35 ± 6.10	30.31 ± 5.64	2.42 ± 0.70	**36.45 ± 5.18 (*P* = 0.001)**	35.32 ± 3.02	33.20 ± 8.68	2.61 ± 0.78
PE (% of total FA)	52.71 ± 1.09	56.08 ± 2.40	55.89 ± 1.14	48.10 ± 8.04	53.24 ± 0.79	**52.70 ± 1.23 (*P* = 0.012)**	**53.09 ± 0.84 (*P* = 0.001)**	48.30 ± 2.86
$\omega6/\omega3$ ratio								
PC	23.61 ± 2.16	24.76 ± 2.26	21.51 ± 1.14	24.75 ± 4.47	27.73 ± 5.33	28.16 ± 5.10	**27.25 ± 3.74 (*P* = 0.01)**	29.96 ± 2.97 (*P* = 0.08)
PE	11.15 ± 1.33	10.57 ± 1.58	9.86 ± 1.19	15.62 ± 4.10	11.51 ± 2.82	11.12 ± 2.38	11.57 ± 2.13	16.63 ± 3.80
C20:4 $\omega6$ PC/PE ratio								
μg/mg protein	3.01 ± 0.73	2.66 ± 0.52	2.49 ± 1.30	3.20 ± 1.12	**1.64 ± 0.16 (*P* = 0.005)**	**1.49 ± 0.11 (*P* = 0.005)**	1.75 ± 0.31 (*P* = 0.066)	2.10 ± 0.33
% of total FA	1.14 ± 0.04	1.10 ± 0.10	1.11 ± 0.06	1.11 ± 1.17	**0.92 ± 0.05 (*P* = 0.005)**	**0.93 ± 0.06 (*P* = 0.008)**	**0.96 ± 0.10 (*P* = 0.02)**	0.96 ± 0.06 (*P* = 0.09)

Values are means ± STD of five determinations. Values in bold, differ significantly ($P < 0.05$) from the corresponding control subcellular fraction (actual *P* values are indicated in brackets)

Quantitatively (μg FA/mg protein), the total PUFA (ω3 and ω6 PUFA) levels in PC decreased significantly in the mitochondria ($P = 0.0001$); plasma membrane ($P = 0.05$) and nuclear ($P = 0.04$) fraction. In the microsomal fraction the total polyunsaturated fatty acid (PUFA) increased significantly ($P = 0.001$) only in PE. Qualitatively, PUFA (expressed as the % of the total FA) decreased significantly in PC of the microsomal ($P = 0.001$), mitochondrial ($P = 0.0001$) and plasma membrane ($P = 0.002$) subcellular fractions. In PE, PUFA was significantly decreased only the mitochondrial ($P = 0.012$) and plasma membrane ($P = 0.001$) subcellular fractions due to FB_1 treatment.

The ω6/ω3 PUFA ratios of the treated rats was significantly increased in the PC fraction of the plasma membrane ($P = 0.01$) and marginally in the nuclei ($P = 0.08$). The 20:4ω6 PC/PE ratio was significantly reduced, quantitatively in the microsomal ($P = 0.005$); mitochondrial ($P = 0.008$) and marginally in the plasma membrane ($P = 0.066$) fractions. Qualitatively, the 20:4ω6 PC/PE ratio qualitative decreased significantly in the microsomal ($P = 0.005$), mitochondria ($P = 0.008$), plasma membrane ($P = 0.02$) and only marginally ($P = 0.09$) in the nuclear fraction as a result of the FB_1 treatment.

Fatty Acids

Fatty acid profiles of the PE and PC phospholipids fractions of the different subcellular fractions are summarized in Tables 2, 3, 4 and 5. Data from the different subcellular fractions is expressed quantitatively (μg/mg protein) and qualitatively as a percentage (%) of total FA content.

Saturated Fatty Acid (C16:0 and C18:0)

The FB_1 treatment significantly increased C16:0 ($P = 0.023$) in the PC phospholipid fraction in the microsomes. The total saturated fatty acid (SFA) content of PE ($P = 0.002$) increased significantly mainly due the increase in both C16:0 ($P = 0.005$) and C18:0 ($P = 0.002$). In the mitochondria, the SFA levels were decreased in PC ($P = 0.005$) due to a significant decrease in C18:0 ($P = 0.001$). In PE, the SFA levels were significantly increased ($P = 0.001$) due to a sig-

Table 2 Effect of FB_1 on the SFA profiles of PC and PE phospholipids of different membrane subcellular fractions from rat liver

Subcellular fractions	Control				FB_1 treated			
	PC (μg/mg protein)	PC (%)	PE (μg/mg protein)	PE (%)	PC (μg/mg protein)	PC (%)	PE (μg/mg protein)	PE (%)
Microsomes								
C16:0	22.19 ± 3.06	20.00 ± 1.97	7.59 ± 1.21	17.93 ± 1.48	**27.17 ± 3.36 (*P* = 0.023)**	**22.42 ± 1.38 (*P* = 0.033)**	**10.71 ± 1.79 (*P* = 0.005)**	**15.60 ± 0.57 (*P* = 0.015)**
C18:0	22.02 ± 4.03	20.13 ± 1.40	9.22 ± 1.75	21.60 ± 1.05	23.11 ± 4.65	18.92 ± 2.11	**15.71 ± 3.46 (*P* = 0.002)**	22.69 ± 2.03
Total	44.20 ± 4.25	40.13 ± 1.93	16.81 ± 2.76	39.54 ± 1.23	50.28 ± 7.65	41.34 ± 2.67	**26.42 ± 5.20 (*P* = 0.002)**	38.28 ± 2.20
Mitochondria								
C16:0	25.63 ± 3.12	19.55 ± 1.64	7.61 ± 0.86	13.95 ± 2.70	24.70 ± 1.56	**22.94 ± 1.42 (*P* = 0.003)**	**10.80 ± 0.54 (*P* = 0.001)**	16.13 ± 1.01 (*P* = 0.09)
C18:0	27.70 ± 2.80	21.13 ± 1.50	12.60 ± 2.90	22.53 ± 0.71	**20.22 ± 2.90 (*P* = 0.001)**	18.75 ± 2.40 (*P* = 0.066)	**15.53 ± 2.42 (*P* = 0.046)**	23.07 ± 2.26
Total	53.32 ± 4.63	40.70 ± 1.33	20.20 ± 1.90	36.09 ± 2.30	**44.92 ± 3.53 (*P* = 0.005)**	41.69 ± 2.75	**26.30 ± 2.75 (*P* = 0.001)**	39.20 ± 2.06 (*P* = 0.056)
Plasma membrane								
C16:0	26.46 ± 7.25	8.71 ± 2.98	7.23 ± 2.64	13.30 ± 0.82	23.00 ± 5.26	21.17 ± 1.39 (*P* = 0.096)	8.94 ± 1.69	14.70 ± 1.90
C18:0	32.93 ± 8.39	22.19 ± 1.67	12.35 ± 1.96	23.35 ± 1.09	24.49 ± 6.28	21.68 ± 1.50	15.20 ± 4.72	23.88 ± 2.22
Total	59.39 ± 14.13	40.90 ± 1.52	19.58 ± 3.51	36.65 ± 1.01	48.17 ± 11.41	42.86 ± 2.20	24.15 ± 6.24	**38.58 ± 1.39 (*P* = 0.02)**
Nuclei								
C16:0	2.83 ± 0.26	20.75 ± 1.53	0.82 ± 0.27	17.33 ± 7.83	2.61 ± 0.53	22.65 ± 1.00 (*P* = 0.061)	0.95 ± 0.50	16.88 ± 4.21
C18:0	2.83 ± 0.35	20.69 ± 1.54	1.04 ± 0.19	21.42 ± 3.82	2.44 ± 0.36	21.27 ± 1.25	1.15 ± 0.30	21.73 ± 3.02
Total	5.66 ± 0.52	41.44 ± 1.82	1.86 ± 0.15	38.75 ± 8.47	5.05 ± 0.88	43.92 ± 1.75 (*P* = 0.065)	2.84 ± 0.70	38.61 ± 2.20

Values are means ± STD of five determinations. Values in bold, differ significantly ($P < 0.05$) from the corresponding control subcellular fraction (actual *P* values are indicated in brackets). Percentage (%) = % of total FA

Table 3 The effect of FB_1 on the monounsaturated FA profiles of PC and PE phospholipids of different membrane subcellular fractions from rat liver

Subcellular fraction	Control				FB_1 treated			
	PC (μg/mg protein)	PC (%)	PE (μg/mg protein)	PE (%)	PC (μg/mg protein)	PC (%)	PE (μg/mg protein)	PE (%)
Microsomes								
C16:1	0.40 ± 0.09	0.85 ± 0.19	0.23 ± 0.06	0.56 ± 0.17	**1.81 ± 0.81 (*P* = 0.008)**	1.55 ± 0.89	0.27 ± 0.07	0.40 ± 0.15
C18:1	8.87 ± 0.78	8.21 ± 0.85	3.09 ± 0.74	7.20 ± 0.58	**13.22 ± 0.66 (*P* = 0.0001)**	**10.97 ± 0.64 (*P* = 0.0001)**	**5.41 ± 0.34 (*P* = 0.001)**	8.07 ± 1.58
Total	9.27 ± 0.77	9.06 ± 0.75	3.32 ± 0.72	7.76 ± 0.45	**15.03 ± 0.55 (*P* = 0.0001)**	**12.52 ± 1.38 (*P* = 0.0003)**	**5.67 ± 0.29 (*P* = 0.0001)**	8.47 ± 1.65
Mitochondria								
C16:1	0.89 ± 0.20	0.68 ± 0.12	0.19 ± 0.08	0.36 ± 0.16	1.67 ± 1.13	1.55 ± 1.06	0.27 ± 0.11	0.42 ± 0.20
C18:1	10.60 ± 1.02	8.12 ± 0.90	3.95 ± 0.80	7.10 ± 0.44	**11.77 ± 0.54 (*P* = 0.033)**	**10.94 ± 0.64 (*P* = 0.0001)**	**5.11 ± 0.64 (*P* = 0.019)**	7.68 ± 1.23
Total	11.50 ± 1.10	8.80 ± 0.89	4.15 ± 0.80	7.43 ± 0.50	**13.44 ± 1.60 (*P* = .032)**	**12.49 ± 1.60 (*P* = 0.0005)**	**5.40 ± 0.70 (*P* = 0.015)**	8.10 ± 1.40
Plasma membrane								
C16:1	0.79 ± 0.37	0.50 ± 0.27	0.25 ± 0.18	0.38 ± 0.32	1.03 ± 0.24	**0.98 ± 0.42 (*P* = 0.037)**	0.16 ± 0.09	0.28 ± 0.15
C18:1	11.16 ± 2.43	8.17 ± 1.14	3.74 ± 0.24	7.08 ± 0.52	11.62 ± 2.30	**10.46 ± 0.49 (*P* = 0.001)**	4.89 ± 0.92 (*P* = 0.08)	8.05 ± 1.19 (*P* = 0.096)
Total	11.95 ± 2.64	8.67 ± 1.07	3.99 ± 0.92	7.46 ± 0.62	12.65 ± 2.25	**11.44 ± 0.84 (*P* = 0.0005)**	5.05 ± 0.93	8.33 ± 1.26
Nuclei								
C16:1	0.23 ± 0.07	1.66 ± 0.53	0.11 ± 0.09	2.11 ± 1.69	0.15 ± 0.03 (*P* = 0.066)	1.27 ± 0.09	0.14 ± 0.11	3.07 ± 2.56
C18:1	1.40 ± 0.40	10.32 ± 3.42	0.56 ± 0.31	11.05 ± 4.39	1.13 ± 0.19	9.83 ± 0.20	0.55 ± 0.34	10.01 ± 3.95
Total	1.62 ± 0.40	11.99 ± 3.41	0.66 ± 0.29	13.16 ± 3.71	1.30 ± 0.22	11.09 ± 0.26	0.70 ± 0.28	13.10 ± 4.26

Values are means ± STD of five determinations. Values in bold, differ significantly ($P < 0.05$) from the corresponding control subcellular fraction (actual *P* values are indicated in brackets). Percentage (%) = % of total FA

nificant increase in both C16:0 ($P = 0.0001$) and C18:0 ($P = 0.046$). Similar patterns were also noticed in the plasma membrane PE and PC fractions although differences were not significant. In the nuclear subcellular fraction no effects were observed.

Qualitatively total SFA in the microsomal fraction of the treated rats were not altered despite a significant decrease and increase of the percentage C16:0 in the PC ($P = 0.033$) and PE ($P = 0.015$) fractions, respectively. In the mitochondrial fraction, the percentage total SFA was marginally increased ($P = 0.056$) due to a marginal increase in C16:0, whereas in the plasma membranes it was significantly increased ($P = 0.02$) in the PE fraction. In the nuclei, C16:0 ($P = 0.061$) and the total SFA marginally ($P = 0.065$) increased in PC.

Monounsaturated Fatty Acid (C16:1 and C18:1)

The total monounsaturated fatty acid (MUFA) content of PC and PE was significantly elevated in the microsomal fraction ($P = 0.0001$) due to an increase in C16:1 ($P = 0.008$) and C18:1 ($P = 0.0001$) in PC and C18:1 ($P = 0.0001$) in PE. In the mitochondria, the total MUFA was increased in PC ($P = 0.032$) and PE ($P = 0.015$) due to an increase in C18:1 in PC ($P = 0.033$) and PE ($P = 0.019$) fractions. Only a marginally ($P = 0.066$) decrease of C16:1 was noticed in the nuclear PC. Except for a marginal ($P = 0.08$) increase in C18:1 in PE, no changes was noticed in the plasma membrane in the nuclear fraction.

In the PC fractions, the qualitative levels of the MUFA was significantly increased in the microsomal ($P = 0.0003$), mitochondrial ($P = 0.0005$) and plasma membrane ($P = 0.0005$) subcellular fractions due to an increase in C18 ($P < 0.001$ to $P < 0.0001$). In the plasma membrane fraction C16:1 ($P = 0.037$) was increased in PC. In the PE fraction C18:1 was marginally ($P = 0.096$) increased only in the plasma membrane fraction.

Polyunsaturated Fatty Acid

ω6 PUFA: (C18:2, C18:3, C20:3, C20:4, C22:4 and C22:5): In the microsomal subcellular fraction C18:2

Table 4 FB_1 modulation of the ω-6 FA profiles of the PC and PE phospholipids of different membrane subcellular fractions from rat liver

Subcellular fractions	Control					FB_1 treated			
	FA	PC (μg/mg protein)	PC (%)	PE (μg/mg protein)	PE (%)	PC (μg/mg protein)	PC (%)	PE (μg/mg protein)	PE (%)
Microsomes	C18:2	9.54 ± 0.40	8.86 ± 0.92	2.84 ± 0.76	6.57 ± 0.64	**15.13 ± 1.77 (*P* = 0.0004)**	**12.54 ± 1.42 (*P* = 0.0003)**	**5.20 ± 0.63 (*P* = 0.0001)**	**7.65 ± 0.85 (*P* = 0.033)**
	C20:4	36.76 ± 4.72	33.80 ± 1.20	12.71 ± 2.71	29.66 ± 1.18	32.48 ± 3.60	**26.83 ± 1.35 (*P* = 0.0001)**	**20.06 ± 3.27 (*P* = 0.002)**	29.23 ± 0.90
	C22:4	1.02 ± 0.11	0.94 ± 0.06	1.06 ± 0.19	2.50 ± 0.11	0.93 ± 0.07	**0.78 ± 0.11 (*P* = 0.009)**	**1.73 ± 0.13 (*P* = 0.0001)**	2.56 ± 0.36
	C22:5	4.84 ± 1.40	4.38 ± 0.76	3.80 ± 0.76	8.98 ± 1.37	4.17 ± 0.78	**3.43 ± 0.41 (*P* = 0.023)**	**6.08 ± 1.10 (*P* = 0.002)**	8.83 ± 0.05
	Total	53.00 ± 6.65	48.74 ± 1.41	20.68 ± 4.20	48.33 ± 1.26	53.81 ± 5.00	**44.50 ± 1.62 (*P* = 0.001)**	**33.44 ± 4.92 (*P* = 0.001)**	48.83 ± 1.06
Mitochondria	C18:2	11.35 ± 1.0	8.68 ± 0.80	3.71 ± 0.81	6.64 ± 0.70	**13.39 ± 1.35 (*P* = 0.014)**	**12.45 ± 1.39 (*P* = 0.0002)**	**4.80 ± 0.17 (*P* = 0.022)**	**7.21 ± 075 (*P* = 0.022)**
	C20:4	44.63 ± 2.96	34.09 ± 1.20	17.33 ± 3.65	30.98 ± 1.88	**28.82 ± 1.60 (*P* = 0.0001)**	**26.80 ± 1.27 (*P* = 0.0001)**	19.35 ± 1.32	**28.89 ± 0.06 (*P* = 0.041)**
	C22:4	1.20 ± 0.10	0.91 ± 0.05	1.52 ± 0.37	2.72 ± 0.38	**0.83 ± 0.11 (*P* = 0.0001)**	**0.77 ± 0.11 (*P* = 0.015)**	1.60 ± 0.20	2.41 ± 0.35
	C22:5	5.40 ± 1.10	4.11 ± 0.74	5.59 ± 1.10	10.04±1.42	**3.13±0.45 (*P* = 0.004)**	**3.34±0.36 (*P* = 0.044)**	6.17±1.24	9.15±1.16
	Total	63.56 ± 3.91	48.55 ± 0.85	28.61 ± 5.62	51.16 ± 2.20	**47.58 ± 1.43 (*P* = 0.0001)**	**44.19 ± 1.40 (*P* = 0.0001)**	32.29 ± 2.42	**48.21 ± 0.62 (*P* = 0.02)**
Plasma Membrane	C18:2	11.95 ± 2.93	8.33 ± 0.56	3.56 ± 0.78	6.66 ± 0.58	12.64 ± 2.86	**11.38 ± 1.19 (*P* = 0.0002)**	4.56 ± 0.96	7.44 ± 0.68 (*P* = 0.056)
	C20:4	49.50 ± 12.98	33.73 ± 1.83	16.38 ± 3.17	30.32 ± 0.76	**31.08 ± 7.04 (*P* = 0.02)**	**27.78 ± 1.58 (*P* = 0.0001)**	18.32 ± 5.17	29.18 ± 1.65
	C22:4	1.47 ± 0.40	0.97 ± 0.10	1.57 ± 0.31	2.91 ± 0.14	**0.90 ± 0.19 (*P* = 0.02)**	**0.82 ± 0.15 (*P* = 0.075)**	1.62 ± 0.39	**2.61 ± 0.11 (*P* = 0.003)**
	C22:5	6.51 ± 1.29	4.41 ± 0.94	5.50 ± 0.85	10.00 ± 1.28	**3.59 ± 0.85 (*P* = 0.003)**	**3.21 ± 0.37 (*P* = 0.016)**	5.77 ± 1.65	9.15 ± 0.79
	Total	70.65 ± 17.42	48.20 ± 2.00	27.48 ± 5.07	50.71 ± 0.77	**49.16 ± 10.55 (*P* = 0.05)**	**44.05 ± 1.57 (*P* = 0.002)**	30.52 ± 8.13	**48.76 ± 1.41 (*P* = 0.014)**
Nuclei	C18:2	1.36 ± 0.30	10.03 ± 2.21	0.47 ± 0.37	8.96 ± 5.52	1.27 ± 0.20	11.09 ± 1.01	0.54 ± 0.37	9.25 ± 3.60
	C20:4	4.11 ± 0.70	30.04 ± 4.25	1.40 ± 0.46	27.95 ± 7.40	**2.95 ± 0.50 (*P* = 0.22)**	25.70 ± 0.35 (*P* = 0.054)	1.30 ± 0.36	24.51 ± 4.40
	C22:4	0.08 ± 0.01	0.62 ± 0.11	0.10 ± 0.03	1.79 ± 0.48	0.14 ± 0.06	**1.15 ± 0.29 (*P* = 0.003)**	**0.20 ± 0.05 (*P* = 0.001)**	**3.83 ± 1.10 (*P* = 0.002)**
	C22:5	0.46 ± 0.11	3.36 ± 0.66	O.30 ± 0.20	5.80 ± 3.75	0.56 ± 0.11	**4.85 ± 0.75 (*P* = 0.011)**	0.40 ± 0.15	7.34 ± 0.96
	Total	6.11 ± 0.64	44.70 ± 2.70	2.26 ± 0.63	44.99 ± 7.35	**5.01 ± 0.84 (*P* = 0.044)**	43.52 ± 1.54 (*P* = 0.089)	2.46 ± 0.76	45.44 ± 3.16

Values are means ± STD of five determinations. Values in bold, differ significantly ($P < 0.05$) from the corresponding control subcellular fraction (actual *P* values are indicated in brackets). Percentage (%) = % of total FA

Table 5 FB_1 modulation of the ω-3 FA profiles of the PC and PE phospholipids of different membrane subcellular fractions from rat liver

Subcellular fractions	Control				FB_1 treated			
	PC (μg/mg protein)	PC (%)	PE (μg/mg protein)	PE (%)	PC (μg/mg protein)	PC (%)	PE (μg/mg protein)	PE (%)
Microsomes								
C22:5	0.19 ± 0.04	0.17 ± 0.03	0.14 ± 0.06	0.33 ± 0.09	**0.14 ± 0.01 (*P* = 0.019)**	**0.12 ± 0.02 (*P* = 0.004)**	**0.23 ± 0.05 (*P* = 0.033)**	0.34 ± 0.10
C22:6	1.97 ± 0.40	1.80 ± 0.20	1.68 ± 0.38	3.92 ± 0.40	1.71 ± 0.33	**1.42 ± 0.27 (*P* = 0.018)**	**2.66 ± 0.66 (*P* = 0.01)**	3.90 ± 0.76
Total	2.27 ± 0.42	2.08 ± 0.21	1.88 ± 0.45	4.38 ± 0.46	1.99 ± 0.34	**1.65 ± 0.29 (*P* = 0.014)**	**3.00 ± 0.67 (*P* = 0.006)**	4.41 ± 0.82
Mitochondria								
C22:5	0.22 ± 0.04	0.17 ± 0.03	0.22 ± 0.07	0.40 ± 0.10	**0.13 ± 0.04 (*P* = 0.002)**	0.12 ± 0.04	0.23 ± 0.04	0.34 ± 0.08
C22:6	2.22 ± 0.23	1.70 ± 0.20	2.44 ± 0.50	4.39 ± 0.57	**1.48 ± 0.28 (*P* = 0.0005)**	**1.37 ± 0.27 (*P* = 0.038)**	2.70 ± 0.80	4.00 ± 1.00
Total	2.58 ± 0.22	1.97 ± 0.19	2.74 ± 0.60	4.92 ± 0.70	**1.73 ± 0.29 (*P* = 0.0002)**	**1.61 ± 0.28 (*P* = 0.027)**	3.03 ± 0.80	4.50 ± 0.92
Plasma membrane								
C22:5	0.28 ± 0.10	0.18 ± 0.04	0.22 ± 0.05	0.42 ± 0.08	**0.14 ± 0.03 (*P* = 0.05)**	**0.13 ± 0.03 (*P* = 0.032)**	0.18 ± 0.03	**0.29 ± 0.06 (*P* = 0.012)**
C22:6	2.73 ± 0.75	1.80 ± 0.27	2.37 ± 0.48	4.31 ± 0.54	**1.46 ± 0.38 (*P* = 0.009)**	**1.31 ± 0.20 (*P* = 0.005)**	2.34 ± 0.66	3.77 ± 0.75
Total	3.31 ± 0.92	2.23 ± 0.23	2.83 ± 0.63	5.17 ± 0.61	**1.83 ± 0.45 (*P* = 0.012)**	**1.65 ± 0.25 (*P* = 0.002)**	2.68 ± 0.70	4.33 ± 0.70 (*P* = 0.051)
Nuclei								
C22:5	0.02 ± 0.01	0.12 ± 0.04	0.02 ± 0.02	0.32 ± 0.36	0.01 ± 0.01	0.13 ± 0.05	0.02 ± 0.01	0.32 ± 0.17
C22:6	0.18 ± 0.03	1.32 ± 0.21	0.12 ± 0.06	2.43.09	**0.13 ± 0.03 (*P* = 0.046)**	1.16 ± 0.07	0.11 ± 0.04	2.05 ± 0.58
Total	0.26 ± 0.06	1.86 ± 0.40	0.16 ± 0.06	3.11 ± 1.11	**0.17 ± 0.04 (*P* = 0.041)**	1.46 ± 0.12	0.15 ± 0.03	2.86 ± 0.67

Values are means ± STD of five determinations. Values in bold, differ significantly ($P < 0.05$) from the corresponding control subcellular fraction in controls (actual P values are indicated in brackets). Percentage (%) = % of total FA

was significantly increased ($P = 0.0004$) in the PC fraction. The total ω6 PUFA content in the PE fraction was significantly ($P = 0.001$) increased due to a significant increase in the levels of C18:2 ($P = 0.0001$), C20:4 ($P = 0.002$), C22:4 ($P = 0.0001$) and C22.5 ($P = 0.002$).

In the mitochondria, the total ω6 PUFA content was significantly reduced in PC ($P = 0.0001$) due to a decrease in C20:4 ($P = 0.0001$), C22:4 ($P = 0.0001$) and C22:5 ($P = 0.004$) despite the fact that C18:2 ($P = 0.014$) increased. Except for a significant increase in C18:2 ($P = 0.022$), no other changes were noticed with respect to the PE fraction. In the plasma membrane PC, the total ω6 PUFA content was marginally ($P = 0.05$) decreased due to a decrease in C20:4 ($P = 0.02$), C22:4 ($P = 0.02$) and C22:5 ($P = 0.003$). In the nuclei, the total ω6 FA was decreased significantly in PC ($P = 0.044$) due to a decrease in C20:4 ($P = 0.022$). In PE, C22:4 ($P = 0.001$) increased significantly. Due to the low levels of C18:3 and C20:3 it was not included in Table 4 however; they were included in determining the total PUFA values.

Qualitatively the total ω6 PUFA decreased significantly ($P < 0.005$) in PC in the microsomal, mitochondrial and plasma membrane fractions due to a significant ($P < 0.05$) decrease in the long-chain ω6 PUFA (C20:4; C22:4 and C22:5), in spite of significant increases in C18:2 in the microsomes ($P = 0.0003$), mitochondria ($P = 0.0002$) and plasma membrane ($P = 0.0002$) fractions. In the nuclei the total ω6 PUFA only decreased marginally ($P = 0.089$) due to a marginal ($P = 0.054$) and significant ($P = 0.011$) decrease in C20:4 and C22:5, respectively, while C22:4 significantly ($P = 0.003$) increased. The total ω6 PUFA in the PE fraction was decreased significantly in the mitochondrial ($P = 0.02$) and plasma membrane ($P = 0.014$) fractions due to a decrease in the level of C20:4 ($P = 0.041$) and C22:4 ($P = 0.003$), respectively. C18:2 was significantly increased in both the microsomal fraction ($P = 0.033$) as well as the mitochondrial fraction ($P = 0.022$) and marginally in the plasma membrane ($P = 0.056$). In the nuclear fraction, only C22:4 ($P = 0.002$) was increased.

ω3 PUFA: (C22:5, C22:6): The total ω3 FA in PC significantly decreased quantitatively in the mitochondrial ($P = 0.0001$) and plasma membrane ($P = 0.012$) due to a decrease in both C22:5 and C22:6 ($P < 0.05$). In the microsomes, only C22:5 was significantly ($P = 0.019$) decreased while in the nuclear fraction, the total ω3 FA decreased significantly ($P = 0.041$) due to a decrease ($P = 0.046$) in C22:6. In the microsomal PE the total ω3 FA ($P = 0.006$) significantly increased due to an increase in C22:5 ($P = 0.033$) and C22:6 ($P = 0.01$).

Qualitatively the total ω3 PUFA decreased significantly in PC in the microsomal ($P = 0.014$), mitochondria ($P = 0.027$) and plasma membrane ($P = 0.002$) fractions. This is due to a significant decrease in both C22:5 and C22:6 in the microsomal (C22:5, $P = 0.004$; C22:6, $P = 0.018$), mitochondria (C22:6, $P = 0.038$) and plasma membrane (C22:5, $P = 0.032$; C22:6, $P = 0.005$) fractions. The total ω3 FA in the PE fraction, was marginally decreased ($P = 0.051$) in the plasma membrane subcellular fraction due to a decrease in C22:5 ($P = 0.012$). No changes were observed in PE for any of the other subcellular fractions.

Discussion

FB_1 disrupts sphingolipid, phospholipids, FA and cholesterol metabolism in the liver and kidneys of different animal species. The disruption of the critical balance between proliferation and apoptosis by these different lipid parameters has been associated with cancer promotion by FB_1 in the liver and kidney of rats [25, 41]. However, it is unclear how changes in the lipid components of the different subcellular membrane fractions could create a growth differential in the liver that selectively stimulate the outgrowth of initiated cells. The current model and FB_1-dose used promotes cancer in the liver [19] while a similar dose over a period of 5 weeks induces hepatocellular carcinoma in Fischer 344 rats [17].

Alterations to lipid components in the liver are associated with changes in membrane fluidity reflected by changes in the cholesterol/phospholipid (PC + PE) ratio, PC/PE and P/S ratios [42–44]. In the present study, the PC/PE ratio fluidity indicator, decreased significantly in the microsomal and mitochondrial subcellular fractions, mainly due to a significant increased in PE. The P/S ratio was decreased in PC from the microsomal, mitochondrial and plasma membrane subcellular membrane fractions and in PE in the mitochondrial and plasma membrane subcellular fractions due to a significant decrease and increase in the qualitative levels of PUFA and SFA, respectively. Changes in these lipid parameters, which differ depending on the subcellular fraction, are likely to alter membrane fluidity. A reduction in the PC/PE and P/S ratios is associated with a more rigid membrane structure [42, 43], which could adversely affect many critical biological processes occurring at the membrane. Although the cholesterol/phospholipid (PC + PE) ratio was not altered significantly in the present study, the cholesterol was increased in the microsomal and nuclear membrane fraction, which will further increase the rigidity of the membrane structure as it stabilizes the fatty acyl groups [45].

Regarding the maintenance of cellular homeostasis, the organization of lipids and proteins into specialized clusters or microdomains on the outer leaflet in the plasma membrane is of importance. These microdomains, including lipid rafts and caveolae, are enriched with SM, cholesterol, C20:4ω6-containing plasmanylethanolamine and glycolipids serve as platforms for vesicular trafficking and the initiation and regulation of cell signalling processes [46, 47]. Many different growth factor receptors, including the endothelin, EGF, insulin receptor, insulin-like growth factor, platelet-derived growth factor, tumour necrosis factor (TNF) and folic acid receptor are lodged in lipid rafts/caveolae [48–51]. Lipid rafts/caveolae-associated signalling events affected by FB_1, include the inhibition of folate receptor-mediated vitamin uptake [52], increased expression of TNF-alpha, TNF receptor-1, TNF-related apoptosis-induced ligand [53] and the inhibition of the EGF-induced mitogenic response [25]. The effect of FB_1 on these signalling processes has been related to the disruption of membrane integrity involving alterations in the sphingolipid [52, 53], cholesterol, phospholipid and FA metabolism [54]. Although, the plasma membrane concentrations of SM and cholesterol were not altered, the P/S ratio and PUFA were significantly reduced suggesting, as discussed above, a more rigid membrane structure, likely to affect membrane receptor and enzyme responses. The ω6/ω3 ratio was also increased due to a decrease in ω3 PUFA in PC which will impact on the prostaglandin synthesis by directing prostanoids synthesis more towards the E2-series associated with sustained cell proliferation [55]. In this regard the C20:4ω6 PC/PE ratio was significantly decreased, possibly due to the FB_1-induced increase in the concentration of PE and the decrease in C20:4ω6 in PC. The increased level of C20:4ω6 in PE relative to PC has been implied as an important growth stimulus in hepatocyte nodules [44]. Changes in the content of C20:4ω6-enriched ethanolamine plasmalogens could

also be important during raft-mediated receptor responses and should be further investigated.

Changes to the major lipid parameters in the microsomal membrane fraction are similar to that reported previously [14]. Altered lipid parameters are known to impact on the activity of membrane enzymes relating to the synthesis of proteins, lipids and sterols [56]. The activity of cytochrome P450 isozymes [57], ceramide synthase [58], and the delta-6 desaturase [14] has been reported to be inhibited by FB_1. Although FB_1 increased the level of cholesterol in the rat liver microsomal membrane fraction [14] the affect on 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme in endogenous cholesterol biosynthesis, has not been elucidated. The level of SM was not altered in the microsomal membrane fraction, which is in agreement with a previous report [24].

In the mitochondrial subcellular fraction, the PC/PE ratio was significantly reduced due to a decrease and increase in PC and PE, respectively. Disruption of the typical asymmetric lipid distribution of cell membranes could impact negatively on important physiological processes such as the induction of apoptosis [59]. In addition to the changes in PC and PE, SM was also reduced, presumably due to the disruption of the de novo sphingolipid biosynthesis by FB_1. In this regard the presence of both ceramide synthase and ceramide has been observed in the mitochondrial membrane fraction [60, 61]. Ceramide regulates the generation of reactive oxygen species and the activation of mitochondrial apoptosis via several mechanisms, including glutathione peroxidase depletion and increased lipid peroxidation [62]. One of these mechanisms involves the down regulation of Bcl-2, an anti-apoptotic protein, which leads to the opening of the mitochondrial permeability transition pores (PTP) and apoptosis. Resistance to mitochondrial PTP opening is responsible for the promotion of initiated cells and an important event in the 2-acetylaminofluorene-induced hepatocarcinogenesis [63]. A moderate increase in lipid peroxidation was noticed in rat liver mitochondria after a dietary exposure of 250 mg FB_1/kg [22], which could be related to the disruption of ceramide synthase.

It is unclear whether the depletion of ceramide in the mitochondria of altered hepatocytes is related to the cancer promoting properties of FB_1. However, it was suggested that FB_1-induced apoptosis may be due to the inhibition of ceramide synthase resulting in the depletion of ceramide and other complex sphingolipids and the accumulation of sphinganine and sphingosine [41, 64]. Cells sensitive to the proliferative effects of decreased ceramide and increased sphingosine 1-phosphate may have a selective growth advantage. Although the liver is not normally a proliferative organ, it seems likely that the disruption of lipid and sphingolipid metabolism in the mitochondria by FB_1 could result in the impairment of apoptosis in the altered hepatocytes. The differential effect of FB_1 on mitochondrial oxidative damage in normal and initiated hepatocytes, therefore, could play an important role in their altered growth pattern. It has been postulated that, depending on the cell type, the disruptive effect of FB_1 on the sphingolipid metabolism, i.e. a decrease in ceramide and increased sphingosine 1-phosphate, will either favour proliferation or induce cell death [41, 65]. A recent study indicated that sphingosine accumulates in FB_1-induced hepatocyte nodules suggesting it may be involved in the enhanced growth characteristics of these lesions via the formation of sphingosine 1-phosphate [66].

Lipid metabolism in the nuclear membrane fraction is considered to play an important role in signalling events that occur in this cellular compartment [67]. The effect of FB_1 on nuclear-associated membrane enzymes regarding lipid metabolism has not been established. In the present study the nuclear membrane fraction behaved very similarly to the microsomes regarding the increase in cholesterol with the exception that SM also increased. The presence of an SM cycle has been established in the rat liver nuclei [67], however the increase in SM in the nuclei due to FB_1 is unknown. As it was reported that similar levels of cholesterol and SM occur in the rat liver nuclei [67], the increase in SM could result from the corresponding increase in cholesterol. This increase and decrease of SM in the nuclei and mitochondria, respectively, could also explain why the level of SM was not affected by FB_1-exposure when analysing whole liver [29]. The decrease in both ω3 and ω6 PUFA in the PC fraction could be due to an increase in lipid peroxidation in rat liver nuclei [23]. FB_1-induced peroxidation of the membrane lipids was reported to induce oxidative DNA damage in isolated liver nuclei [68].

When considering the FA parameters, the observed changes differ for each subcellular membrane fraction as a result of the FB_1 exposure. Apart from the relative increase and decrease in SFA and PUFA, respectively, the total MUFA were moderately to significantly increased in PC and PE in the different subcellular fractions, except for the nuclear fraction. Of interest is the increase in C18:1ω9, the most abundant MUFA in membranes [69], which is associated with the modulation of the function of membrane-bound proteins in normal cells [1]. In addition, the increased in MUFA, specifically C18:1ω9 and C18:2ω6 is associated with the disruption of the delta-6 desaturase enzyme known to

be inhibited by FB_1 [30]. The resultant increase in MUFA could also be due to an increase in delta-9 desaturase as observed in tumour growth in mouse mammary carcinoma cells [70], hepatoma cells [71], human leukemia and lymphoma cells [72]. The growth of mammary carcinogenesis in vitro was blocked by the addition of an inhibitor of delta-9 desaturase [73]. It is not known at present whether FB_1 affects the activity of delta-9 desaturase. As C18:1ω9 is suggested to exhibit anti-oxidative properties [74] the accumulation thereof is likely to protect against the increased lipid peroxidation induced by FB_1 in rat liver and subcellular fractions [74, 75]. The significant reduction in PUFA in most of the subcellular fractions indicated that apart from the inhibition of the delta-6 desaturase, the FB_1-induced lipid peroxidation also impacted on the status of the long chained FA such as C22:5ω6 and C20:6ω3. The increase lipid peroxidation and the resultant lipid breakdown products, especially in the nuclei, could be important in the cancer initiating properties of FB_1 and in determining the extent of necrotic and/or apoptotic cell death.

Arachidonic acid (C20:4ω6) has been associated with the growth regulatory effects of FB_1 in primary hepatocytes and cancer cells [24, 76]. It was hypothesized that the increase of C20:4ω6 in the PE phospholipids fraction together with the decrease of long-chain PUFA and associated low levels of lipid peroxidation are early events in the neoplastic transformation of hepatic nodules [44]. In the present study the C20:4ω6 PC/PE ratio was decreased in all the subcellular membrane fractions, due to an increased level of C20:4ω6 in PE. The importance of C20:4ω6 is further highlighted by the dual effect it has on cell proliferation or apoptosis via the formation of prostanoids or ceramide, respectively [77–79]. Recently a member of the phospholipase A_2 (PLA_2) enzyme-family, the type VI calcium-dependent $iPLA_2$, involved in C20:4ω6-generation and associated with C20:4ω6-induced apoptosis, was detected in close proximity of cyclooxygenase-2 (COX-2) in the mitochondria [80]. It has also been shown that FB_1 stimulates cytoplasmic PLA_2 activity, resulting in an increase in C20:4ω6 and its metabolites [81]. The suppression of apoptosis seems to be related to the conversion of C20:4ω6 to prostanoids by two isoforms of the enzyme COX, shown to be overexpressed in numerous human neoplasms [82]. In this regard the elevated expression of these COX isoform enzymes could be responsible for reduced availability for ceramide generation via the sphingomyelinase pathway [79]. The current study indicated that SM synthesis is also impaired by FB_1 in the mitochondria through the inhibition of ceramide synthase.

The interaction between COX-2, PLA_2 and ceramide synthase play a determining role in the regulation of apoptosis and cell proliferation during FB_1 exposure. Therefore, the differential regulation of C20:4ω6 and ceramide levels in normal and altered hepatocytes are likely to be important determinants in the selective stimulation of preneoplastic lesion in the liver by FB_1.

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INTERACTION OF ^{14}C-LABELLED FUMONISIN B MYCOTOXINS WITH PRIMARY RAT HEPATOCYTE CULTURES

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Abstract—An *in vitro* study on the interaction and biotransformation of the [^{14}C]fumonisin B mycotoxins was conducted, using primary rat hepatocyte cultures and subcellular enzyme preparations. At the same concentration, fumonisin B_2 (FB_2) exhibited a higher cytotoxicity and specific binding to primary rat hepatocytes than fumonisin B_1 (FB_1). However, if the effective dose level (EDL) is considered (i.e. the lowest level of toxin that binds to the hepatocytes to elicit a cytotoxic effect), FB_1 and FB_2 exhibited a similar cytotoxic effect. FB_1 was found to be associated with both the soluble and insoluble compartments within the cell. As assessed by the radioactivity associated with the cellular preparations, very little (approximately 0.01%) FB_1 and/or FB_2 bound to hepatocytes. In the subsequent fractionation of the culture medium using amberlite XAD-2 and silica-gel chromatography, no metabolites were detected, indicating that the fumonisin molecule was not metabolized by primary hepatocytes. The latter aspect was confirmed by the fact that incubation of FB_1 with microsomal enzyme preparations also failed to indicate any metabolism of the fumonisins by the esterases or by cytochrome *P*-450 monooxygenase. FB_1 was also found not to be a substrate for the triglyceride hepatic endothelial lipase, nor for a lipase from porcine pancreas. This study supports further the hypothesis that the intact molecule of the fumonisins is required for biological activity.

INTRODUCTION

In long- and short-term studies in male BD IX rats conducted with culture material of the fungus *Fusarium moniliforme*, the liver appears to be the primary target organ. Pathological lesions such as cirrhosis, cholangiofibrosis and hepatocellular carcinoma were induced (Jaskiewicz *et al.*, 1987). The causative agent, the fumonisin B (FB) mycotoxins and their structurally related analogues, have been identified (Cawood *et al.*, 1991; Gelderblom *et al.*, 1988a) and have since been the topic of intensive research efforts with respect to their biological effects in different animal species (Gelderblom *et al.*, 1991; Harrison *et al.*, 1990; Kellerman *et al.*, 1990).

Fumonisins proved to be non-mutagenic in the Salmonella test (Gelderblom and Snyman, 1991) and also lack genotoxic effects in the *in vivo* and *in vitro* DNA repair assays in primary rat hepatocytes (Gelderblom *et al.*, 1992; Norred *et al.*, 1992). In addition, FB_1 has been shown to be cytotoxic to certain mammalian cell lines (Abbas *et al.*, 1993; Shier *et al.*, 1991) and also inhibits sphingolipid biosynthesis in rat hepatocytes (Wang *et al.*, 1991) and renal epithelial cells (Yoo *et al.*, 1992). Studies on the cytotoxicity of the FB mycotoxins indicated that they exhibit a low cytotoxic effect to rat hepatocytes compared with the potent aflatoxin B_1 (Gelderblom *et al.*, 1992). With respect to their carcinogenic potential, FB_1, FB_2 and FB_3 initiate cancer in the liver whereas FA_1, an *N*-acetyl derivative of FB_1, and the aminopolyols (AP_1 and AP_2), the hydrolysis products of FB_1 and FB_2, lack any cancer-initiating activity (Gelderblom *et al.*, 1993). It was suggested that the free amino-group, as well as the intact molecule, is required for cancer initiation (Gelderblom *et al.*, 1993). However, a recent study has implicated that the hydrolysis products could be responsible for the phytotoxic effect in plants (Abbas *et al.*, 1993). *In vitro* studies in primary hepatocyte cultures also indicated that the aminopolyols are more cytotoxic than the parent molecules (Gelderblom *et al.*, 1993).

Toxicokinetic studies in rats indicated that FB_1 was rapidly absorbed into the bloodstream after ip administration and that 16% was excreted unmetabolized in the urine within the first 24 hr. When a similar dose was administered by gavage, 0.4% was recovered in the urine while only trace amounts were detected in the plasma (Shephard *et al.*, 1992a). In a subsequent study, using ^{14}C-labelled FB_1, 32% of the

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Abbreviations: DME = dimethylesters; EDL = effective dose level; EDTA = ethylenediaminetetraacetic acid; FB_1 = fumonisin B_1; FB_2 = fumonisin B_2; G-6-P = glucose-6-phosphate; MME = monomethylesters; TLC = thin-layer chromatography; LDH = lactate dehydrogenase; SDS = sodium dodecyl sulfate; TCA = trichloroacetic acid; WE = Williams' E.

radioactivity, of which 25% was unmetabolized FB_1, was recovered in the urine 24 hr after ip dosing, while the bulk of the radioactivity (66%) was recovered in the faeces of the rats (Shephard *et al.*, 1992b). In contrast almost all the radioactivity was recovered in the faeces after the animals received a similar dose by gavage. Although no metabolites of FB_1 were identified in either of these studies, the presence of some metabolites were suggested as only 78% of the radioactivity present in the urine after the ip dose could be accounted for as unmetabolized FB_1. The study using radiolabelled FB_1 indicated that, 24 hr after dosing, only trace amounts of FB_1 or some metabolites were retained in the liver and kidneys (Shephard *et al.*, 1992b).

At present very little is known about the biotransformation of the fumonisins by hepatic metabolizing enzymes. This study was conducted to investigate the *in vitro* interaction of the fumonisin B mycotoxins with primary rat hepatocyte cultures, including the binding of radiolabelled FB_1 and FB_2 to rat liver microsomal and plasma membrane fractions. In addition, the metabolism of the fumonisins by two microsomal enzyme systems, the esterases and the cytochrome *P*-450-dependent monooxygenase, was investigated. As the aminopolyol derivatives could be an important metabolic product of the fumonisins, the role of the triglyceride hepatic endothelial lipase as well as a lipase from porcine pancreas in the metabolic conversion of the fumonisins was investigated.

MATERIALS AND METHODS

Mycotoxin standards

[G-21,22-^{14}C]FB_1 and -FB_2 were prepared by spiking corn cultures of *F. moniliforme* MRC 826 with L-[*methyl*-^{14}C]methionine (more than 50 μCi mmol^{-1}; Amersham International, Amersham, Bucks., UK) during the active production phase of the mycotoxins (Alberts *et al.*, 1993). The specific activities varied between different batches and were 22.1, 36.0 and 18.6 μCi/mmol for FB_1, while the specific activity of the FB_2 preparation used was 17 μCi/mmol. The unlabelled toxins were purified as described by Cawood *et al.* (1991), yielding FB_1 and FB_2 with a purity of 95%. Analytical standards of fumonisin A_1 (FA_1), the aminopentol (AP_1, hydrolysis product of FB_1) and the monomethyl and dimethyl esters (MMEs and DMEs) of FB_1 were prepared as described by Cawood *et al.* (1991) and Gelderblom *et al.* (1993).

Preparation of hepatocyte cultures

Rat hepatocytes, with a viability of approximately 90%, were isolated from male Fischer rats according to the collagenase perfusion technique as described by Hayes *et al.* (1984). Cells were plated at a density of 4×10^5 viable cells/culture dish (5 cm) for 3 hr in 4 ml modified Williams' E (WE) medium containing foetal bovine serum (10%), insulin (20 U/litre), L-glutamine (2 mM), HEPES (10 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). After plating, the cells were washed with Hanks' buffer solution and supplemented with 4 ml serum-free modified WE medium containing L-proline (2 mM) and sodium pyruvate (10 mM) in addition to the components described above.

Preparation of liver fractions

Liver homogenate fractions (S-9) were prepared from male Fischer rats as described by Ames *et al.* (1975). Centrifugation of the S-9 fraction at 40,000 rpm for 1 hr delivered a microsomal fraction with cytochrome *P*-450 concentration of 0.3 nmol/mg protein. The microsomal preparation obtained from Aroclor-induced rats (Ames *et al.*, 1975) had a cytochrome *P*-450 concentration of 2.27 nmol/mg protein. Rat liver plasma membranes were fractionated on a self-forming Percoll gradient (Sigma Chemical Co., St Louis, MO, USA), according to the technique of Loten and Redshaw-Loten (1986). The purity of the preparation was considered to be satisfactory, based on the high specific activity (2.88 μmol/min/mg protein) of the marker enzyme 5′-nucleotidase for plasma membranes (Aronson and Touster, 1974). Only slight contamination (0.075 μmol/min/mg protein) with the microsomal marker enzyme, glucose-6-phosphatase (Baginski *et al.*, 1974) and a small amount of DNA (14.4 μg DNA/mg protein) was recorded by the method of Giles and Myers (1965).

Preparation of rat hepatic lipase

Rat hepatic triglyceride lipase was prepared as described by Grosser *et al.* (1981). Rats, under barbiturate anaesthesia, were injected with a heparin (Sigma Chemical Co.) solution (500 IU/animal) through the femoral vein. 2 min after the heparin treatment they were bled by heart puncture and the plasma was separated from the blood cells by centrifugation (10 min at 1500 *g*). Control blood drawn from the animals before heparin treatment contained no lipase activity. The total lipase activity of the plasma (774 nmol free fatty acid/min/ml) was measured by the method of Iverius and Brunzell (1985). Commercially available lipase (EC 3.1.1.3) from porcine pancreas (110–220 units/mg protein, using olive oil as substrate) was obtained from Sigma Chemical Co.

Incubations

Binding assays. Stock solutions of the ^{14}C-labelled FB_1 and FB_2 were prepared in WE medium and the pH set between 7 and 8 with 1 N NaOH. An aliquot of 50 μl was used to monitor the binding of the compounds to the hepatocyte cultures and the membrane fractions.

Primary hepatocytes. Studies of binding to the primary hepatocytes and of the respective cytotoxic

effects of the fumonisins were monitored by incubating 150, 300 and 600 μM FB_1 and 50, 150 and 300 μM FB_2 with hepatocyte cultures for 48 hr. The culture medium was collected separately (see below) and the plated cells washed with saline (2 × 3 ml), trichloroacetic acid (TCA, 3 × 3 ml) and absolute alcohol (3 × 3 ml). The precipitated cells were dissolved in 2 ml 2% sodium dodecyl sulfate (SDS) containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 20 mM $NaHCO_3$, and the radioactivity and protein concentration determined. In a separate experiment where the binding of FB_1 (600 μM) and FB_2 (300 μM) was determined after 48 hr, the cells of 4–8 dishes were scraped from the dishes in saline, combined and washed by centrifugation (10 min at 50 *g*) until the supernatant was free of any radioactivity. The cells were dissolved in 2 ml 2% SDS and the radioactivity and protein concentration determined. The detached (dead) cells in the culture medium were recovered by centrifugation (50 *g*), washed with saline to remove radioactivity and treated as described above for the quantitation of protein and radioactivity. The binding of FB_1 to the different fractions was expressed as μg FB_1/48 hr/mg protein. Cell cytotoxicity was monitored after 48 hr by measuring the release of lactate dehydrogenase (LDH) from the hepatocytes into the culture medium according to the method of Hayes *et al.* (1984). The percentage ratio of LDH released in the growth medium, expressed as a function of the total LDH release by Triton X-100 (final concentration 1%), was used as the index of hepatocytocidal injury. Control dishes without fumonisin were included.

Membrane fractions. FB_1 (600 μM) was incubated with microsomes (1 mg protein/ml) and plasma membranes (1 mg protein/ml) containing 50 mM Tris–HCl buffer (pH 7.5) at 37°C for 1 hr. The microsomes were pelleted by centrifugation (40,000 rpm for 1 hr) and washed (approximately six times with Tris–HCl buffer) until the supernatant was free of any radioactivity. The plasma membranes were treated similarly except that the membranes were pelleted and washed at 4000 rpm. The resultant membrane fractions were dissolved in 2% SDS solution (2 mM EDTA and 20 mM $NaHCO_3$) and aliquots used for the determination of protein and radioactivity. Specific binding was expressed as μg FB_1/mg protein.

In vitro metabolism

Primary rat hepatocytes. In an attempt to determine whether any metabolites were formed during incubation of hepatocytes with 600 μM FB_1 (see above) the culture media of eight dishes were combined, the dead cells removed by centrifugation and the medium fractionated on an Amerlite XAD-2 column after the pH was adjusted to 3.5 with 1 N HCl. The column (2.5 × 15 cm) was equilibrated with $CH_3OH:H_2O$ (1:3) and after the sample was applied, washed with 100 ml of the latter solvent followed by 100 ml $CH_3OH:H_2O$ (1:1) and finally CH_3OH. Quantification of FB_1 in the fractions (50 ml) was achieved by HPLC analysis using a C_{18} column, as described by Alberts *et al.* (1990) and the radioactivity of each fraction monitored. After the solvents were removed *in vacuo*, the combined extract containing the bulk of the radioactivity from the XAD-2 column was fractionated on a silica-gel column (2.5 × 7 cm) using: $CHCl_3:CH_3OH:H_2O:CH_3COOH$ (55:38:8:1, by vol.) as developing solvent. The radioactivity of each fraction (2 ml) was determined and each fraction analysed by TLC on silica-gel, using the same solvent system. The developed plates were sprayed with *p*-anisaldehyde solution (Gelderblom *et al.*, 1988a) and spots visualized by heating at 120°C.

Radioactivity on TLC plates (including control areas) was determined by scraping individual sections (5 mm), from the origin up to the solvent front zone, from the plate into counting vials containing Ready Value scintillation cocktail (Beckman, South Africa). The identity of the visualized spots and areas containing radioactivity was examined by comparing their R_f values with authentic FB_1 and structurally related analogues including FA_1, AP_1, and MMEs and DMEs of FB_1.

Microsomes. The microsomal metabolism of FB_1 (600 μM) was monitored by incubation with a mixture containing (per ml) $MgCl_2$ (8 μmol), KCl (150 μmol), NADP (1 μmol), glucose-6-phosphate (G-6-P, 5 μmol), G-6-P dehydrogenase (1 U), Tris–HCl (50 μmol) and microsomes (1 mg protein) at 37°C for 1 hr (Gelderblom *et al.*, 1988b).

Lipases. Incubation with the different lipase enzyme preparations was carried out by incubating FB_1 (600 μM) with mixtures containing the porcine lipase (0.28 mg, 500 U/ml) or rat hepatic triglyceride lipase (0.2 ml plasma/ml) in 500 mM TrisHCl (pH 7.5) at 37°C for 1 hr.

The different incubation media obtained from the microsomal and enzyme incubations were fractionated by XAD-2 and silica-gel chromatography as described for the culture medium obtained from the hepatocyte culture incubations. The microsomal incubation mixture was centrifuged at 40,000 rpm for 1 hr prior to the chromatographic fraction procedures. The different fractions obtained from the silica-gel column were monitored for the presence of possible metabolites by TLC as described above. The sensitivity of the TLC screening technique was determined by analysing different amounts of FB_1 (10–1000 ng) on TLC and determining the radioactivity of the visualized spots that corresponded with the R_f value of authentic FB_1.

Scintillation counting and protein determination

Radioactivity was determined by liquid scintillation counting in Ready Value scintillation cocktail (Beckman, South Africa) on a Packard (Downers Grove, IL, USA) Tri-carb 460 CD instrument. The calculations (disintegrations per minute) were

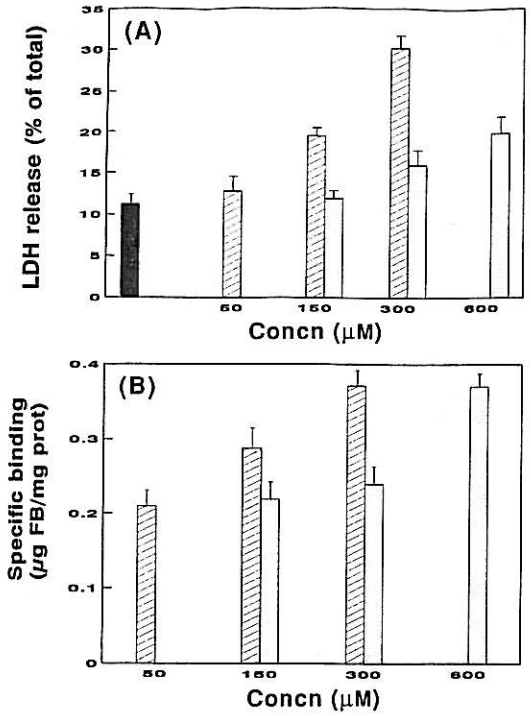

Fig. 1. Comparative (A) cytotoxic and (B) binding potential of varying concentrations of fumonisin B_1 (FB_1) (□) and FB_2 (▨) after 48 hr to rat hepatocytes. Values are means ± SD of triplicate determinations. LDH = lactate dehydrogenase; ■ = control.

determined by external standardization to correct for any quenching. Protein determinations were performed according to the method described by Kaushal and Barnes (1986) with bicinchoninic acid using bovine serum albumin as protein standard.

RESULTS AND DISCUSSION

Both FB_1 and FB_2 exhibited a low cytotoxic effect to primary hepatocytes (Fig. 1A), which is in agreement with the findings of Gelderblom *et al.* (1992). A significant ($P < 0.04$) cytotoxic effect was noticed after 48 hr in cultures containing 300 μM FB_1. FB_2 is more cytotoxic than FB_1 and

Table 1. Binding of ^{14}C-labelled fumonisin B_1 and B_2 to primary rat hepatocyte cultures and rat liver membrane fractions

Liver fraction	Toxin	Concn (μM)	Specific binding* (μg FB_1/mg protein)†
Hepatocytes (intact)	FB_1	600	2.09 ± 0.49
	FB_2	300	2.29 ± 1.42
Hepatocytes (precipitated)	FB_1	600	0.37 ± 0.04
	FB_2	300	0.36 ± 0.01
Hepatocytes (dead cells)	FB_1	600	2.30 ± 1.580
	B_2	300	3.10 ± 0.420
Microsomes	FB_1	600	0.42 ± 0.01
Plasma membranes	FB_1	600	0.35 ± 0.03

*Means of triplicate determination ± SD.
†Specific binding in hepatocytes was monitored after 48 hr and for the membrane fractions after an incubation period of 1 hr.

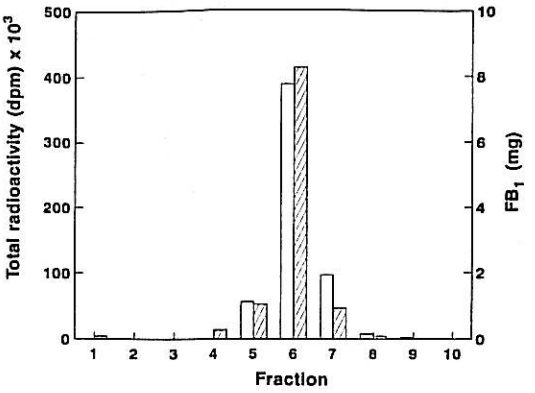

Fig. 2. Elution profile of fumonisin B_1 (FB_1) (▨) and the radioactivity (□) present in the culture medium of hepatocytes from an Amerlite XAD-2 column using an aqueous/methanol eluting solvent mixture.

showed a significant increase in LDH release ($P < 0.02$) above the control at a concentration of 150 μM, whereas FB_1 showed no cytotoxic effect at this level.

Binding of the respective toxins to the primary hepatocytes correlated well with their cytotoxicity (Fig. 1A,B). As suggested previously (Gelderblom *et al.*, 1993), the higher cytotoxicity of FB_2 could be ascribed to its less polar nature, which is evident from the higher specific binding to hepatocytes than occurs with FB_1 at a similar concentration (Fig. 1B). However, if the effective dose level (EDL) is considered [i.e. the lowest level (μg/mg protein) of the toxin that binds to the hepatocytes to elicit a cytotoxic effect], FB_1 and FB_2 appear to be equally cytotoxic. This can be deduced from the fact that for FB_1 a cytotoxic effect is obtained at an EDL range of 0.22 μg/mg protein < EDL < 0.24 μg/mg protein, compared with that of FB_2 (0.21 μg/mg protein < EDL < 0.28 μg/mg protein) (Fig. 1B).

From Table 1 it is evident that binding of FB_1 to microsomes and plasma membranes after 1 hr corresponds well with the binding obtained in the precipitated cells after 48 hr. However, a higher binding of both FB_1 and FB_2 to the detached (dead) cells present in the medium after incubation, and to the intact cells scraped from the dishes, was noticed. This can be ascribed to the fact that not all the fumonisins in intact cells are bound to cellular constituents and that some of the radioactivity was washed from the cells with TCA and ethanol. It was also evident (since FB_1 binds very tightly to cellular membranes, as indicated) that some of the ^{14}C-labelled FB_1 remained in the plasma membranes and microsomes even after extensive washing of these fractions. These data imply that the fumonisins are associated with both the soluble and insoluble (membranous) compartment within the hepatocytes. As judged by the amount of radioactivity added to the cells, only a small fraction (0.01%) was eventually associated with the hepatocytes.

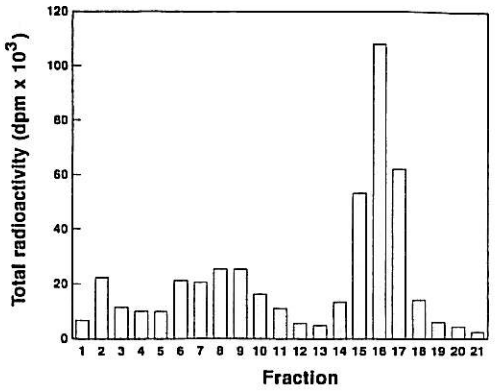

Fig. 3. Elution of radioactivity on fractionation of the combined Amberlite XAD-2 column eluate on a silica gel column using $CHCl_3$:CH_3OH:H_2O:CH_3COOH (55:36:8:1, by vol.) as developing solvent.

During the fractionation of the different incubation media on Amberlite XAD-2, all the radioactivity co-elutes with FB_1 as monitored by TLC ($R_f = 0.23$) and (in the case of the hepatocyte culture medium) also by HPLC (Fig. 2). A non-polar compound ($R_f = 0.64$) was noticed after silica-gel chromatography (Fig. 3), but as less than 1% of this compound was formed, structural analysis was not feasible. A high percentage recovery of radioactivity was obtained from both the XAD-2 (97%) and silica-gel (89%) columns, which are of the same order as previously recorded during the purification of FB_1 (Cawood *et al.*, 1991). The only other radioactive compounds that were characterized were the structurally related monomethyl esters of FB_1, with R_f values of 0.35 (Fig. 4), which are artefacts of the purification procedure (Cawood *et al.*, 1991). No radioactivity could be detected at the R_f values that correspond with the aminopentol (AP_1) or the *N*-acetyl (FA_1) analogues of FB_1. The same applies to incubations of FB_1 with liver microsomes, plasma membranes, pancreatic lipase and rat hepatic triglyceride lipase. However, as the detection limit of radioactive FB_1 on TLC was between 50 and 100 ng, the formation of minor metabolites at concentrations that fall beyond the detection limit of the present method cannot be ruled out.

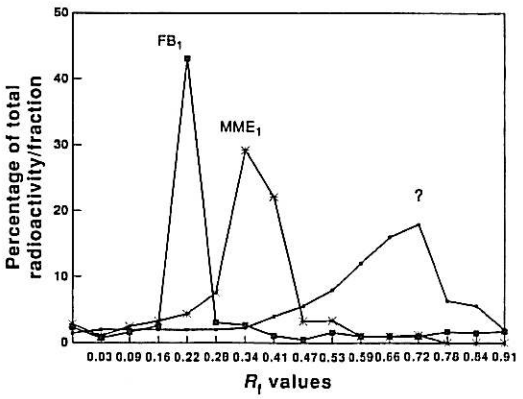

Fig. 4. Illustration of the distribution of the percentage radioactivity from different fractions of a silica gel column [Fractions 2 (■), 8 (✳) and 16 (■)] on a TLC plates, with respect to their R_f values. MME_1 = monomethyl esters of FB_1.

It can be concluded from the microsomal incubation that FB_1 is not a substrate for the microsomal *P*-450 monooxygenase. In addition, metabolic conversion of the fumonisins through hydrolysis of the C-14 and C-15 ester bonds by microsomal esterase or hepatic triglyceride lipase does not occur *in vitro* in the liver of rats. It appears, therefore, that the aminopolyols are not the active metabolites responsible for the toxicological effects of the fumonisins in primary rat hepatocytes and hence in rat liver. These data are in accordance with those from toxicokinetic studies (Shephard *et al.*, 1992a,b) of FB_1 in rats, which indicated that the bulk of the radiolabel is excreted, unmetabolized, in the faeces of the rats. The present study supports the finding of a previous study on the structure–activity relationships of the fumonisins (Gelderblom *et al.*, 1993) indicating that the intact molecule appears to be responsible for the biological activity of the fumonisins.

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Mutagenicity of potentially carcinogenic mycotoxins produced by Fusarium moniliforme

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Abstract

The mutagenic behaviour of two potentially carcinogenic mycotoxins produced by *Fusarium moniliforme* was investigated in the *Salmonella* mutagenicity test using tester strains TA97a, TA98, TA100, and TA102. The mutagenic response obtained with fusarin C (1, 5, and 10 μg/plate) against tester strains TA98 and TA100 in the presence of microsomal activation confirmed previous observations on the mutagenic behaviour of this mutagen while that obtained against TA97a is reported for the first time. No dose - response relationship could be detected for the concentration levels (0.2, 0.5, 1, 5, 10 mg/plate) tested for FB_1, FB_2, and FB_3 against any of the tester strains used in either the plate incorporation and / or the pre - incubation tests. A cytotoxic effect was obtained at concentration levels of 5 and 10 mg/plate in the absence of the microsomal activation mixture. From the studies it became evident that *F moniliforme* produces two compounds, a mutagenic compound, fusarin C which has been shown to lack carcinogenic activity in rats and the non - mutagenic fumonisin B mycotoxins of which FB_1 is known to be responsible for the hepatocarcinogenicity of the fungus in rats.

Introduction

The carcinogenic activity of corn cultures of *Fusarium moniliforme* Sheldon in experimental animals has received considerable attention, particularly since the fungus is associated with human esophageal cancer (1, 2) and produces metabolites that are hepatocarcinogenic to rats (3, 4). As many carcinogens are also mutagens (5), investigations were directed towards the screening of various isolates of *F moniliforme* for their ability to produce mutagenic metabolites. Several isolates of the fungus obtained from a variety of foods and feeds were found to exhibit a mutagenic response in the *Salmonella* mutagenicity test (6). Subsequent studies led to the isolation and characterization of the mutagenic metabolite, fusarin C (7, 8, 9), from corn cultures of the fungus. In addition, fusarin C is produced in culture by several other *Fusarium* species (10) and also occurs naturally in corn intended for human and animal consumption (11, 12).

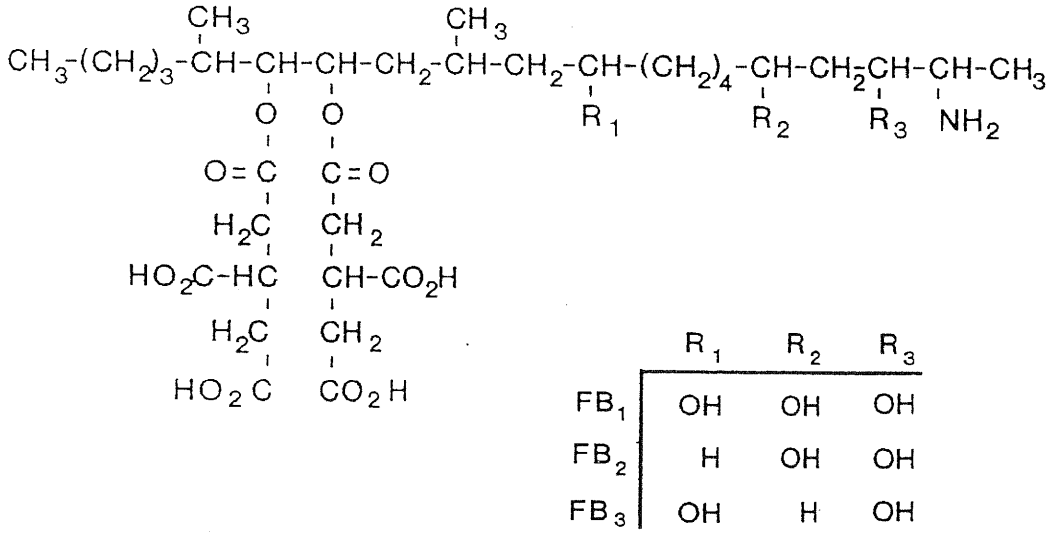
Apart from the mutagenic effects of fusarin C in the *Salmonella* test it induces clastogenic effects including sister chromatid exchanges, micronuclei, chromosomal aberrations, and 6 - thioguanine - resistant mutants in V79 cells (12). These data strongly suggest that fusarin C could act as a potential carcinogen. Extensive studies on the biochemical (13, 14, 15) and carcinogenic (4, 16, 17) properties were performed to evaluate the role of fusarin C in the hepatocarcinogenic activity of corn cultures of *F moniliforme* strain MRC 826 in rats. However, shortterm *in vivo* cancer initiation / promotion assays in rats and mice (16) failed to indicate any cancer initiating activity by fusarin C. Long - term studies in rats (4) with culture material of strain MRC 826 and a non - toxic strain MRC 1069 containing 3 times as much fusarin C failed to indicate any causative role for fusarin C in the hepatotoxic and - carcinogenic effects of strain MRC 826. It was suggested that the hepatotoxicity and hepatocarcinogenicity of the fungus could be caused by the same compound.

Recently several strains of *F moniliforme* were found to exhibit cancer promoting activity in a short - term cancer initiation / promotion assay in rat liver (18). A relationship between the toxicity of the different strains and their cancer promoting potential was established. An active cancer promoting compound, fumonisin B_1 (FB_1), was isolated and characterized from corn cultures of *F moniliforme* strain MRC 826 (19, 20) and found to be responsible for the major hepatotoxic and -carcinogenic effects of the fungus in rats (19, 21). In the present study the mutagenic behaviour of fumonisin B_1 and two structurally related compounds fumonisins B_2 (FB_2) and B_3 (FB_3) was investigated to obtain more information about their carcinogenicity.

Materials and methods

Mycotoxins

The fumonisin B mycotoxins, FB_1, FB_2, and FB_3 (Fig 1) were purified according to the method described by Gelderblom et al (19) with some modifications (22). The purity, as determined by HPLC (23) using pure analytical standards, was 98% for FB_1 and FB_2 and 90% for FB_3. Fusarin C was purified as described previously (7).



	R_1	R_2	R_3
FB_1	OH	OH	OH
FB_2	H	OH	OH
FB_3	OH	H	OH

Fig 1: Chemical structures of fumonisins B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3)

Tab 1: Mutagenicity of fusarin C, fumonisins B_1, B_2, and B_3 in the Salmonella mutagenicity test

Mycotoxin	Concentration (mg/plate)	Histidine revertants per plate[a] (+) S-9				(−) S-9			
		TA97a	TA98	TA100	TA102	TA97a	TA98	TA100	TA102
FB_1	1	190 ± 15	63 ± 11	163 ± 5	393 ± 48	156 ± 13	37 ± 3	173 ± 13	320 ± 31
	5	200 ± 39	66 ± 11	185 ± 11	404 ± 25	216 ± 12	51 ± 5	182 ± 9	326 ± 10
	10	45 ± 33[c]	56 ± 15	109 ± 21[c]	159 ± 32[c]	–	–	–	–[b]
FB_2	1	187 ± 5	64 ± 6	209 ± 22	321 ± 18	156 ± 15	40 ± 7	187 ± 24	288 ± 31
	5	246 ± 52	60 ± 6	200 ± 16	323 ± 25	104 ± 20[c]	31 ± 10	79 ± 6[c]	337 ± 86
	10	210 ± 29	42 ± 10	137 ± 8	152 ± 29[c]	–	–	–	–[b]
FB_3	5	178 ± 26	56 ± 11	144 ± 8	247 ± 6	150 ± 11	38 ± 5	91 ± 3[c]	126 ± 14[c]
Fusarin C (µg/plate)	1.0	253 ± 41	52 ± 9	387 ± 26	371 ± 23	210 ± 32	27 ± 8	143 ± 26	203 ± 29
	5.0	675 ± 47	186 ± 49	1442 ± 53	298 ± 17	189 ± 16	32 ± 9	141 ± 19	209 ± 41
	10.0	1082 ± 171	218 ± 61	1740 ± 212	317 ±25	172 ± 14	36 ± 9	109 ± 16	183 ± 39
Control (DMSO)	0.1mL	261 ± 27	49 ± 9	158 ± 9	369 ± 60	221 ± 11	47 ± 9	174 ± 42	293 ± 47

a Values represent the means ± standard deviation of triplicate determinations.
b No revertant counts due to a toxic effect.
c Slight toxic effect manifested by a decrease in the revertant counts due to a sparse background growth.

Mutagenicity assays

The bacterial tester strains TA97a, TA98, TA100, and TA102, generally recommended for screening of the mutagenic behaviour of compounds, were used. All the strains were tested for the different genotypic markers as described previously (24). The assays were carried out in the presence and absence of Aroclor 1254 (Monsanto) induced microsomal preparations (S-9) which were incorporated in the S-9 mixture at a concentration of 2.16 mg protein/mL S-9 mixture. As the mutagenicity of certain compounds is poorly detected by the standard plate incorporation assay, the mutagenicity of the fumonisins was also tested by utilizing the pre - incubation test (11) during which the compounds were pre - incubated with the S - 9 mixture and the bacteria for 30 minutes at 37°C before plating (24). Various concentrations of FB_1 and FB_2 (0.2, 0.5, 1.0, 5.0, and 10 mg/plate), using DMSO as solvent, were tested in the plate incorporation test. Only two concentrations (0.5 and 10 mg/plate) of both FB_1 and FB_2 were tested in the pre - incubation test. Due to the limited quantities available of the newly discovered FB_3 (22) it was tested only in the plate incorporation test at concentrations of 0.5 and 5.0 mg/plate. The mutagen fusarin C was tested in both the absence and presence of the S-9 mixture at concentrations of 1, 5, and 10 μg/plate. As fusarin C is light sensitive (7) the assay was carried out in subdued light.

Results and discussion

A positive mutagenic response is generally indicated by a dose - response relationship over a narrow concentration range of the compound to be tested (25). Such a relationship is clearly demonstrated with the mutagenic response obtained with fusarin C towards tester strains TA98 and TA100. This is in agreement with previous reports (7, 12) while the dose response obtained against strain TA97a is given for the first time (Tab 1). As described previously fusarin C is mutagenic only in the presence of microsomal activation (7). In contrast to that, no dose response for the fumonisins could be detected at the concentration levels tested against any of the tester strains in both the standard plate incorporation (Tab 1) and pre - incubation mutagenesis assays (Tab 2). Only the revertant counts of the highest concentration levels tested for FB_1 and FB_2 (1.0, 5.0, 10.0 mg/plate) and FB_3 (5.0 mg/plate) were included in Tab 1.

FB_1 and FB_2 exhibited a cytotoxic effect against strains TA97a, TA100, and TA102 in the absence of the S-9 mixture when incorporated at a concentration of 10 mg/plate (Tab 1). At this concentration no background bacterial growth was observed. A slight toxic effect was noticed, manifested as a reduction in the revertant counts due to a sparse background growth, at a concentration of 5 mg/plate with FB_2 against TA97a and TA100 and FB_3 against strains TA100 and TA102. No reduction in the revertant counts of these strains was noticed at this concentration in the presence of S-9 (Tab 1). Similarly, the cytotoxic effect obtained against TA97a, TA98, TA100, and TA102 at FB_1 and FB_2 concentrations of 10 mg/plate was also reduced in the presence of the S-9 mixture (Tab 1). In the case of TA97a and TA98 the toxic effect of FB_1 and FB_2 was respectively completely inhibited while lower revertant counts were scored with the other strains with both FB_1 and FB_2. As stated above, the lower revertant counts are due to a sparse background bacterial growth as compared to the controls.

Whether this inhibition of toxicity by the S-9 mixture is related to metabolism and / or a non - specific interaction of these compounds with components of the activation mixture is presently not known. With regard to this it has been noticed that at the concentrations of 5.0 and 10.0 mg/plate the fumonisins tend to precipitate when added to the top agar resulting in slightly turbid suspensions. When

Tab 2: Mutagenicity of FB_1 and FB_2 (10 mg/plate) in the pre-incubation Salmonella assay[1]

Tester strain	Revertant counts[2]		
	FB_1	FB_2	Control/DMSO
TA 97a	226 ± 5	269 ± 21	250 ± 24
TA 98	56 ± 5	61 ± 8	62 ± 5
TA 100	170 ± 26	111 ± 11	128 ± 15
TA 102	251 ± 38	312 ± 15	357 ± 30

1 The respective fumonisin was pre - incubated with the S-9 mixture at 37°C for 20 min after which the top agar was added and the plates prepared.
2 Values represent the means ± standard deviation of triplicate determinations.

the S-9 mixture was added to the top agar clear suspensions were obtained, indicating that the microsomal preparation solubilized the fumonisins at these concentrations.

Short - term feeding studies in rats using a purified diet indicated that FB_1, the major fumonisin produced in culture, induces hyperplastic foci and hepatocyte nodules in the absence of any added carcinogen (19). These data seem to imply that FB_1 can effect both the cancer initiation and promotion stages in rat liver carcinogenesis and is thus likely to be responsible for the hepatocarcinogenicity of the fungus in rats. Long - term feeding trials using pure FB_1 indicated that this mycotoxin is indeed responsible for the carcinogenic behaviour of the fungus in rats (21). Apart from the lack of mutagenicity of FB_1 and FB_2, they also fail to induce unscheduled DNA synthesis in primary hepatocyte cultures (25). As far as their ability to interact with DNA is concerned, as monitored with the *Salmonella* test and the hepatocyte genotoxicity assay, the fumonisins appear to be non-genotoxic in contrast to the genotoxicity of fusarin C (7, 17).

F moniliforme produces two types of compounds which are potentially carcinogenic, viz the mutagen fusarin C and the cancer promoter, FB_1. Fusarin C exhibits no carcinogenic behaviour in rats (4, 16) while FB_1, although lacking mutagenic activity in the *Salmonella* test and genotoxicity in primary hepatocyte cultures (25), is hepatocarcinogenic in rats (21). The failure of fusarin C to induce any neoplastic lesions in rat liver has been ascribed to the cell defense mechanisms and more specifically to the rat liver microsomal and cytosolic detoxifying enzyme systems (13, 14). At present very little is known about the *in vivo* biotransformation of the fumonisins or whether they can be classified as non-genotoxic, like the peroxisome proliferators, which are known to induce cancer by apparent non - genotoxic means (26). The mechanism of cancer induction by FB_1 and the carcinogenic behaviour of FB_2 and FB_3 are presently under investigation.

The present study emphasized the importance of short - term *in vivo* carcinogenicity bioassays other than standard *in vitro* mutagenicity assays measuring only genotoxicity of chemicals when screening foods and feeds for the presence of potential carcinogens. The lack of mutagenicity of the fumonisins in the *Salmonella* test is the main reason why previous attempts to isolate the fumonisin mycotoxins by screening culture extracts of the fungus for mutagenicity failed (6, 7).

Apart from fusarin C, extracts of *F moniliforme* were found to contain direct-acting mutagens as monitored by the *Salmonella* mutagenicity test and the formation of DNA adducts in the ^{32}p - postlabelling assay (17). The identity of these compounds has yet to be determined in order to evaluate their role in the carcinogenic behaviour of the fungus.

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The cancer-initiating potential of the fumonisin B mycotoxins

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The cancer-initiating potential of the fumonisin B (FB) mycotoxins produced by *Fusarium moniliforme* was screened in rat liver for their ability to induce rare hepatocytes with an acquired resistance to the mitoinhibitory effect of 2-acetylaminofluorene (2-AAF). Two different initiating protocols were used: a feeding regimen during which FB_1 was fed at a dietary level of 0.1% for 26 days, and another where single or multiple doses of FB_1 and FB_2 (varying from 200 to 50 mg/kg) were administered (by gavage) to hepatectomized rats. In both cases promotion was effected by a 2-acetylaminofluorene/carbontetrachloride treatment. Cancer initiation was only obtained after the prolonged feeding regimen, indicating that the fumonisins are poor cancer initiators. FB_1 and FB_2 also lack genotoxic effects in the *in vivo* and *in vitro* DNA repair assays in primary hepatocytes. Although FB_1 primarily affects the liver, it is not very cytotoxic to primary hepatocytes when compared to aflatoxin B_1.

Introduction

The fumonisin B mycotoxins (FB_1 and FB_2*) were purified from corn cultures of *Fusarium moniliforme* as rat liver cancer promoters (1). Subsequent investigations into the long-term toxigenic properties of FB_1 in rats (2) indicated that it accounts for the hepatocarcinogenicity of corn cultures of *F.moniliforme* strain MRC 826 (3) in rats. In addition to the carcinogenic effects, FB_1 also induces cirrhosis and cholangiofibrosis—two major pathological effects of the fungal culture material (3) in rat liver.

In a short-term carcinogenesis study (1) FB_1 induced enzyme-altered liver foci in BD 1X rats after chronic feeding for 26 days at a dietary level of 0.1% in the absence of any initiating carcinogen. This property, as well as the long-term feeding study, which proves that FB_1 is a complete carcinogen in rat liver (2), implies that it can act as a cancer initiator. The present study was therefore directed to investigate the mechanism of the cancer-initiating step.

Several models have been described to investigate the cancer-initiating and cancer-promoting stages of liver cancer development (4,5). A common feature of all these models, despite the diversity of the initiating carcinogen and promoting regimen used, is a focal proliferation of hepatocytes constituting the liver nodule. In the present study the 'resistant hepatocyte' model (6) was utilized as it has been used effectively to evalute the cancer-initiating potential of a variety of chemical carcinogens (7). The induction of resistant hepatocytes during initiation is determined by the ability of these cells to proliferate in an environment that inhibits the growth of normal hepatocytes, resulting in the formation of hepatocyte nodules. It has been shown that in five different models, including the RH model, cancer arises inside the hepatocyte nodule (8).

Recent studies showed that FB_1, though hepatocarcinogenic, lacks mutagenic effects in the *Salmonella* mutagenicity test (9). This is in contrast to fusarin C, a highly mutagenic compound produced by *F.moniliforme* (10), which lacks any carcinogenic effects in short-term and long-term carcinogenesis assays in rats (3,11). In order to obtain more information about the genotoxic effects of FB_1 and FB_2, their ability to induce DNA repair was monitored in primary rat hepatocyte cultures.

Materials and methods

Chemicals

FB_1 and FB_2 were purified as previously described to a purity of 90–95% (1). The respective monomethyl ester derivatives of FB_1 and FB_2, which are artefacts of the isolation process (12), constitute the remainder of the toxin preparations. Diethylnitrosamine (DENA) was obtained from Sigma Chemical Co (St Louis, MO), while 2-acetylaminofluorene (2-AAF) was obtained from Eastman Kodak (Rochester, NY).

Animals

Male Fischer rats (Charles River Breeding Laboratories, Kingston, NY) weighing 100–120 g were used in all the experiments and were maintained on a standard cereal-based diet (5001; Ralston Purina Co., St Louis, MO). When a purified basal diet (101, Dyetts Inc Bethlehem, PA) was used, the cereal-based diet was replaced with the former diet 1 week before commencing with the experiment. The rats were subjected to daily cycles of 12 h light and 12 h dark and received their food and water *ad libitum*.

Tissue examination. The rats were killed under ether anaesthesia and the livers screened, macroscopically, for the presence of hepatocyte nodules. Freshly cut slices (2–4 mm thick) were prepared from each liver lobe and fixed in cold acetone and 10% buffered formalin for γ-glutamyltranspeptidase (GGT) and H&E staining respectively. The slices fixed in acetone were embedded in low melting paraffin and sections stained for GGT according to the method of Ogawa *et al.* (13). The number and size of the foci and/or nodules were quantitated with a Hewlett Packard image analysis system with customized software.

Cancer initiation by the fumonisins

Two experimental protocols were used to investigate the cancer initiating activity of FB_1 and FB_2.

Protocol 1. The experimental design is outlined in Figure 1. FB_1, dissolved in methanol, was incorporated into the diet at a concentration of 0.1%, the methanol evaporated at room temperature for 12 h, and the diet stored at 4°C. An equal volume of methanol was incorporated into the control diet and subsequently treated in the same manner as the fumonisin-containing diet. Rats were divided at random into a control and treated groups and fed the above diets. They were weighed three times per week after commencement of the experiment. After a feeding period of 26 days all the rats were subjected to partial hepatectomy (PH) and the liver sections prepared for H&E and GGT analyses as described above. The drinking water of the rats was supplied with 5% glucose for 24 h post PH while they were maintained on the control (basal) diet for the remainder of the experiment. The promoting regimen (14), which was introduced 2 weeks after PH, consisted of one daily dose of 2-AAF (20 mg/kg; by gavage) on three consecutive days (15) while the stimuli for cell proliferation was effected on day 4 by a necrotizing dose of CCl_4 (0.2 ml/100 g body wt; by gavage). All the rats were killed 10 days after the promoting treatment and the livers examined histologically as described above.

Protocol 2. The experimental design is outlined in Figure 2. Rats were fed a cereal-based diet throughout the experiment. Application of the fumonisins was

*Abbreviations: FB, fumonisin B; DENA diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; GGT+, γ-glutamyltranspeptidase-positive; PH, partial hepatectomy; WE, Williams E; AFB_1, aflatoxin B_1; LDH, lactate dehydrogenase.

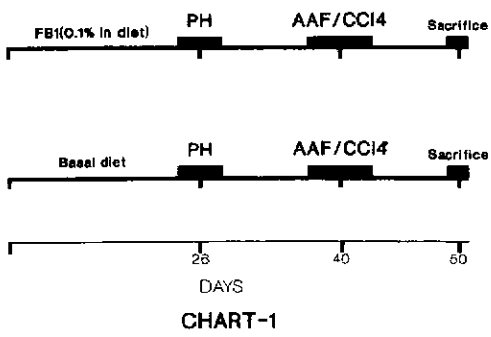

Fig. 1. The experimental regimen for studying the cancer-initiating potential of FB_1. The mycotoxin was fed at a dietary level of 0.1% over a period of 26 days to non-initiated rats. Rats were subjected to PH and after a further 2 weeks to the 2-AAF/CCl_4 selection step as described above. All the rats were killed 10 days after selection.

by gavage in DMSO (0.2 ml/100 g body wt) while DENA was administered by i.p. injection in saline (0.2 ml/100 g body wt). The dosage regimens were as follows: (i) single dose of FB_1 and FB_2 (groups 1 and 2) ranging from 50 to 100 mg/kg administered 18 h after PH; (ii) a single dose as in (i) followed by a second dose (50 mg/kg) 24 h after PH (groups 4 and 5); (iii) a single dose as in (i) but to rats treated with the FB mycotoxin (200 mg/kg) 4 h prior to PH (group 3). For comparison, one positive control group (group 6) was given a known initiating carcinogen, namely DENA (30 mg/kg; i.p.), 18 h after PH. DMSO (group 7) was administered by gavage (0.2 ml/100 g body wt) as a solvent control for the FB-treated groups. After a further 2 weeks the rats received a selection treatment (2-AAF/CCl_4) as described in protocol 1. All the rats were killed 2 weeks after the promoting treatment and the livers processed for histological analyses.

The dosages selected for this study were based on acute toxicity studies in BD 1X male rats (1) in which the rats died within 3 days following a daily dose (per gavage) of 240 mg FB_1/kg body wt.

Measurement of cell cytotoxicity and DNA repair

Primary hepatocyte cultures. Hepatocytes were isolated from male Fischer rats according to the collagenase perfusion technique and the monolayers prepared as previously described (16). In short, viable hepatocytes (200 000/dish) were plated on collagen-coated dishes (30 mm) at 37°C in modified Williams E (WE) median containing fetal bovine serum (10%), insulin (20 U/l), L-glutamine (2 mM), HEPES (10 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were allowed to attach for 3 h, after which they were washed and supplemented with serum-free modified WE containing L-proline (2 mM) and sodium pyruvate (10 mM) in addition to the components described above. The viability of the cells was of the order of 90%.

Cell cytotoxicity. For the cell cytotoxicity assay 4 ml of the cells (1×10^6), suspended in modified WE containing 10% FBS, were transferred to a tissue culture flask (25 ml) and allowed to attach for 2 h. The cells were washed with Hank's washing solution and then supplemented with modified WE medium containing 10% FBS and three different concentrations of FB_1 and FB_2 (87.5, 175, 350 μM). Two concentrations of aflatoxin B_1 (AFB_1) (1, 5 μM) were used as positive controls and DMSO as the solvent control. The cytotoxicity was monitored at 24 h (22 h after plating) and 48 h (46 h after plating) by measuring the release of lactate dehydrogenase (LDH) from the hepatocytes into the culture media according to the method described by Hayes *et al.* (16). The percentage ratio of LDH released between 2 and 24 h or 2 and 48 h to the total LDH released by Triton X-100 (final concentration 1%) was used as the index of hepatocytocidal injury.

DNA repair. The genotoxicity of the fumonisins was monitored by the *in vitro* and *in vivo* rat hepatocyte DNA repair assays as described by Butterworth *et al.* (17,18). The cells were isolated and plated (2×10^5 cells/dish) for 2 h after which they were cultured in serum-free modified WE medium containing the toxins and [^{3}H]thymidine (5 μCi/dish). Various concentrations of FB_1 (ranging from 80 to 0.04 μM/plate) and FB_2 (ranging from 40 to 0.04 μM) were tested in triplicate, while 2-AAF (1 and 0.1 μM) was used as the positive control. After an incubation period of 18 h the cells were washed and the plates subjected to autoradiograph. Net grain counts/nucleus were obtained by subtracting the number of grains in a nuclear-sized area in the cytoplasm from the number scored in the nucleus on the same cell. The nuclear grains of at least 20 nuclei were scored in each dish.

For the *in vivo* assay the rats were treated, in duplicate, with either FB_1 (100 mg/kg) or FB_2 (100 mg/kg) which represents the highest single dose used

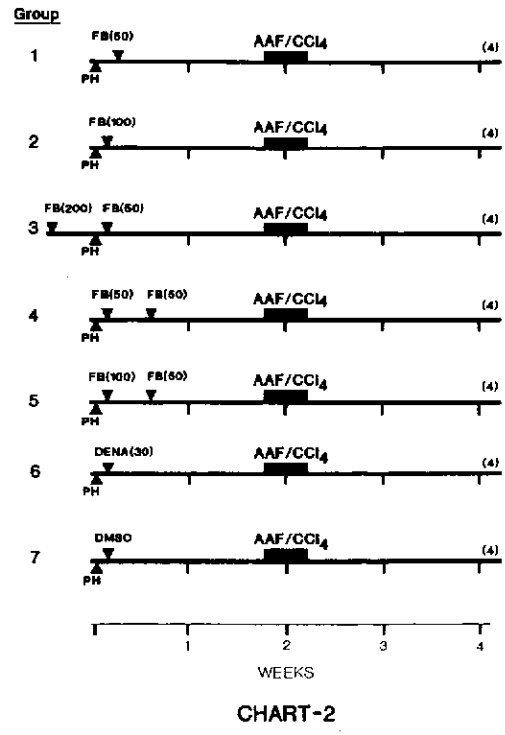

Fig. 2. Experimental regimens for studying the cancer-initiating potential of FB_1 and FB_2. The fumonisins were administered at various time points at dosages (g/kg body wt) indicated in parentheses 18 h after pH (groups 1 and 2); 4 h prior to followed by another dose 18 h after pH (group 3); or 18 h after followed with another dose at 24 h after PH (groups 4 and 5). Positive controls were initiated with DENA (group 6), while the uninitiated controls received DMSO (group 7).

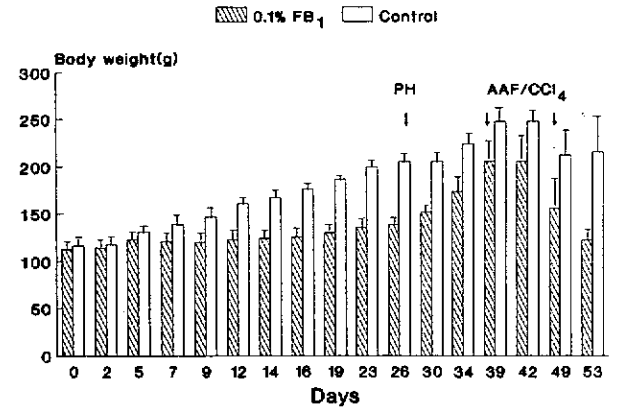

Fig. 3. Body weight gains during chronic feeding of FB_1 (0.1% in the diet) over a period of 26 days. Subsequently the rats were subjected to a PH treatment followed by the 2-AAF/CCl_4 selection regimen between days 29 and 32. The rats were weighed three times weekly and values are means ± SD of four animals per treatment group.

in the initiating regimen described in protocol 2. The hepatocyte cultures were prepared 13–14 h later, the cells plated (in triplicate) for 2 h, after which the [^{3}H] thymidine (5 μCi/dish) was added and the plates incubated for a further 4 h. The cells were washed and incubated with the growth medium containing cold thymidine (pulse chase) for another 12 h, after which the dishes were subjected to autoradiography and the grains/nucleus scored as described above. The viability of the hepatocytes of the toxin-treated (2-AAF and the fumonisins) animals was between 70 and 80% in comparison to the 90–95% of the control animals. For the quantitation of the percentage of cells in the S-phase a minimum of 500 cells were scored (10× objective) from each dish of randomly selected fields.

Table I. Cancer initiation by the fumonisins (protocol 1)

Treatment	Duration (days)	Liver wt (% of body wt)	GGT$^+$ foci and nodules[a]		
			No./cm^2	Mean area (mm^2)	Mean % area/ liver section
FB_1 (0.1% in diet)	26	–	2.9 ± 0.7*	0.14 ± 0.11**	0.42 ± 0.34***
Basal diet[b]	26	–	1.0 ± 0.2	0.01 ± 0.00	0.01 ± 0.00
PH at day 27 and selection (2-AAF/CCl_4) 2 weeks later[c]					
FB_1 (0.1% in diet)	53	2.5 ± 0.28†	7.1 ± 0.8††	2.15 ± 1.00†††	15.64 ± 8.22†††
Basal diet[b]	53	3.1 ± 0.18	1.2 ± 0.3	0.06 ± 0.05	0.07 ± 0.05

[a]Values are means ± SD of four animals/group. Values differ significantly from control at day 26; *$P < 0.0025$, **$P < 0.01$, ***$P < 0.0025$, while after 53 days †$P < 0.0025$, ††$P < 0.0005$ and †††$P < 0.005$.
[b]Basic 101 diet.
[c]All the rats were killed 10 days after the selection regimen.

Table II. Cancer initiation of the fumonisins (protocol 2)

Treatment[a] (initiation)[b]		Liver wt (% of body wt)	GGT$^+$ foci and nodules[c]		
			No./cm^2	Mean area (mm^2)	Mean % area/ liver section
FB_1	(50 mg/kg)	3.7 ± 0.20	1.0 ± 0.26	0.1 ± 0.02	0.1 ± 0.04
FB_1	(100 mg/kg)	3.9 ± 0.28	1.6 ± 0.39	0.1 ± 0.09	0.2 ± 0.10
FB_1	(200 mg/kg)[d]				
	(50 mg/kg)	4.4 ± 0.04	1.2 ± 0.16	0.2 ± 0.19	0.3 ± 0.25
FB_1	(50 mg/kg)[e]				
	(50 mg/kg)	3.4 ± 0.23	1.0 ± 0.27	0.1 ± 0.01	0.1 ± 0.01
FB_1	(100 mg/kg)[e]				
	(50 mg/kg)	3.5 ± 0.07	1.0 ± 0.18	0.1 ± 0.02	0.1 ± 0.01
DENA (30 mg/kg)		4.3 ± 0.18	32.2 ± 14.0*	0.5 ± 0.11	15.5 ± 3.63*
Control (DMSO)		3.6 ± 0.23	0.9 ± 0.03	0.1 ± 0.01	0.1 ± 0.01

[a]FB_2 was tested in a similar way (data not shown).
[b]Rats initiated 18–20 h after PH.
[c]Values are means ± SD of three animals/group.
[d]Rats treated with FB_1 (200 mg/kg) 4 h prior to PH followed by initiation (50 mg/kg) dose 18 h after PH.
[e]The second 50 mg/kg treatment 24 h after the first dose, which was applied 24 h after the initiating dose.
*Significantly different from the control and the FB-treated groups, $P < 0.05$.

Results and discussion

When FB_1 was fed at a dietary level of 0.1% over a period of 26 days (protocol 1) the body weight gains of the rats were significantly lower ($P < 0.0025$) when compared to the control group (Figure 3) from day 7 until the PH treatment at day 26. A marked increase in the rat weight gains was obtained when the FB_1-containing diet was replaced with the basal diet after the PH treatment. Upon termination, 10 days after the 2-AAF/CCl_4 treatment, the rat liver weights of the fumonisin-treated group (2.45 ± 0.28 g) were significantly ($P < 0.025$) smaller than that of the control rats (3.11 ± 0.18 g). The reduction in rat weight gains is in agreement with the previous observation in BD 1X rats (1) fed a fumonisin-containing diet (0.1%) for 25 days.

The livers of the treated animals showed early pathological lesions very similar to that described previously for the BD 1X rats (1,2). A few discrete focal changes in hepatocytes were noticed, which can be regarded as early hepatocyte nodules. The cells in these focal areas were somewhat bigger, displayed more mitotic figures than the cells in the surrounding liver, and also showed vacuolization consistent with glycogen deposition. These areas were not localized in any particular region of the acinus or lobule but could be seen in either zone 1, zone 2 or zone 3. Another prominent pathological feature was the mild to moderate proliferation of bile ductules, which fan out in the surrounding parenchyme to produce dissociation, disorganization and piecemeal necrosis of hepatocytes. This progressive proliferation resulted in some distortion of the normal architecture of the liver. The hepatocytes between the fine ductules showed either hypertrophy or atrophy. Some of the nuclei were quite dark and they

Table III. Measurement of DNA repair by the FB mycotoxins

Compound	Concentration	Grains/nucleus[a]	DNA repair[b]
In vitro assay			
FB_1	80 μM	−11.5 ± 5.1	–
FB_2	40 μM	−11.1 ± 4.4	–
2-AAF	0.1 μM	+8.5 ± 2.9	+
DMSO	25 μl/dish	−19.1 ± 10.4	
In vivo assay			
FB_1	100 mg/kg	−8.63 ± 3.01*	–
FB_2	100 mg/kg	−9.69 ± 4.15*	–
2-AAF	50 mg/kg	+11.57 ± 3.69**	+
Control	DMSO	−12.52 ± 3.61***	

[a]Mean ± SD of triplicate coverslips. With 2-AAF and the DMSO control, 78 and 0.5% of cells responded respectively for the *in vitro* assay. For the *in vivo* assay, 56% of the cells were in the repair with the 2-AAF treatment, 0.3% for the control. Cells in S-phase: *2%; **<0.5%; ***<0.01%.
[b]+, induced DNA repair; −, failed to induce DNA repair.

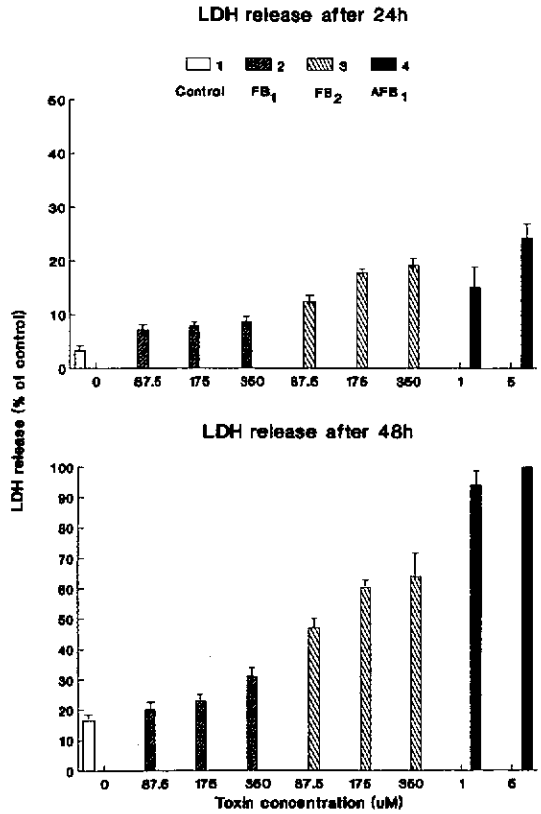

Fig. 4. The cytocidal response of rat hepatocyte to varying concentrations of FB_1 and FB_2 at 22 and 24 h. AFB_1 was used as the positive control with DMSO as the solvent control. Bars represent the means ± SD (triplicate determinations) of the percentage LDH released into the medium.

showed acidophilic cytoplasm. A small degree of fatty change was also noticed. The livers of the untreated animals showed an occasional small degree of fatty change, while no ductular proliferation or changes in hepatocytes were seen.

Upon selection with 2-AAF/CCl_4, three to five hepatocyte nodules (0.3−0.6 cm) were visible macroscopically, scattered randomly throughout the liver of each rat treated with FB_1. Histologically, the nodules were fairly sharply demarcated from the surrounding liver while the hepatocytes in the nodules showed increased mitotic figures and glycogen accumulation. No hepatocyte nodules were visible in the livers of the control animals. The treated animals showed more extensive ductular proliferation and islands of early cholangiofibrosis were present. In contrast, the control animals showed minimal ductular proliferation with some fanning out between the hepatocytes in zone 3. A marked increase in the number and size of GGT^+ foci and/or nodules (Table I) was also noticed in the FB_1-treated animals after the selection treatment.

The presence of the GGT^+ liver foci after the feeding regimen indicated that initiation did occur and that the growth of the initiated cells was selectively stimulated by FB_1. The presence of these hepatocyte foci is in agreement with previous studies performed in BD 1X rats (1) and again indicated that FB_1 can effect both cancer initiation and promotional phases in chemical carcinogenesis. The cancer-promoting property of FB_1 is probably due to its toxic effect, which selectively inhibited the proliferation of normal hepatocytes and not of the initiated cells. The subsequent selection of the foci into hepatocyte nodules with 2-AAF/CCl_4 treatment indicated that FB_1 induces resistant hepatocytes in the liver with respect to the mitoinhibitory effect of 2-AAF. Whether these nodules also comprise the complete resistant phenotype induced by many other carcinogens (3,7) with respect to drug metabolism is not known.

The cancer-initiating activity of FB_1 as well as FB_2 was also monitored under different conditions of exposure (protocol 2) as compared to the chronic feeding regimen (protocol 1). In the present study PH was introduced as the stimulus for cell proliferation and, since very little is known about the metabolism of the fumonisins, various dosage treatments were applied at different time points with respect to the PH treatment (Figure 2). Despite the different treatments, no induction of hyperplastic foci and/or nodules was observed after the 2-AAF/CCl_4 selection treatment by either FB_1 (Table II) or FB_2 (data not shown).

From these data it became clear that FB_1 is a poor cancer initiator as prolonged exposure to a relatively high dietary level resulted in the induction of three to five hepatocyte nodules in the liver. These data seem to imply that FB_1, which is hepatocarcinogenic in rats (1), has a strong effect on the post-initiation events with greater emphasis on promotion and the selection of initiated hepatocytes. Furthermore, the induction of hepatocyte nodules and the proliferation of ductular epithelial cells induced by FB_1 are very similar to those seen with other carcinogens such as 2-AAF and ethionine (19), and the aflatoxins (20). The latter carcinogens are strong inhibitors of hepatocyte proliferation but are weak cancer initiators and cause nodule and cancer development after a chronic exposure for many weeks. The role of FB_1 as an inhibitor of hepatocyte proliferation, which is likely to play an important role during promotion, is presently under investigation. An inhibitory effect of FB_1 on hepatocyte proliferation could also contribute to the lack of cancer initiation by FB_1 and FB_2 (protocol 2) as cell proliferation is essential for initiation (21).

Recent investigations indicated that FB_1 and FB_2 lack mutagenicity in the *Salmonella* assay (8). In the present study the genotoxicity of the fumonisins was monitored in the *in vitro* and *in vivo* DNA repair assays utilizing rat hepatocytes. The latter assay has been shown to be a good measure for predicting the carcinogenicity of chemicals (22). However, the fumonisins lack genotoxicity in both the DNA repair assay systems (Table III) at the concentrations tested. The *in vivo* dosage level also closely resembles the dosages used in the cancer-initiating regimen (protocol 2), which suggests that no DNA damage occurs after a single dosage of the fumonisins. The lower viability of the hepatocytes isolated from the fumonisin- and 2-AAF-treated animals could possibly be attributed to a slight toxic effect as a result of the treatment. The higher percentage of cells that occur in the S-phase of the cell cycle (Table III) could therefore be a result of the compensatory hepatic cell proliferation. The presence of labelled cells in the S-phase is recognized when performing the *in vivo* DNA-repair assay (18), which emphasizes the importance of avoiding excessive high-dosage regimens. However, in the *in vivo* UDS assay the cells in S-phase contain intensely labelled nuclei and can easily be distinguished from non-replicating cells even if they contain a high level of UDS (23).

Based on the present data concerning the genotoxic effects in the hepatocyte DNA repair assays and the lack of mutagenicity in the *Salmonella* test (8), the fumonisins appear to be non-genotoxic. These short-term assays therefore cannot be applied directly to predict the carcinogenic potential of the fumonisins. The ability of the fumonisins to induce DNA repair after a prolonged feeding regimen, similar to that used in protocol 2, which effects cancer initiation, would provide more insight into

the *in vivo* genotoxicity of the fumonisins. However, the hepatotoxic effects induced by FB_1 during the prolonged feeding regimen in the present study (protocol 2) complicate a mechanistical evaluation of the cancer-initiating properties of the fumonisins, such as their ability to induce DNA repair *in vivo*. Several aspects regarding the smallest dose needed and the shortest time required for initiation are presently under investigation in order to minimize those toxic effects of the fumonisins unrelated to the carcinogenic effects.

Although FB_1 primarily affects the liver in both BD 1X (1) and Fischer rats (present study), it exhibits a low toxic effect on primary hepatocytes as compared to the mycotoxin AFB_1 (Figure 4). A significant ($P < 0.025$) toxic effect was noticed after 24 h in the cultures containing FB_1 concentrations of 175 and 350 μM. FB_2 is more cytotoxic than FB_1: a significant increase in the release of LDH was observed after 24 h ($P < 0.05$) and 48 h ($P < 0.0025$) at a concentration of 87.5 μM. Acute toxicity studies of FB_1 in male BD 1X rats (100 g body wt) indicated that the rats only died after three dosages of 250 mg FB_1/kg body wt on 3 consecutive days (1). When compared to the reported oral LD_{50} value of AFB_1 in male rats (100 g body wt) of 7.2 mg/kg (24), FB_1 is not very toxic to rats. Whether this moderate toxic effect of FB_1 on primary hepatocytes in culture could explain the low toxicity in rats is not known.

The biochemical characterization of the hepatocyte nodules induced by FB_1 in the present study should provide more information about the nature of the cancer-initiating step and whether the characteristic enzyme pattern regarding xenobiotic metabolism, appropriate to the resistant phenotype (3,7), is induced. It would also indicate whether cancer initiation by FB_1 has any mechanistic similarity to that seen with other non-genotoxic compounds such as the peroxisome proliferators, which are carcinogenic but lack genotoxicity in a variety of short-term assays (25).

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STRUCTURE–ACTIVITY RELATIONSHIPS OF FUMONISINS IN SHORT-TERM CARCINOGENESIS AND CYTOTOXICITY ASSAYS*

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Abstract—A short-term rat liver cancer initiation/promotion model was used to monitor the cancer-initiating activity of the mycotoxins fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) as well as the *N*-acetyl derivatives of FB_1 and FB_2, and their respective hydrolysis products the aminopolyols. The induction of resistant hepatocytes, which develop into hepatocyte nodules on selection by the 2-acetylaminofluorene–partial hepatectomy promoting treatment, was taken as the endpoint for cancer initiation. When fed at a level of 1000 mg/kg diet for 21 days, only the fumonisins B were found to initiate cancer. In addition, these mycotoxins caused a marked reduction in the rat body weight during the initiating treatment. Comparative cytotoxicity studies in primary rat hepatocytes indicated that FB_2 exhibited the highest cytotoxic effect followed by FB_3 and FB_1. In general, the fumonisin B mycotoxins exhibited a low cytotoxic effect in hepatocyte cultures, and the concentrations of FB_1 and FB_2 that caused a 50% (CD_{50}) release of the total lactate dehydrogenase (LDH) were in the order of 2000 and 1000 μM, respectively. The *N*-acetyl derivatives also exhibited a cytotoxic effect, but were not as cytotoxic as the parent molecules at high concentrations. The respective aminopolyols exhibited a higher cytotoxicity than did the parent compounds, while tricarballylic acid (TCA) exhibited no dose–response effect despite the fact that it had a higher background cytotoxicity compared with the control. The apparent inability of the aminopolyols to act as cancer initiators could be related to a lack in absorption from the gut. An active role of the TCA moiety in the absorption of the fumonisins from the gut was proposed. The present study indicated that the intact molecule and the presence of a free amino group determine the cancer-initiating activity of the fumonisins, and need to be considered in the detoxification procedures of these compounds in foods and feeds.

INTRODUCTION

Fumonisin B_1 (FB_1) is hepatocarcinogenic in rats when fed chronically over a period of approximately 2 yr at a level of 50 mg/kg diet (Gelderblom *et al.*, 1991). In addition to the induction of liver cancer, which was found to develop in 66% of the animals, all treated rats invariably developed cirrhosis and cholangiofibrosis of the liver. With the exception of the kidneys, which were only affected towards the end of the experiment, pathological changes were not observed in any other organs. The liver, therefore, proves to be a major target organ in rats affected by the fumonisins. A recent study in vervet monkeys also implicated the fumonisins in the hepatotoxic effects of corn cultures of *Fusarium moniliforme* MRC 826 when fed chronically over an extended period (Fincham *et al.*, 1992). In other animal species, FB_1 shows remarkable organotropism and, although the liver is constantly affected in a dose-dependent manner, the brain and lungs are major target organs in horses (Kellerman *et al.*, 1990) and pigs (Colvin and Harrison, 1992), respectively.

During the isolation and purification of FB_1 from corn cultures of *F. moniliforme*, several structurally related compounds were purified and chemically characterized (Cawood *et al.*, 1991). These include FB_2, FB_3, FB_4 and the *N*-acetyl derivatives of FB_1 and FB_2, namely FA_1 and FA_2, respectively. The latter structural analogues are produced by the fungus in culture (Cawood *et al.*, 1991), while only FB_1, FB_2 and FB_3 are known to occur under natural conditions (Sydenham *et al.*, 1992). Apart from a preliminary report on the biological activities of these structurally related compounds in rats (Gelderblom *et al.*, 1992a), and few *in vitro* studies on the comparative phytotoxicity and cytotoxicity in plants and mammalian cell cultures (Abbas *et al.*, 1993; Shier

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Abbreviations: AAF = 2-acetylaminofluorene; AP_1 = aminopentol; AP_2 = aminotetraol; FA_1 = fumonisin A_1; FA_2 = fumonisin A_2; FB_1 = fumonisin B_1; FB_2 = fumonisin B_2; FB_3 = fumonisin B_3; FBS = foetal bovine serum; GGT = γ-glutamyl transpeptidase; IC_{50} = inhibition of 50% cell proliferation; LDH = lactate dehydrogenase; MME = monomethyl esters; PH = partial hepatectomy; TCA = tricarballylic acid; TLC = thin-layer chromatography.

et al., 1991), no information on their possible additive effects is available with respect to the toxicological effects of FB_1 in animals.

The aim of the present study was therefore to evaluate the cancer-initiating potential of the different compounds in a short-term rat liver cancer initiation/promotion bioassay (Gelderblom *et al.*, 1992b). The ability of a compound to induce 'resistant hepatocytes', which on selection (promotion) develop into hepatocyte nodules, was taken as a measure of cancer-initiating activity (Tsuda *et al.*, 1987). In addition to the compounds known to be produced by the fungus, the hydrolysis products of FB_1 and FB_2 [the aminopentol (AP_1) and aminotetraol (AP_2) derivatives, respectively] and the tricarballylic acid (TCA) moiety were tested for cancer-initiating activity. The monomethyl ester of FB_1 (MME), which is an artefact of the isolation procedures (Cawood *et al.*, 1991) was also included. As the hepatotoxicity of FB_1 is closely associated with its hepatocarcinogenicity (Gelderblom *et al.*, 1991), the cytotoxicities of the different compounds were compared in primary hepatocyte cultures.

MATERIALS AND METHODS

Fumonisin mycotoxins and derivatives. FB_1, FB_2, FB_3, FA_1, FA_2 and the monomethyl esters of FB_1 were purified as described previously (Cawood *et al.*, 1991). AP_1 and AP_2 were prepared by boiling preparations of FB_1 and FB_2 in a 10% KOH solution under reflux for 12 hr. The respective reaction mixtures were extracted with $CHCl_3$ (3 × 100 ml) after the oily droplets, which represented the bulk of the AP derivatives, were removed with a glass rod. These were combined with the $CHCl_3$ solutions and then evaporated to dryness. Subsequent purification was achieved by column chromatography on silica gel using $CHCl_3$–CH_3OH–CH_3COOH–H_2O (65:25:6:4, by vol.) as developing solvent. Final purification was obtained on an XAD-2 column equilibrated in CH_3OH–H_2O (1:1) containing 1% NH_3 solution. After application, the column was thoroughly washed with the latter solvent, after which the respective aminopolyol derivatives were eluted with CH_3OH–H_2O (3:1). The purity of the aminopolyols was verified by thin-layer chromatography (TLC) and ^1H and ^{13}C nuclear magnetic resonance spectroscopy as described previously (Cawood *et al.*, 1991), and was considered to be 100% pure for the purpose of the present study. TCA was obtained from Sigma Chemical Co. (St Louis, MO, USA). As a result of the small amount of FA_2 present in corn cultures of *F. moniliforme*, this mycotoxin was excluded from the cancer-initiating protocol. The chemical structures of the different compounds are shown in Fig. 1. The R_f values determined by TLC using $CHCl_3$–CH_3OH–H_2O–CH_3COOH (55:36:8:1, by vol.) as developing solvent (Cawood *et al.*, 1991), which are a reflection of the polarity of the aminopolyols with respect to the fumonisins B, were as follows: 0.51, 0.56, 0.23, 0.30 and 0.30 for AP_1, AP_2, FB_1, FB_2 and FB_3, respectively.

Animals. Male Fischer rats weighing between 140 and 160 g were used. They were fed a purified basal diet formulated on the modified AIN-76 diet (American Institute of Nutrition, 1980) immediately after weaning, and were housed in a controlled environment at 25°C with a 12-hr light/dark cycle. Prior to the study, they were randomly divided into treatment groups. The rats had free access to food and water.

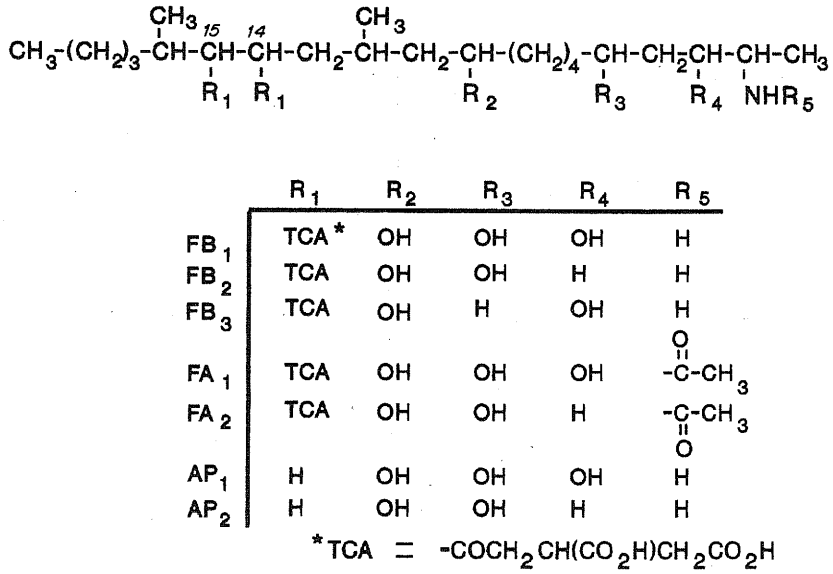

Fig. 1. Chemical structures of the fumonisins FB_1, FB_2, FB_3, FA_1 and FA_2, the hydrolysis products AP_1 and AP_2, and tricarballylic acid (TCA).

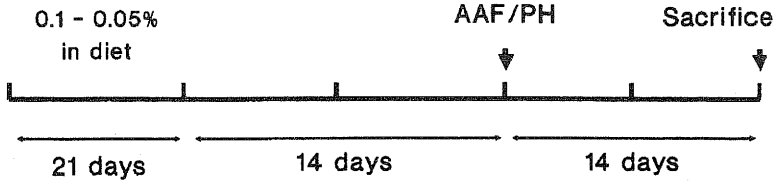

Fig. 2. Experimental protocol used for the screening of the cancer-initiating activity of the different fumonisins and their structurally related derivatives. Each compound was fed at a dietary level of between 0.05 and 0.1% for a period of 21 days followed by the 2-acetylaminofluorene/partial hepatectomy (PH) selection treatment after a further 14 days. Rats were killed 14 days after PH.

Treatments and diets. Each toxin dissolved in CH_3OH (50 ml) was evaporated on a subsample (200 g) of the diet. The samples were allowed to dry for 4 hr in a fume cupboard, and the weight was adjusted to the required amount with untreated diet. The control diet was prepared in a similar way by adding an equal amount of CH_3OH as described above. In the first experiment the fumonisins B, FA_1 and MME were incorporated at a concentration of 0.1% (1000 mg/kg of diet), whereas AP_1, AP_2 and TCA were at a concentration of approx. 0.05% (500 mg/kg diet) since they represent half the molecular weight of the parent molecules (Fig. 1). The respective diets were stored under nitrogen at 4°C until used. Each treatment group consisted of five rats, while eight rats were used in the control group. In the second experiment FB_1, FB_2 and FB_3 were incorporated into the diet at a concentration similar to that of the aminopolyols and TCA (0.05%, 500 mg/kg diet). Three rats per treatment group were used because of the limited quantities of the different toxins available. In the third and final experiment, the cancer-initiating activity of AP_1 at a dietary level of 0.1% (1000 mg/kg diet) was investigated using five rats per group. The cancer-initiation/promotion bioassay used was basically the same as that used in the study of the cancer-initiating activity of FB_1 (Gelderblom *et al.*, 1992a). Briefly, the assay consisted of a feeding regimen of 21 days with the respective fumonisin-containing diets. After a period of 2 wk, during which the rats were fed the control diet, all the animals were subjected to a cancer-promoting treatment with 2-acetylaminofluorene (AAF) (20 mg/kg body weight) on 3 consecutive days followed by partial hepatectomy (PH) on day 4. All the animals were killed 2 wk after the selection treatment. A schematic representation of the short-term bioassay is illustrated in Fig. 2.

Histological examination. Rats were anaesthetized with pentobarbital. Liver slices (2–5 mm thickness) were prepared from each liver lobe and preserved in cold acetone and buffered formalin for histochemical analysis of γ-glutamyltranspeptidase (GGT) and staining with haematoxylin and eosin. The liver slices preserved in acetone were embedded in soft paraffin and 5-μm sections were stained for GGT (Ogawa *et al.*, 1980). The number and size of the nodules and/or foci were quantified with an image analyser with customized software.

Primary rat hepatocytes. Hepatocytes were isolated from male Fischer rats receiving the basal diet using the collagenase-perfusion technique as described previously (Hayes *et al.*, 1984). Hepatocyte monolayers were prepared by plating cell suspensions at a density of 200,000 viable cells per collagen-coated dish (30 mm) in modified Williams E medium containing 10% heat-inactivated foetal bovine serum (FBS) for 3 hr. After the cells were washed with Hanks' buffer solution, complete Williams E medium with FBS was added to the dishes. The viability of the isolated hepatocytes determined by the dextran blue exclusion was in the order of 85 to 90%.

Cytotoxicity assays. The different toxins dissolved in saline–dimethyl sulphoxide (1:1), which was used as the solvent control, were added in 50-μl quantities to the hepatocyte-culture dishes at different concentrations. Cytotoxicity measured by the percentage of lactate dehydrogenase (LDH) released (Hayes *et al.*, 1984) was monitored at 24 and 48 hr. The percentage of LDH released in the growth medium for the periods of 3 to 24 hr and 3 to 48 hr, expressed as a function of the total LDH release after the addition of Triton X-100 (final concentration 1%), was used as the index of cytocidal injury.

Statistical analysis. Analysis of variance and determination of the coefficient of variation were carried out for the cytotoxicities of the mycotoxins and their respective derivatives at the different concentrations tested (125, 250, 500 and 1000 μM). Tukey's Studentized range Q test (Zar, 1984) was used to determine whether the mean cytotoxicities at a specific concentration differed significantly ($P < 0.05$) from each other. Student's t-test was used to determine the differences in cytotoxicity between the various concentrations tested of the individual toxins.

RESULTS

Cancer initiation/promotion bioassay

The effect of FB_1, FB_2, FB_3 and FA_1 on rat body weights during the initiating treatment of 21 days is illustrated in Fig. 3. All animals fed diets containing 0.1% FB_1, FB_2 or FB_3 started to lose weight within the first wk after commencement of the feeding regimen (Fig. 3). Although the toxin-containing diets were diluted twice with the control diet after 1 wk and used for the remainder of the study, body weights of

treated rats did not recover satisfactorily and were still significantly lower ($P < 0.05$) than initial body weights. The marked decrease in body weights of fumonisin B-treated rats resulted in the death of some animals 1 wk after the AAF/PH selection treatment (Table 1). For this reason the study was repeated with a level of the fumonisin B in the diet reduced to 0.05%. Although a similar pattern of reduction in body weight was noticed, it was markedly lower than in the first experiment; this resulted in a higher survival rate after PH. In both experiments, body weight losses were noticed first in rats receiving FB_2, a significant reduction ($P = 0.008$), with respect to controls, was obtained after 4–5 days (Fig. 3). In the case of FB_1 and FB_3, a significant reduction ($P = 0.01$) in body weight was noticed after 7–8 days. In both experiments, body weight loss induced by FB_3 was significantly lower ($P < 0.001$) than that induced by FB_1 and FB_2 (Table 1; Fig. 3). Similar results were obtained when MME was fed to rats at a dietary level of 0.1% for 1 wk and 0.05% for a further 2 wk; body weight loss was also markedly ($P < 0.001$) smaller compared with rats receiving FB_1 (Table 1). Rats given diets containing AP_1, AP_2 and TCA did not show any significant ($P > 0.05$) loss in body weight compared with controls, while those treated with FA_1 showed a significantly lower ($P = 0.01$) gain in body weight compared with controls (Table 1). In the third experiment where the concentration of AP_1 was increased to 0.1% (1000 mg/kg diet), which is equivalent to 0.2% (2000 mg/kg diet) of the intact FB_1, no reduction in body weight was observed compared with controls (Table 1).

2 wk after the selection treatment, four to eight hepatocyte nodules (2–5 mm) were observed macroscopically in the livers of rats receiving FB_1, FB_2, FB_3 or MME in the diet. In contrast, no hepatocyte nodules were present in the livers of rats receiving FA_1, AP_1, AP_2 or TCA. The GGT results are summarized in Table 1. The hepatocyte nodules, which occurred at random throughout the liver, were well demarcated from the surroundings and showed increased mitotic figures and vacuolization consistent with glycogen deposition. Other pathological features in the livers of fumonisin B- and MME- treated rats subjected to the AAF/PH treatment included ductular proliferation and early signs of cholangiofibrosis, which were more visible than those observed in control rats. Similar effects have been described previously in FB_1-treated rats subjected to the AAF/CCl_4 treatment (Gelderblom *et al.*, 1992b).

Cytotoxicity in primary rat hepatocytes

Of the fumonisins B, FB_2 exhibited the highest cytotoxic effect ($P < 0.05$) after 48 hr, followed by FB_3 and FB_1, at all the concentrations tested (Table 2). There was no significant increase in the cytotoxicities of FB_1, FA_1 or MME at 250 μM as compared with the control (background) value of 15.1 ± 1.3 (Table 2). No significant differences in cytotoxicity were observed between FA_1 and FA_2 and their respective parent compounds at the concentrations of 250 and 500 μM (Table 2). However, at 1000 μM both FA_1 and FA_2 exhibited a significantly lower cytotoxic effect ($P < 0.05$) than did FB_1 and FB_2, respectively. AP_1 exhibited a significantly higher cytotoxicity ($P < 0.05$) than did FB_1 at 500 and 1000 μM while AP_2 exhibited a much higher cytotoxic effect ($P < 0.0001$) than did the parent molecule as indicated by the comparative cytotoxicities at 125 μM (Table 2). The

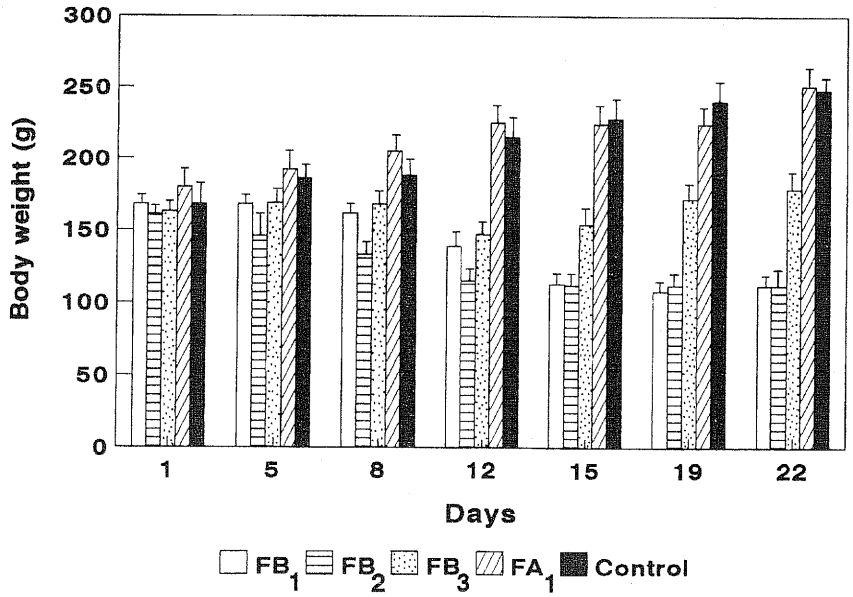

Fig. 3. Effect of the fumonisins FB_1, FB_2, FB_3 and FA_1 on body weight gains of rats during the initiating treatment of 21 days (experiment 1). Each bar represents the mean body weight ± SD of five animals per group.

Table 1. Comparative cancer-initiating potential of the fumonisins and different structurally related derivatives

Compound	Treatment* (initiation)	Experiment	No. of rats with nodules	Body weight gain or loss‡ (g)	GGT foci and nodules§ No./cm²	Mean area	Mean% area/ liver section
FB_1	0.1% (7 days)						
	0.05% (14 days)	1	3/3†	−71.6 ± 2.8 A	1.3 ± 1.2	0.01 ± 0.005	0.85 ± 0.82
	0.05% (21 days)	2	3/3	−55.3 ± 9.4 A	0.5 ± 0.1	0.01 ± 0.01	0.40 ± 0.02
FB_2	0.1% (7 days)						
	0.05% (14 days)	1	3/3†	−74.4 ± 6.6 A	0.5 ± 0.2	0.07 ± 0.08	2.70 ± 0.25
	0.05% (21 days)	2	3/3	−49.0 ± 3.5 A	0.5 ± 0.2	0.03 ± 0.02	2.20 ± 1.85
FB_3	0.1% (7 days)						
	0.05% (14 days)	1	4/4†	−41.4 ± 7.1 A	0.9 ± 0.6	0.04 ± 0.02	2.90 ± 1.80
	0.05% (21 days)	2	3/3	+15.6 ± 6.8 A	0.7 ± 0.4	0.01 ± 0.01	1.00 ± 0.40
MME	0.1% (7 days)						
	0.05% (14 days)	1	5/5	−4.8 ± 14.2 A	2.7 ± 1.3	0.05 ± 0.01	13.3 ± 8.9
FA_1	0.1% (21 days)	1	0/5	+56.5 ± 2.9 a	nd	nd	nd
	0.1% (21 days)	2	0/3	+71.0 ± 2.6 a	nd	nd	nd
AP_1	0.05% (21 days)	1	0/5	+56.6 ± 10.1 b	nd	nd	nd
	0.05% (21 days)	2	0/3	+78.6 ± 16.0 b	nd	nd	nd
	0.1% (21 days)	3	0/5	+50.6 ± 8.0 b	nd	nd	nd
AP_2	0.05% (21 days)	1	0/5	+56.6 ± 12.7 b	0.1 ± 0.003	0.01 ± 0.01	0.02 ± 0.01
	0.05% (21 days)	2	0/3	+73.6 ± 6.4 b	nd	nd	nd
TCA	0.05% (21 days)	1	0/5	+61.2 ± 8.25 b	nd	nd	nd
	0.05% (21 days)	2	0/3	+79.3 ± 9.7 b	nd	nd	nd
Control	Normal diet	1	0/8	+67.1 ± 7.9 b	0.1 ± 0.001	0.003 ± 0.02	0.01 ± 0.01
Control	Normal diet	2	0/4	+80.0 ± 3.4 b	nd	nd	nd
Control	Normal diet	3	0/5	+46.8 ± 9.9 b	nd	nd	nd

nd = not detected

*Each experiment (1, 2 and 3) was compared separately with its appropriate control.

†One to two rats died within 1 wk after partial hepatectomy.

‡Means followed by the same letter are not significantly different; means followed by a and those followed by b are different at $0.05 < P < 0.01$; means followed by A and those followed by a or b are different at $0.01 < P < 0.001$.

§Values are means ± SD of three to five rats.

MME derivative of FB_1 exhibited a cytotoxic effect that was similar ($P > 0.05$) to that of the parent compound at all the concentrations tested, whereas TCA showed only a higher background cytotoxicity compared with the control.

With respect to the dose–response effects of the fumonisins B, MME, AP_1 and AP_2, significant increases in the respective cytotoxicities with increasing concentrations of the individual toxins were recorded (Table 2). FA_1 exhibited a significantly higher cytotoxicity only at 1000 μM, whereas in the case of FA_2 no significant difference was noted between 500 and 1000 μM, although the cytotoxicity at these concentrations was significantly higher ($P < 0.001$) than that at 250 μM. No dose–response effect was noticed with TCA, despite the fact that significantly higher ($0.01 > P > 0.001$) cytotoxic effect were obtained at all the concentrations tested compared with the

Table 2. Comparative cytotoxicities in primary rat hepatocytes of the fumonisins A and B and their structurally related derivatives

Compound	Lactate dehydrogenase release at 48 hr (% of total) Compound concentration . . . 125 μM	250 μM	500 μM	1000 μM
FB_1		16.6 ± 1.5 a (c)	21.8 ± 1.5** a (c)	38.7 ± 1.9** B (c)
FB_2	28.3 ± 1.7** aA (a)	39.8 ± 2.0** bB (a)	47.2 ± 2.7** cB (a)	56.5 ± 1.5** dB (a)
FB_3	20.9 ± 1.0** aA (b)	27.9 ± 1.2** bB (b)	37.4 ± 1.9** cB (b)	
MME	—	16.8 ± 1.2 aA (c)	22.3 ± 2.3* aA (c)	40.9 ± 1.9** bB (c)
FA_1	—	20.3 ± 2.3 aA (bc)	21.8 ± 1.8* aA (c)	28.6 ± 1.1** bA (d)
FA_2	—	33.6 ± 1.3** aA (ab)	45.5 ± 1.3** bB (a)	47.9 ± 2.0** bB (b)
AP_1	—	19.4 ± 1.0* aA (c)	35.3 ± 2.1** bB (b)	57.9 ± 1.9** cC (a)
AP_2	90.7 ± 1.9** (c)	100	100	100
TCA	—	22.9 ± 1.6** a (bc)	22.3 ± 1.6** a (c)	21.8 ± 0.9** a (d)

Values are means ± SD of triplicate determinations of two experiments. The percentage of lactate dehydrogenase released in the control solvent was 15.1 ± 1.3. Asterisks indicate that means are significantly different from the control value (*$0.05 < P < 0.01$; **$0.01 < P < 0.001$). For the same compound (values on the same row), comparisons were made by Student's t-test: means followed by the same letter are not significantly different ($P > 0.05$); different lower case letters indicate $0.05 > P > 0.01$; different capital letters indicate $P < 0.001$. For the same compound concentration (values within the same column), comparisons were made by the Tukey test: different letters in parentheses indicate that means are significantly different ($P < 0.05$).

control. The acidic nature of TCA, which would have affected the pH of the growth medium during the incubation period, could explain the slightly higher background cytotoxicity induced by the compound.

With the exception of AP_1 and AP_2, only a slight increase in the cytotoxicities of the different compounds could be detected after 24 hr. For AP_1 and especially AP_2 morphological changes such as the rounding of the hepatocytes, which leads to an increase in detachment of the cells, became evident after 24 hr.

DISCUSSION

Structural differences within a specific group of related mycotoxins have been used successfully to study structure–activity relationships with respect to a specific biological effect. This approach has led to the elucidation of the role of the *bis*-hydrofuran moiety and the requirement of a double bond in the terminal furan ring in the toxic and carcinogenic effects of the aflatoxin molecule in rats (Wogan *et al.*, 1971). A study on the mutagenic behaviour of the fusarins (mutagenic compounds produced by *Fusarium moniliforme*) has indicated that the presence of the C_{13}–C_{14} epoxide determines the mutagenic behaviour of the molecule (Gelderblom *et al.*, 1988b). In the present study, the different fumonisin mycotoxins and compounds derived chemically from the parent molecules have been compared with respect to their biological activities in both an *in vivo* cancer initiation/promotion bioassay using the rat liver (Gelderblom *et al.*, 1992b) and an *in vitro* cytotoxicity assay in primary rat hepatocytes in an attempt to obtain more information about the structural requirements for the toxic and carcinogenic behaviour of the fumonisins.

Of the fumonisins B, FB_2 and FB_1 exhibited a significantly higher reduction ($P < 0.001$) in rat body weights than did FB_3 during the feeding regimen of 21 days (Table 1). Preliminary data on the specific cause of the reduction in body weights have indicated that the fumonisins induce a feed-refusal effect (W. C. A. Gelderblom, M. E. Cawood, D. Snyman and W. F. O. Marasas, unpublished data, 1993). With respect to the cancer-initiating potential, all three fumonisins B have the ability to induce resistant hepatocytes in the liver and thus are likely to act additively with respect to cancer initiation in rats. As both FB_2 and FB_3 induce similar toxic effects in the liver with respect to proliferation of bile ducts and induction of cholangiofibrosis, as described previously for FB_1 (Gelderblom *et al.*, 1991), they must also be considered in the toxicological effects of *F. moniliforme* in rats and other animal species such as monkeys (Fincham *et al.*, 1992), pigs (Colvin *et al.*, 1992) and horses (Kellerman *et al.*, 1990). The present study indicated that MME, an artefact of the isolation procedure (Cawood *et al.*, 1991) and a minor contaminant of the FB_1 preparations (3–5%) used in long-term carcinogenicity studies in rats (Gelderblom *et al.*, 1991), also exhibited cancer-initiating activity and similar hepatotoxic effects in the short-term cancer initiating/promoting bioassay, as described above for the mycotoxins, and hence could have contributed to the carcinogenic effects of FB_1.

None of the other compounds tested (FA_1, TCA, AP_1 and AP_2) exhibited cancer-initiating potential in the rat liver under the present experimental conditions. Among these compounds, only FA_1 caused a significant ($P = 0.01$) decrease in rat body weights as compared with the control. As FA_1 and FA_2 exhibited a lower cytotoxic effect than did the parent fumonisin B molecules at high concentrations (Table 2), the free amino group is likely to be involved in the cytotoxicity of the fumonisins *in vitro*. These data are in accordance with the *in vivo* data, which imply a specific role for the free amino group in the toxicity and cancer-initiating activity of the fumonisins. An important role for the amino group in the *Alternaria alternata* f. sp. *lycopersici* toxins, which are structurally related to the fumonisins, was also indicated in the phytotoxicity in tomato plants (Siler and Gilchrist, 1983). The present study provides additional proof that the cancer-initiating ability of the fumonisins is directly associated with the toxicity of the compounds *in vivo*; this is in agreement with previous observations (Gelderblom *et al.*, 1988a and 1992a,b). Long-term effects of low dose levels that fail to exhibit toxic effects are presently under investigation, and will provide more insight into the relationship between the toxicity and carcinogenicity of the fumonisins in rats.

The lack of *in vivo* toxicity and cancer-initiating activity in rats of AP_1 and AP_2, the respective hydrolysis products of FB_1 and FB_2, is of interest. Conversely, AP_2 and AP_1 exhibited a higher cytotoxic effect in primary rat hepatocyte cultures than did the respective parent molecules. The reason for the apparent lack of cancer-initiating activity of these compounds *in vivo* could be related to their absorption from the intestine and/or metabolism. At present, very little is known about the metabolism of the fumonisins, and it is unclear whether these compounds are detoxified in the liver. A recent study in rats using ^{14}C-labelled FB_1 indicated that the bulk of FB_1 was excreted unmetabolized in the faeces after a single gavage dose; this indicates that the fumonisins are poorly absorbed from the gut (Shephard *et al.*, 1992). As no metabolites of the fumonisins could be detected in the urine and/or faeces, it seems that the inability of the aminopolyol derivatives to initiate cancer can be ascribed to a lack of absorption from the gut; this in turn could point to a role for the TCA moiety in the absorption of the fumonisins.

This study emphasizes that the cytotoxicity of the fumonisins and their structurally related derivatives in primary hepatocytes cannot be directly related to *in vivo* toxicity and/or cancer-initiating activity of

the compounds. Therefore the development of more sensitive bioassay systems for the fumonisins using mammalian cell lines (Shier *et al.*, 1991), although useful for studying structure–activity relationships with respect to a specific effect such as inhibition of cell proliferation, could be misleading with respect to the mechanism involved in cancer initiation and/or promotion. However, a specific role for the aminopolyols in the toxicological effects of the fumonisins in animals and plants cannot be ruled out since they could be formed *de novo* by the action of esterases as suggested elsewhere (Abbas *et al.*, 1992).

The fumonisins exhibited an extremely low cytotoxicity in primary hepatocytes, and the concentrations of FB_1 and FB_2 that caused a 50% release of the total LDH (CD_{50} values) were in the order of 2000 and 1000 μM, respectively. This is in accordance with a previous study on the cytotoxicity in primary rat hepatocytes and the relative low toxic effect of FB_1 in the short-term cancer initiation/promotion bioassay (Gelderblom *et al.*, 1992b). The inhibitory effect of the fumonisins on *de novo* sphingolipid biosynthesis (Wang *et al.*, 1991), which has been correlated with the cytotoxic effect of the fumonisins in a renal epithelial cell line (LLC-PK1), has been suggested to precede toxicity in these cells (Yoo *et al.*, 1992). A time lag of 24 hr prior to cell death, during which the LLC-PK1 cells appear to be functionally normal, was noticed. This observation is in agreement with a previous study (Gelderblom *et al.*, 1992b) and the present study, where, except for AP_1 and AP_2, very little cytotoxicity was monitored after 24 hr compared with 48 hr in the hepatocyte cultures at all the concentrations tested.

The fumonisins have been found to inhibit cell proliferation in different cell lines, including rat hepatoma (H4TG) and dog kidney (MDCK) (Abbas *et al.*, 1992), as well as in renal epithelial cells (LLC-PK_1) (Yoo *et al.*, 1992). The reported inhibition of 50% cell proliferation (IC_{50}) in the H4TG and MDCK cell lines ranges from 1.7 to 56 μM for the individual fumonisins (Abbas *et al.*, 1992). This IC_{50} compares well with that ranging from 20 to 30 μM observed with FB_1 in the LLC-PK_1 cell lines. Although no direct comparisons can be made, these values are well below those that induce cell death in primary hepatocytes. An inhibitory effect of the fumonisins on hepatocyte cell proliferation *in vivo*, which is presently under investigation, has been suggested to be the reason why these compounds are regarded as poor cancer initiators in the rat liver (Gelderblom *et al.*, 1992b).

From the *in vitro* cytotoxicity data of the fumonisins B and their respective aminopolyol derivatives, it would appear that polarity is an important determinant in the cytotoxic behaviour of the molecule. The less polar the molecule the higher its cytotoxicity, as indicated by the response obtained with FB_1 in comparison with the less polar FB_2 and FB_3 as well as with AP_1 and AP_2 in comparison with the more polar parent molecules FB_1 and FB_2, respectively (Table 2). The lower cytotoxic effect of the fumonisins A, which are less polar than the fumonisins B, can be, however, ascribed to a specific role of the free amino group. Therefore in addition to polarity, other determinants such as the presence of a free amino group and presumably the location of a specific hydroxyl group could also affect the biological activities of these compounds. The latter determinant (specific hydroxyl group) can be deduced from the effect of FB_2 and FB_3 on the rat body weight during initiation. Although these compounds exhibit similar polarities (Cawood *et al.*, 1991), FB_2 has a more drastic effect on body weight than FB_3 (Fig. 3; Table 1). In addition, FB_2 is also significantly more cytotoxic ($P > 0.05$) to primary hepatocytes than FB_3 (Table 2).

The present study indicates that the amino group as well as the intact molecule play an important role in both the toxic and cancer-initiating activities of the fumonisins *in vivo*. Therefore in studies directed at the inactivation of the fumonisins with respect to their toxic and carcinogenic effects, two sites on the molecule need to be considered for modification: the free amino group and/or the hydrolysis of the C_{14} and C_{15} ester bonds.

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Fumonisin B_1 dosimetry in relation to cancer initiation in rat liver

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Dose response studies regarding the cancer initiating potential of fumonisin B_1 (FB_1) were conducted as a function of time. Feeding studies over 21 days indicated that FB_1 induced a feed refusal effect in rats at dietary levels of 250, 500 and 750 mg FB_1/kg diet. This effect was overcome after 14 days and the feed intake profiles reached a level which was equivalent to that of the controls after 21 days. Based on the feed intake records the effective dosage level (EDL) for cancer initiation over a period of 21 days is $14.2 < EDL < 30.8$ mg FB_1/100 g body wt. This is equivalent to a daily intake of $0.7 < EDL < 1.5$ mg FB_1/100 g body wt. Over a period of 14 days the amount of FB_1 required for cancer initiation is $9.6 < EDL < 23.3$ mg FB_1/100 g body wt. The latter values were markedly higher than the EDL values obtained in a gavage study where a fixed amount of FB_1 was dosed to rats over 14 days ($5.39 < EDL < 11.56$ mg FB_1/100 g body wt). The dietary level of FB_1 required for cancer initiation is dependent on the duration of exposure as a dosage of 29.7 mg/100 mg body wt over 7 days did not initiate cancer whilst a similar dose (30.8 mg/100 body wt) over 21 days did. FB_1 effectively delayed hepatocyte cell proliferation when fed at a dietary level of 250 mg FB_1/kg (the lowest dietary level tested to effect cancer initiation over 21 days) or by a single gavage dose of 5 mg FB_1/100 g body wt 6 h following partial hepatectomy. This inhibitory effect of FB_1 on cell proliferation appears to be the reason why the fumonisins are slow cancer initiators. The present data suggest that a balance exists between the compensatory cell proliferation due to the hepatotoxicity induced by FB_1 and the inhibitory effect on the subsequent hepatocyte cell proliferation. Therefore, a threshold level for cancer initiation exists which, as a function of time, will be determined by the dosage used and the subsequent inhibitory effect on cell proliferation.

Introduction

The fumonisin B mycotoxins (FB_1, FB_2 and FB_3*) are a group of structurally related metabolites produced by the fungus *Fusarium moniliforme* in corn (1). FB_1, the major fumonisin produced by the fungus, is responsible for several diseases in animals (2,3) and has been shown, together with FB_2 and FB_3, to be natural contaminants of corn and corn based foods and feeds (4).

The short- and long-term toxic effects of FB_1 in rat liver have been described in BD IX (1,5) and Fischer (6) male rats. The short-term toxic effects in both rat species when FB_1 was fed at dietary level 0.1% (1000 mg FB_1/kg diet) for 21–33 days include pathological changes such as hydropic degeneration, hyaline droplet accumulation, mild fatty changes, single cell necrosis and early signs of fibrosis (1,5). A prominent pathological feature was the mild and moderate proliferation of bile ductules. A few discrete focal changes in hepatocytes which can be regarded as early hepatocyte nodules were observed to occur randomly throughout the liver. The hepatocytes in these focal areas were somewhat bigger, showed vacuolization consistent with glycogen deposition and contained more mitotic figures than the surrounding liver. The foci have the ability to proliferate into hepatocyte nodules after a promotion treatment during which the proliferation of normal hepatocytes is inhibited (6). Based on these data it would appear that the fumonisins behave similarly to most hepatocarcinogens regarding the induction of 'resistant' hepatocytes in the liver (7). At present it is not known whether the characteristic enzyme pattern (8), which constitutes the resistant phenotype, is induced by these compounds. This is of particular interest as short-term mutagenesis (9) and DNA repair assays (6) fail to show any interaction with the DNA.

The long-term toxicological effects of FB_1 (50 mg FB_1/kg diet) were studied in male BD IX rats over a period of ~2 years (5). All the rats that were terminated towards the end of the experiment suffered from micro- and macro-nodular cirrhosis as well as cholangiofibrosis. The livers of all the rats killed at 6 months and onwards indicate the presence of hepatocyte nodules, some of which displayed the typical ground glass appearance and stained positively for gamma-glutamyltranspeptidase (GGT^+) in contrast to the majority of the other nodules which were regarded as regenerative. The hepatocellular carcinoma that developed in the rats after 18–26 months were found to originate within one of these large regenerative nodules. However, the toxic effects of FB_1 complicates a mechanistic evaluation as to whether cancer occurs within nodules commonly induced by hepatocarcinogens in rats.

The present study was directed to examine the dose response effect of FB_1 with respect to induction of resistant hepatocytes in the liver under conditions where the toxic effects of the compound are limited. Under these conditions subsequent studies could be directed to study specific changes related to cancer induction, which is often masked by the toxic effects of a carcinogen.

Materials and methods

Chemicals

FB_1 was purified from corn cultures of *F.moniliforme* strain MRC 826 as described previously (10). The purity as compared to an analytical standard by high performance liquid chromatography (11) was in the order of 92–95%. The monomethylester derivatives of FB_1, which are artifacts of the purification procedure (10) and also exhibited cancer initiating activity (12), constitute the remainder of the FB_1 preparation.

Animals and diet

Male Fischer rats weighing between 150 and 170 g were used in all the experiments. They were caged individually in a controlled environment at

*Abbreviations: FB_1, FB_2, FB_3, fumonisin B_1, B_2, and B_3; GGT^+, gamma-glutamyltranspeptidase positive; 2-AAF, 2-acetylaminofluorene; PH, partial hepatectomy; EDL, effective dosage level.

Table I. Dose dependent effects of dietary fumonisin B_1 versus cancer initiation when fed over various periods

Treatment (mg FB_1/kg diet)	Duration (days)	Body wt (g)[a] (gain or loss)	No. of rats with nodules	GGT$^+$ foci and nodules[b]		
				No./cm^2	Mean area	Mean % area/ liver section
750	21	−18.0 ± 16.6aA	5/5(6−12)[c]	2.1 ± 0.6	0.05 ± 0.01	8.8 ± 1.9
	14	−32.2 ± 17.5aA*	5/5(3−30)	2.4 ± 1.5	0.03 ± 0.005	7.3 ± 4.9
	7	−4.2 ± 4.8a**	0/5	0.1 ± 0.2	0.003 ± 0.004	0.1 ± 0.1
500	21	−7.8 ± 9.6aA	5/5(10−20)	2.8 ± 1.1	0.04 ± 0.01	9.0 ± 2.2
	14	2.8 ± 6.1bB*	7/12(1−5)	1.2 ± 0.4	0.05 ± 0.05	5.1 ± 3.1
250	21	21.2 ± 23.9bA	5/5(3−10)	1.5 ± 1.1	0.02 ± 0.01	1.8 ± 0.9
	14	37.0 ± 6.3cC*	1/5(1)	0.4 ± 0.7	0.001 ± 0.001	0.6 ± 1.0
100	21	35.8 ± 13.5cB	0/5	0.1 ± 0.2	0.001 ± 0.004	0.08 ± 0.2
	14	38.6 ± 2.1cC*	0/5	0.3 ± 0.5	0.001 ± 0.01	0.2 ± 0.4
50	21	50.8 ± 3.7dB	0/5	nd	nd	nd
25	21	57.0 ± 5.6dB	0/5	nd	nd	nd
Control	21	59.4 ± 14.2	0/15	nd	nd	nd
	14	46.2 ± 10.3dC*	0/12	0.2 ± 0.3	0.006 ± 0.01	0.3 ± 0.4
	7	20.0 ± 5.2A**	0/10	nd	nd	nd

[a]Values represent means ± SD. Means followed by the same letter do not differ significantly from each other ($P > 0.05$). If letters (small cases) differ $0.01 < P < 0.05$. When letters (upper case) and cases differ then $0.0001 < P < 0.01$.
*,**Means compared separately with each other.
[b]Values are means ± SD.
[c]Nos in parentheses represent the no. of nodules/liver.
nd, not detected.

23−24°C and 50% humidity with a 12 h artificial light cycle. Rats were weighed on a daily basis and food intake and wastage were determined during the cancer initiating treatment (protocol 1). Food and water were available *ad libitum*. All the rats received the modified AIN 76 diet (13) which was prepared according to the guidelines set in the literature with some modifications. The cornstarch was replaced with glucose/sucrose/cornstarch (1:1:1) while sunflower oil was used instead of corn oil as a fat source. The cellulose was donated by Sappi Saicor, Umkomaas, Natal, South Africa.

Treatments

The cancer initiating activity of FB_1 was studied by monitoring the ability to induce initiated cells with an acquired resistant phenotype (14) with respect to the mitoinhibitory effect of 2-acetylaminofluorene (2-AAF). The induction of initiated cells which on selection (promotion) developed into hepatocyte nodules, visible macroscopically in the liver, was taken as a measure of cancer initiating activity of the fumonisins.

The cancer initiation/promotion bioassay used in the present study to investigate the dosimetry of cancer initiation by FB_1 was the same as described previously (6) and consisted of a feeding regimen during which FB_1 was incorporated in the diet and fed to the animals over a defined period. The required amount of the mycotoxin was dissolved in CH_3OH (50 ml) and mixed into a subsample of the diet (200 g). The sample was dried in a fumehood for 4 h and then adjusted to the exact amount with the control diet. The normal diet was treated with CH_3OH in a similar way whereafter the diets were kept at 4°C. After the initiating treatment the rats were kept on the standard diet for a period of 2 weeks. Selection of initiated cells (promotion) occurred by 2-AAF treatment (20 mg/kg body wt) on three consecutive days followed by a stimulus for cell proliferation, effected by partial hepatectomy (PH), on day 4 (15). The animals were killed 2 weeks after PH (4 weeks after initiation) and the livers processed for histochemical analyses for GGT and routine H&E staining.

Protocol 1. In studying the dosimetry of cancer initiation by FB_1 different dietary levels (750, 500, 250, 100, 50 and 25 mg FB_1/kg diet) were fed to different groups of rats over a period of 21 days. In the second experiment the smallest dose of FB_1 that effects cancer initiation over a period of 14 days was investigated by feeding four dietary levels of FB_1 (750, 500, 250 and 100 mg/kg diet) to various groups of rats. In a third experiment the cancer initiating potential of a diet containing 750 mg FB_1/kg was investigated over a period of 7 days. Control groups of rats, receiving the normal AIN-76^{TM} diet, were incorporated in each experiment. In all the experiments the promotion protocol commenced 2 weeks after the initiating treatment was completed. The feed intake and wastage was monitored during the initiating treatment. To investigate the toxicological effects of FB_1 induced during initiation rats were exposed, for a period of 21 days, to an initiating dosage (250 mg FB_1/kg diet) and two dosage levels that fail to initiate cancer (100 and 50 mg FB_1/kg diet). Rats receiving the normal diet were included as a control group. The rats were killed after 21 days and their livers processed in 10% buffered formalin for routine H&E staining.

Protocol 2. Apart from the dietary studies, experiments were conducted where an exact amount of FB_1 was dosed, per gavage, to the rats over a defined period. In a preliminary experiment a solution of FB_1 (50 mg FB_1/ml) was dosed (0.1 ml/100 g body wt) on every alternative day to two groups of the rats for 7 (4 dosages) and 11 days (6 dosages) respectively. Based on these results a second experiment was performed during which various amounts of FB_1 (5.9, 11.9, 22.6 and 32.3 mg FB_1/100 g body wt) were dosed, on a daily basis, over a period of 14 days. In both experiments selection occurred 2 weeks after initiation as described above. The livers of the rats that died after the initiating treatment during the preliminary experiment were preserved as described above for routine histopathological examination (H&E).

Protocol 3. The effect of FB_1 on hepatocyte cell proliferation was monitored by performing two different experiments. During the first experiment the rats were fed FB_1 at a dietary level of 250 mg/kg for 21 days after which they were subjected to PH and fed the control diet for the remainder of the experiment. Subgroups (3−4 rats) of these rats, as well as controls, were killed at different time points following PH (1, 3 and 7 days) after being treated with ^{3}H-labelled thymidine (20 μCi/rat) 1 h prior to termination (16). As described for studies on the dosimetry of the FB_1 with respect to cancer initiation, the effect of single gavage dosages (25, 50, 100 and 200 mg FB_1/rat) on hepatocyte cell proliferation was monitored in the second experiment. FB_1 was administered 6 h after PH (17) and the rats killed after 24 h, 1 h after the administration of [^{3}H]thymidine (20 μCi/rat). The incorporation of the radiolabelled thymidine into DNA, expressed as d.p.m./μg DNA, was determined in the DNA hydrolysates as described by Neal and Cabral (18). The DNA content of the samples was determined by the method of Giles and Myers (19) and the radioactivity by liquid scintillation counting using Ready Value (Beckmann, SA).

Results

FB_1 dosimetry and cancer initiation

Protocol 1. During the feeding study over 21 days only the rats receiving diets containing 750, 500, 250 mg FB_1/kg diet elicited hepatocyte nodules whereas no hepatocyte nodules were present in those rats receiving the diets containing 100, 50 or 25 mg FB_1/kg (Table I). The rats receiving the diets containing 750 and 500 mg FB_1 showed a significant reduction in their body wt within the first 2 weeks after the feeding regimen commenced (data not shown). The group of rats receiving the diet containing 250 mg FB_1/kg showed no significant increase in body wt during the initiating treatment as compared to the control rats and those receiving the diets containing 100, 50 and 25 mg FB_1/kg. The body wt gains of the rats receiving 750, 500, 250

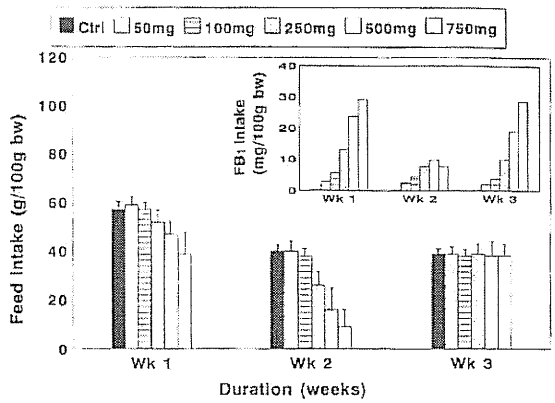

Fig. 1. Feed intake profiles of the rats measured on a daily basis of rats exposed to different dietary levels of FB_1. Data on the dietary intake records are presented on a weekly basis and represent the mean ± SD of five rats per group. Based on the weekly dietary consumption the FB_1 intake was calculated (insert).

Table II. Feed intake profiles of rats fed a diet containing different levels of FB_1 over various time intervals

Treatment (mg/FB_1/kg diet)	Duration (days)	Feed intake (g/100 g body wt)[a]	FB_1 intake (mg/100 g body wt)	Cancer initiation
750	21	87.5 ± 11.3a	65.6 ± 8.4	+
	14	49.9 ± 15.1a	37.4 ± 11.4	+
	7	39.1 ± 3.7a**	29.3 ± 2.7	+
500	21	105.1 ± 24.2a	52.6 ± 12.1	+
	14	67.1 ± 21.6a	33.5 ± 10.8	+
250	21	123.3 ± 22.0a	30.8 ± 5.5	+
	14	94.2 ± 9.2b*	23.3 ± 2.3	+/−
100	21	141.5 ± 8.4b	14.2 ± 0.8	−
	14	96.5 ± 7.3b*	9.6 ± 0.8	−
50	21	145.8 ± 10.6b	7.3 ± 0.5	−
25	21	139.4 ± 14.3b	3.5 ± 0.4	−
Control	21	144.4 ± 8.5b	−	−
	14	100.9 ± 6.6b*	−	−
	7	53.6 ± 5.1b**	−	−

[a]Means ± SD of five rats/group. Means followed by the same letter did not differ significantly ($P > 0.05$). If the letters differ then $P < 0.05$. *,**Means were compared separately with each other.

and 100 mg FB_1/kg diet were significantly lower ($0.01 < P < 0.05$) than the controls as well as those rats receiving 25 and 50 mg FB_1/kg diet (Table I). Based on the weekly feed intake profiles this reduction in the body wt gain was accompanied by a concomitant reduction in food intake and therefore of FB_1 (Figure 1). The reduction in feed intake was overcome after the second week resulting in a feed intake similar to that of the control rats at the end of the initiating treatment. Although the body wt gain of the rats receiving the diet containing 100 mg FB_1 was significantly ($0.01 < P < 0.05$) lower than the control (Table I) the food intake (g/100 g body wt) profile over the feeding period did not differ ($P > 0.05$) significantly (Table II). No reduction in the body wt gain and feed intake as compared to the control was noticed in the rats that received the diets containing 25 or 50 mg/kg FB_1. Based on the feed intake data, the apparent amount of FB_1 consumed during the initiating period of the different groups i.e. 750, 500, 250, 100, 50 and 25 mg FB_1/kg diet, was in the order of 65.6, 52.6, 30.8, 14.2, 7.3 and 3.5 mg FB_1/100 body wt respectively (Table II). The feed wastage determined during initiation was in the order of 0.36% ± 0.21 (SD).

Induction of hepatocyte nodules accompanied by reduction in body wt gain and feed intake was also observed in the rats receiving diets containing 750 and 500 mg FB_1/kg over a period of 14 days (Tables I and II). The respective amounts of FB_1 consumed were in the order of 37.4 and 33.5 mg FB_1/100 g body wt (Table II). The rats receiving a diet containing 100 and 250 mg FB_1/kg also showed a significant reduction in their body wt gain during this period whilst only one rat in the latter group elicited a single nodule in the liver. These rats received an apparent amount of 9.6 and 23.3 mg FB_1/100 g body wt respectively. A similar response with respect to the dietary intake and body wt loss was obtained when the rats were exposed to a diet containing 750 mg of FB_1/kg for a period of 1 week consuming 29.3 mg FB_1/100 g body wt. However, no hepatocyte nodules were observed in the liver of these rats (Table I).

Protocol 2. The rats (~160−180 g in body wt) receiving the gavage treatment (5 mg/100 g body wt) on every alternative day for 7 and 11 days showed a significant reduction ($P < 0.0001$) in their body wt gain (data not shown). Two out of 10 rats subjected to the latter treatment died 2 days after the initiating treatment was completed. Hepatocyte nodules (GGT^+) were induced in all the rats (8/8) that received a total dose of 47.9 ± 2.9 mg FB_1/rat (30 mg FB_1/100 g body wt) i.e. those rats receiving six dosages (5 mg/100 g body wt) over a period of 11 days. No hepatocyte nodules were noticed in the livers of the rats that received a total dosage of 36.1 ± 2.7 FB_1/rat (20 mg FB_1/100 g body wt) over 7 days i.e. four dosages of 5 mg FB_1/100 g body wt.

When dosing the rats (~150 g in body wt) with different amounts of FB_1 on a daily basis for a period of 14 days only the rats that received a total dosage of 11.9 mg FB_1/100 g body wt (daily dosage of ~0.85 mg FB_1/100 g body wt) and more, elicited nodules in the liver after selection (Table III). The livers of the rats receiving the lowest amount of FB_1 (total dosage of 5.9 mg FB_1/100 g body wt or a daily dosage of 0.40 mg FB_1/100 body wt) had no nodules although the body weight gain was significantly ($P < 0.05$) lower than that of the control rats. One out of five and two out of five rats receiving the highest dosage levels of 22.6 and 32.3 mg FB_1/100 g body wt respectively died after the initiating treatment.

Effect on hepatocyte proliferation

Protocol 3. When fed at a dietary level of 250 mg/kg for 21 days FB_1 effectively delays hepatocyte proliferation induced by partial hepatectomy (Figure 2A). The incorporation of [^{3}H]thymidine was significantly ($P < 0.001$) lower after 24 h when compared to the control group. After 3 days the FB_1 treated rats showed a delayed response as the DNA synthesis was significantly ($P < 0.02$) higher than the controls. There was no difference in DNA synthesis between the two groups after 7 days. When administered 6 h after PH, a single dose (50, 100 and 200 mg/kg body wt) significantly ($P < 0.01$) inhibited hepatocyte proliferation (Figure 2B). At a lower dosage level (25 mg FB_1/kg body wt) FB_1 also markedly inhibited the incorporation of [^{3}H]thymidine but the difference was not significant ($P > 0.05$).

Histopathological examination

The livers of the rats that died (protocol 2) after the gavage treatment (50 mg/kg; six dosages over 11 days) showed pathological lesions very similar to that described previously (2,3). These included degenerative changes such as hydropic swelling, as well

Table II. The dose dependent cancer initiating activity of FB_1 during gavage dosing to rats

Treatment (mg FB_1/100 g body wt)	Body wt (g)[a] (gain or loss)	No. of rats with nodules	GGT+ foci and nodules[b]		
			No./cm^2	Mean area	Mean % area/ liver section
Control (saline)	58.0 ± 4.2aA	0/5	nd	nd	nd
5.9	43.2 ± 10.7bA	0/5	nd	nd	nd
11.9	30.4 ± 7.1cB	3/5(2–6)	0.77 ± 0.48	0.02 ± 0.14	1.77 ± 1.75
22.6	−5.8 ± 35.2dC*	4/4(3–6)	1.22 ± 0.61	0.02 ± 0.005	4.56 ± 4.24
32.3	4.0 ± 25.9dC**	3/3(6–24)	2.78 ± 0.78	0.05 ± 0.04	11.06 ± 7.38

[a]Values represent the means ± SD. Means followed by the same letter (lower case) then $P > 0.05$. If letters differ then $0.05 > P > 0.01$. If letters (upper case) differ then $0.0001 < P < 0.01$.
[b]Values represent means ± SD.
nd, not detected.
*1/5 and **2/5 rats died after initiation.

as hyaline droplet accumulation. Single cell necrosis, increased mitotic figures and lipid accumulation were noticed throughout the liver as well as early signs of fibrosis. Proliferation of bile ductules that resulted in the distortion of the normal architecture of the liver lobe was also observed. Similar changes, although far less pronounced, were observed in the livers of the rats that received the initiating dosage of FB_1 (250 mg FB_1/kg diet) over a period of 21 days. The lesions in the liver of the rats receiving the non-initiating dietary treatment (100 mg FB_1/kg) were not as prominent while no proliferation of bile ductules was noticed. Except for a few necrotic cells no differences were noticed between the rats receiving a dietary level of 50 mg FB_1/kg and the control.

Discussion

Dietary intake profiles of the rats fed diets containing 250, 500 and 750 mg FB_1/kg indicated that the reduction in rat body wt gain within the first 2 weeks coincides with a simultaneous reduction of feed intake. This effect was overcome during the third week after which the feed intake profiles reach the same level as that of the control group. At present it is not known whether this effect could be related to metabolism which renders the animals more resistant to the toxicological effects of the fumonisins. In a long-term feeding experiment, rats exposed to a diet containing 50 mg FB_1/kg, also showed a marked reduction in body wt gain (5) as compared to the control group. As no feed intake records were collected it is not known whether the lower body wt gain could be ascribed to a feed refusal effect. The feed refusal effect of FB_1 at lower dietary levels therefore needs to be investigated.

Data regarding the dose response effects of FB_1 with respect to cancer initiation indicated that, when incorporated in the diet at a dosage of 250 mg FB_1/kg and higher (500 and 750 mg FB_1/kg diet), FB_1 induced the formation of hepatocyte nodules when fed over a period of 21 days (Table I). Based on the feed intake records, initiation is effected in rats receiving a dosage level of 30.8 mg FB_1/100 g body wt over a period of 21 days (Table II). As no cancer initiation was effected in the rats fed the diet containing 100 mg FB_1/kg the effective dosage level (EDL) for cancer initiation over 21 days is $14.2 < EDL < 30.8$ mg FB_1/100 g body wt (Table II) or $0.7 < EDL < 1.5$ mg FB_1/100 g body wt when based on a daily feed intake.

When the respective diets were fed over a period of 14 days initiation was obtained in all the rats receiving the diets containing 500 and 750 mg FB_1/kg. Only one rat receiving 250 mg FB_1/kg diet had a single nodule present in the liver and hence was taken as the threshold for initiation over 14 days. Based on these data

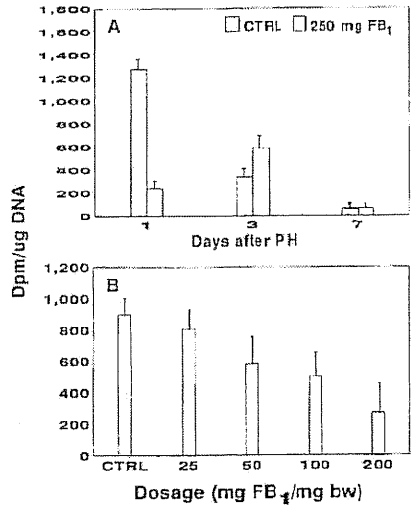

Fig. 2. Inhibitory effect of FB_1 on hepatocyte cell proliferation. (A) Rats fed a diet containing 250 mg FB_1/kg for 21 days were subjected to PH and the incorporation of ^{3}H-labelled thymidine into DNA monitored after 1, 3 and 7 days. (B) Rats were first subjected to PH, treated with a single gavage dosage of FB_1 after 6 h and the incorporation of ^{3}H-labelled thymidine into DNA monitored after 24 h. Values represent the mean of duplicate determinations ± SD of three rats per group.

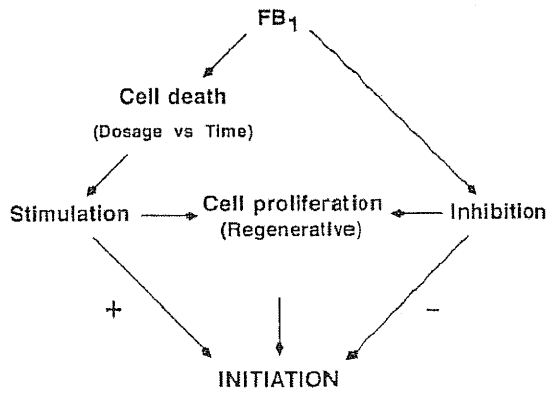

Fig. 3. Diagrammatic representation of the stimulatory effect of FB_1 on cell proliferation via the induction of hepatocyte cell death and the resultant compensatory cell proliferation which is required for cancer initiation. In contrast, the inhibitory effect of FB_1 on cell proliferation is likely to counteract cancer initiation and thus will play a determining role in the amount of FB_1 required to effect cancer initiation within a specific time frame.

the EDL values for cancer initiation during 14 days are of the order of $23.3 < EDL < 33.3$ mg FB_1/100 g body wt (Table II) which is equivalent to a daily dosage of $1.7 < EDL < 2.4$ mg FB_1/100 g body wt. The effective dosage level for cancer initiation [$5.4 < EDL < 11.6$ mg FB_1/100 g body wt over 14 days (Table III) or $0.39 < EDL < 0.83$ mg FB_1/100 g body wt when calculated on a daily basis] obtained from the gavage study is ~3-fold lower than that obtained from the feeding study. As the method of exposure differs from a short pulse exposure during the gavage study to a more gradual exposure during the feeding study it is not known whether these parameters can be directly compared.

No cancer initiation was obtained when rats were fed a diet containing 750 mg FB_1/kg for a period of 7 days. The body wts of these rats were significantly ($0.0001 < P < 0.01$) lower than the controls and based on the dietary intake they received a dosage of 29.3 mg FB_1/100 g body wt (Table II). However, in rats receiving similar dosages of 33.5 or 30.8 mg FB_1/100 g body wt but over a period of 14 and 21 days respectively, cancer initiation was effected (Tables I and II). A similar effect was obtained during the gavage dosing where rats receiving ~36 mg over a period of 7 days did not induce hepatocyte nodules (protocol 2). It can be concluded that, as a function of time, a critical dosage level exists for cancer initiation and that a higher dosage level administered over a reduced period of time does not necessarily initiate cancer.

Hepatocyte proliferation induced either by PH or a toxic effect induced by a compound, is a prerequisite for initiation (20). Therefore, the inhibitory effect of FB_1 on hepatic cell proliferation is likely to play a determining role regarding the amount of FB_1 needed to initiate cancer within a certain time frame. At a dietary level of 250 mg FB_1/kg, which effects cancer initiation after 21 days, hepatic cell proliferation induced by PH was delayed effectively (Figure 2A). The amount of FB_1 consumed over this period is ~30 mg FB_1/100 g body wt or a daily intake of 1.5 mg FB_1/100 g body wt. Even a single dose (5, 10 and 20 mg/100 g body wt) of FB_1 significantly ($P < 0.05$) inhibited hepatocyte cell proliferation (Figure 2B). It is known that FB_1 induces a toxic effect in the liver during dietary exposure (5) and/or single or multiple dosing (1) of rats and hence the resultant cell proliferation could facilitate cancer initiation. However, the inhibitory effect of hepatocyte proliferation is likely to affect initiation as found previously where single (50 and 100 mg FB_1/kg body wt) or multiple (ranging from 200 to 50 mg FB_1/kg body wt) dosing of rats following PH failed to initiate cancer (6).

Therefore, as discussed earlier data obtained from the feeding studies indicated that, as a function of time, a critical dosage level exists ($13.2 < EDL < 30.8$ mg FB_1/100 g body wt over 21 days and $23.3 < EDL < 33.5$ FB_1/100 g body wt over 14 days) for cancer initiation. The inhibitory effect of FB_1 on hepatocyte proliferation is likely to play a determining role in the amount required for cancer initiation. This can be deduced when considering the following aspects: when a rat is exposed to 29.3 mg FB_1/100 g body wt (feeding study, Table II) or 36 mg/100 g body wt (gavage study, protocol 2) over 7 days hepatocyte proliferation is likely to be inhibited due to the relatively high dosage level in a comparatively short time as compared to the exposure levels mentioned above. At low dosage levels (3.5, 7.3 and 14.2 mg FB_1/100 g body wt over 21 days, Table II) no initiation occurs probably because of a low toxicity of the compound and hence a low background level of hepatocyte cell proliferation. Histopathologically the livers of the rats exposed to these dietary levels showed far less toxicological lesions as compared to the livers of the rats exposed to the higher dietary levels. These data provide proof that, in addition to a previous paper (6), the toxic effects play an important role in the cancer initiating activity of the fumonisins. The lack of hepatotoxicity during the initiating treatment could therefore be the reason for the *N*-acetyl analogue of FB_1, FA_1 not to initiate cancer in rat liver (12).

A balance is likely to exist between the compensatory cell proliferation due to the hepatotoxicity induced by FB_1 and the inhibitory effect of the compound on hepatocyte cell proliferation, the outcome of which will determine the threshold for cancer initiation by FB_1 in the liver (Figure 3). This is in agreement with the studies using different carcinogens that an increase in cell proliferation induced by a carcinogen resulted in an increase in the amount of initiated cells and tumor prevalence in a specific organ (21). Therefore, in relation to the cancer initiating potential of FB_1, the inhibitory effect on hepatocyte proliferation could explain the fact that FB_1 is regarded as a slow cancer initiator (6) and that prolonged exposure at high dosage levels is required to facilitate cancer induction.

The hepatotoxicity of FB_1 has been suggested as playing an important role in the cancer promoting activity of the compounds (1,5). The present study indicated, that in addition to the hepatotoxic effects, the inhibitory effect of FB_1 on hepatocyte proliferation is likely to be the major determinant during cancer promotion. On the one hand the compensatory cell proliferation as a result of the hepatotoxicity of FB_1 could selectively stimulate the outgrowth of initiated cells resistant to the toxic effects of FB_1 while on the other hand the proliferation of normal cells is inhibited. In this regard FB_1 simulates the cancer promoting effect of 2-AAF in the resistant hepatocyte model (7).

Cancer induction by FB_1 in rat liver is masked by the cirrhosis (5) resulting in hepatocellular carcinoma being shown to occur within large regenerative nodules. However, it was noted that these regenerative nodules contained focal areas of hepatocytes with a typical ground glass appearance which stained positively for GGT. The fact that FB_1 induces initiated cells and also closely mimics the properties of many carcinogens (22) with respect to the induction of cell death and inhibition of hepatocyte proliferation suggests that, during the chronic feeding study (5), hepatocyte nodules similar to those induced in the short-term carcinogenesis protocol by FB_1 could be the site for the development of liver cancer during the long-term feeding study.

The present study on the dosimetry of FB_1 with respect to cancer initiation indicated that the hepatotoxic effects of the compound cannot be separated from the carcinogenicity of the compound and that threshold for cancer initiation by FB_1 exists as a function of time. This is in agreement with the hypothesis that cancer initiation by non-genotoxic carcinogens which act solely through a cytotoxic mechanism is likely to have a no-effect threshold related to toxicity and regeneration (21).

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Mitoinhibitory effect of fumonisin B_1 on rat hepatocytes in primary culture

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The inhibitory effect of fumonisin B_1 (FB_1) on epidermal growth factor (EGF)-induced DNA synthesis in primary rat hepatocytes was investigated by monitoring the incorporation of [^{3}H]thymidine in the DNA. A pulse-labelling technique was adapted to determine the incorporation of the radioactivity in the DNA (S-phase) quantitatively. FB_1 inhibits the EGF-induced DNA synthesis up to 90% when incorporated at concentrations of 150 to 300 μM for a period of 44 h. A continued presence of FB_1 is required to exhibit this inhibition as (i) the subsequent removal of FB_1 resulted in a reversal of the effect, (ii) a higher stimulatory response in EGF-treated hepatocytes was found when the exposure period of hepatocytes to FB_1 was reduced, and (iii) pretreatment of hepatocytes with FB_1 only slightly reduced (not significantly) DNA synthesis induced by EGF. Whilst the growth inhibitory effect of FB_1 was not associated with a cytotoxic effect, binding studies using [^{125}I]EGF indicated that the growth factor–receptor interaction was not altered. No relationship was found between the disruption of the sphingolipid biosynthesis by FB_1 and (i) the mitoinhibitory effect on the EGF response and (ii) the cytotoxicity of FB_1 in primary hepatocytes.

Introduction

Fumonisin B_1 (FB_1*), a naturally occurring mycotoxin produced by *Fusarium moniliforme* in corn, is hepatotoxic and hepatocarcinogenic when fed chronically to rats (1). Many questions exist with respect to the mechanisms involved during cancer induction by the fumonisins as recent short-term mutagenesis and DNA repair studies (2,3) indicated that FB_1 lacks genotoxic effects. However, short-term carcinogenesis studies (2,4), utilizing a cancer initiating/promoting model in rat liver, indicated that FB_1 closely mimics the properties of many genotoxic carcinogens (5,6) with respect to the induction of initiated cells resistant to the mitoinhibitory effect of 2-acetylaminofluorene and its inhibitory effect on hepatocyte cell proliferation.

FB_1 is a weak cancer initiator in rat liver as high dosage levels over an extended period of time are required (4). It would appear that the events involved during initiation occur at a much slower rate compared to the majority of hepatocarcinogens which require a single or brief exposure in the presence of cell proliferation (7). The reason why the fumonisins are regarded as slow cancer initiators (4) could be due to their role as inhibitors of hepatocyte cell proliferation, a prerequisite for initiation (8). Conversely, the inhibitory effect on cell proliferation has been suggested to play a critical role in the cancer-promoting potential of the fumonisins (3,4). This was deduced from the fact that the outgrowth of diethylnitrosamine (DEN)-initiated cells into hyperplastic foci was selectively stimulated by FB_1, indicating that differential inhibition could be an important mechanism in the cancer promoting activity of the fumonisins (9). The exact mechanism involved in the inhibitory effect on hepatocyte proliferation is not currently known.

Recent studies indicated that FB_1 is a potent inhibitor of sphingolipid biosynthesis (10) which results in the rapid accumulation of the sphingoid base, sphinganine, in different cell culture systems. It has been suggested that the accumulation of sphinganine is involved in the toxicity of the fumonisins to a renal epithelial cell line (11, 12) and could be an important mechanism explaining the toxicological effects of *F.moniliforme* in various animal species. As the disruption of the sphingolipid biosynthetic pathway affects various aspects regarding cellular growth and differentiation (13), it was suggested that the latter could play an important role in the carcinogenicity of FB_1 (10,13,14). In this regard the inhibitory effect of sphingolipids on protein kinase C (PKC) (15) and the stimulation of both the affinity and quantity of the epidermal growth factor (EGF) receptor (16) appear to be important with respect to the carcinogenic behaviour of the fumonisins. A recent study has shown that FB_1 exhibited a growth stimulatory effect in Swiss 3T3 cells, suggesting that the fumonisins play a mitogenic role via the accumulation of sphingoid bases (14). However, presently very little is known about the role of the disruption of sphingolipid metabolism in cancer induction by the fumonisins *in vivo*.

The present study investigates the mechanism involved in the inhibitory effect of FB_1 on cell proliferation *in vitro* by monitoring the effect on the EGF induced DNA synthesis in primary rat hepatocyte cultures.

Materials and methods

Mycotoxin standards and chemicals

FB_1, FB_2, FA_1 (the *N*-acetyl derivative of FB_1) and AP_1 (a hydrolysis product of FB_1) were purified as described previously by Cawood *et al.* (17) and Gelderblom *et al.* (18). Solutions of the different toxins were prepared in saline for FB_1 and in saline/DMSO (1:1) for FA_1, AP_1 and FB_2 on the day used. Fifty microlitres of the respective solutions was added per dish. EGF, sphinganine and sphingosine were obtained from Sigma Chemical Company (St Louis, MO, USA) and [^{125}I]EGF and [^{3}H]thymidine from Amersham, London, UK.

Preparation of hepatocyte cultures

Primary hepatocytes were prepared from male Fischer 344 rats, weighing 150–200 g, by the collagenase perfusion technique as described previously (19). The cells were seeded in collagen-coated dishes for 3 h in modified Williams' E (WE) medium containing fetal bovine serum (10%), insulin (20 U/l), L-glutamine (2 mM), HEPES (10 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). The cells were washed with Hank's buffer solution and supplemented with serum-free, modified WE medium containing L-proline (2 mM) and sodium pyruvate (10 mM) and incubated at 37°C for the desired

Abbreviations: FB_1, fumonisin B_1; DEN, diethylnitrosamine; PKC, protein kinase C; EGF, epidermal growth factor; LDH, lactate dehydrogenase; PB, phenobarbital.

period (see below). Cells were plated at a density of 200 000 (3 cm) or 600 000 (6 cm) per dish.

Pulse-labelling

The technique was adapted from the method of Wiley *et al.* (20) in order to determine quantitatively the labelling index (incorporation of [^{3}H]thymidine into the DNA) of the hepatocytes exposed to a growth factor, such as EGF. The period when the majority of cells are in the S-phase of the cell cycle, using EGF as the growth stimulator, was determined in a series of experiments during which the [^{3}H]thymidine was incubated with the hepatocytes for 4 h intervals beginning at 36–40 h up to 44–48 h after the addition of EGF (50 ng/dish). The non-specific binding of the [^{3}H]thymidine was monitored in cultures in which DNA synthesis was inhibited by the addition of hydroxyurea (25 mM) 30 min prior to the treatment with [^{3}H]thymidine (21). Following the pulse-labelling treatment the dishes were washed with saline (3×3 ml), 5 % trichloroacetic acid (3×3 ml) and finally with absolute ethanol (3×3 ml). The cells were solubilized in 2% SDS solution containing 2 mM EDTA and 20 mM $NaHCO_3$. Aliquots of this solution were used to determine the radioactivity (200 µl) and protein (30 µl) concentration. Scintillation counting (d.p.m.) was performed using Ready Value scintillation cocktail (Beckman, SA) on a Packard (Downers Grove. IL, USA) Tri-carb 460 CD instrument. The protein concentration was determined by the method of Kaushal and Barnes (22) with bicinchoninic acid using bovine serum albumin as protein standard. The specific incorporation of the radiolabel in the hepatocytes (DNA) was expressed as d.p.m./h/mg cellular protein.

Effect on EGF-induced DNA synthesis

Dose–response effects. The effect of FB_1 on DNA synthesis was monitored by incorporating different concentrations (12.5, 25, 75, 150 and 300 µM FB_1/dish) of the compound in the incubation medium in the presence or absence of EGF. The effect of lower concentrations of FB_1 (1, 5, 10 µM FB_1/dish) on the EGF response was monitored in a separate experiment. Structure–activity relationships with respect to the effect on the growth stimulatory effect of EGF were investigated by including FB_2 (27.75 and 150 µM) and two structural analogues of FB_1, i.e. FA_1 (25, 75, 150 µM) and AP_1 (25, 75, 150 µM). The cytotoxicity of the different compounds to the hepatocyte cultures was monitored as described previously (19).

Time-dependent effects. EGF (50 ng/dish) was incubated with the hepatocytes after plating, while FB_1 (75 µM) was added at varying time intervals, i.e. together with EGF and 6, 12 and 24 h after the addition of EGF. In a similar experiment cells were treated with EGF and FB_1 (75 µM) for 12 h after which the medium was replaced with the control medium, either with or without FB_1 (75 µM), for the remainder of the incubation period (32 h). Cultures receiving only EGF for a period of 12 h were used as the controls.

Effect of pretreatment. The hepatocyte cultures were treated with FB_1 (75 µM) for 12 h after plating. Following the 12 h period, culture media of both the FB_1-treated and untreated dishes (controls) were replaced with culture media containing (i) FB_1, (ii) EGF, (iii) FB_1 and EGF and (iv) control medium. The DNA synthesis was monitored at 40–44 h after the treatment with EGF, i.e. 52–56 h after plating.

[^{125}I]EGF binding to primary hepatocyte cultures

The effect of FB_1 on the interaction of EGF with its receptor was monitored (23) by incubating the monolayers with different concentrations of the mycotoxin (50, 75, 150 µM) for 24 h. The cells were rapidly cooled to 4°C and incubated with 2 ng [^{125}I]EGF for 4 h, after which the dishes were carefully washed (4–5×2 ml cold saline) and the cells solubilized in 2% SDS solution. The radioactivity was quantitated with a Beckman gamma counter using Ready Value scintillation fluid, while the cellular protein was determined by the method of Kaushal and Barnes (22). The non-specific binding was estimated in parallel incubations in the presence of 500-fold excess unlabelled EGF.

Effect of sphinganine and sphingosine on EGF-induced DNA synthesis

The effect of sphinganine (1, 2.5 and 5 µM) and sphingosine (1, 2.5 and 5 µM) on the EGF-induced response was monitored over a period of 44 h (pulse-labelling) as described for FB_1. The sphingoid bases were complexed to bovine serum albumin (16) prior to addition to the cultured dishes. The release of lactate dehydrogenase (LDH) in the cultured medium was monitored at 44 h to determine whether any cytotoxic effects occurred.

Sphinganine/sphingosine ratios in primary hepatocytes treated with FB_1

The sphingoid base concentrations in the primary hepatocytes were determined according to the method described by Riley *et al.* (24) with minor modifications, using eicosasphinganine (kindly supplied by A.H.Merril, Atlanta, GA) as internal standard. The cultured dishes (6 cm) were washed with ice-cold saline (3×2 ml), after which the plated cells were harvested (saline; 3×0.5 ml) by scraping with a rubber policeman. An aliquot (0.1 ml) was removed for protein determination prior to lipid extraction. The cell suspension was extracted with $CH_3OH/CHCl_3$ (2:1; sample to solvent ratio = 8) containing 0.01% butylated hydroxytoluene as an antioxidant. Quantitation was performed by HPLC using a gradient [CH_3OH and CH_3OH/0.005 M potasium phosphate buffer, pH 7 (8:2)] solvent system with a Radial Pak C_{18} column and fluorescence detection (Perkin–Elmer 650S).

Statistical analysis

Student's *t*-test was used to determine the statistical differences between treatment means.

Results

Pulse-labelling experiments

The majority of hepatocytes treated with EGF immediately after plating and exposed to [^{3}H]thymidine for 4 h at various time intervals entered the S-phase of the cell cycle between 40 and 44 h after plating (data not shown). Subsequent experiments were therefore conducted using a pulse-labelling schedule between 40 and 44 h. When monitoring the incorporation of [^{3}H]thymidine in EGF-treated hepatocytes a specific labelling of 361 489 ± 18 030 d.p.m./h/mg cellular protein (mean of seven experiments) was obtained. Untreated hepatocytes exhibited a specific labelling of 82 239 ± 8230 d.p.m./h/mg cellular protein (mean of seven experiments), giving a DNA synthesis stimulatory ratio (control versus EGF) of ±1:4. Despite the fact that the EGF-induced stimulatory response varies between different isolation batches and whilst the pulse time schedule of 40–44 h is not always in phase with respect to the exact period when the majority of cells are in the S-phase, the ratio of EGF-treated to untreated cells was found to vary between 3 and 4 thoughout the study. The non-specific binding of the [^{3}H]thymidine to the hepatocytes, determined by using hydroxyurea, was of the order of 11 493 ± 820 and 22 746 ± 1540 for the control and the EGF-treated hepatocytes respectively.

Dose-dependent effects on the EGF-induced response

When incorporated in the incubation medium at a concentration of 25 µM and 12.5 µM respectively, FB_1 significantly ($P < 0.05$) to marginally significantly ($P < 0.1$) inhibited the incorporation of [^{3}H]thymidine in EGF-treated hepatocytes (Figure 1A). The inhibitory effect was considerably greater at higher FB_1 concentration levels, while no effect was obtained below 12.5 µM, i.e. 5 and 1 µM (data not shown). No cytotoxic effect could be detected at FB_1 concentrations up to 150 µM in the absence of EGF, while a significant increase ($P = 0.001$) was noticed at 300 µM. The FB_1 inhibition of the EGF response was achieved at concentrations 10-fold lower than the cytotoxic dosage.

In the presence of EGF a slightly, but significantly ($P < 0.05$), lower cytotoxicity was observed at all FB_1 concentrations as well as the controls (Figure 1B), implying that EGF renders hepatocyte cultures more resistant to cytotoxic effects of chemicals. This is clearly demonstrated as the EGF-treated cells showed no cytotoxic effects towards 300 µM FB_1 as compared to the controls. The baseline labelling of cells in the absence of EGF was also significantly ($P < 0.05$) decreased by FB_1 (Figure 1A).

Time-dependent effects of FB_1 treatment

The time-dependent inhibition and the reversibility of the FB_1 effect on the EGF-induced stimulatory response were investigated. When FB_1 (75 µM) was added at different intervals, i.e. at the same time as EGF, 6, 12 and 24 h following the addition of EGF, the inhibition declined progressively (Figure 2A). The highest inhibition (83–84%) was observed

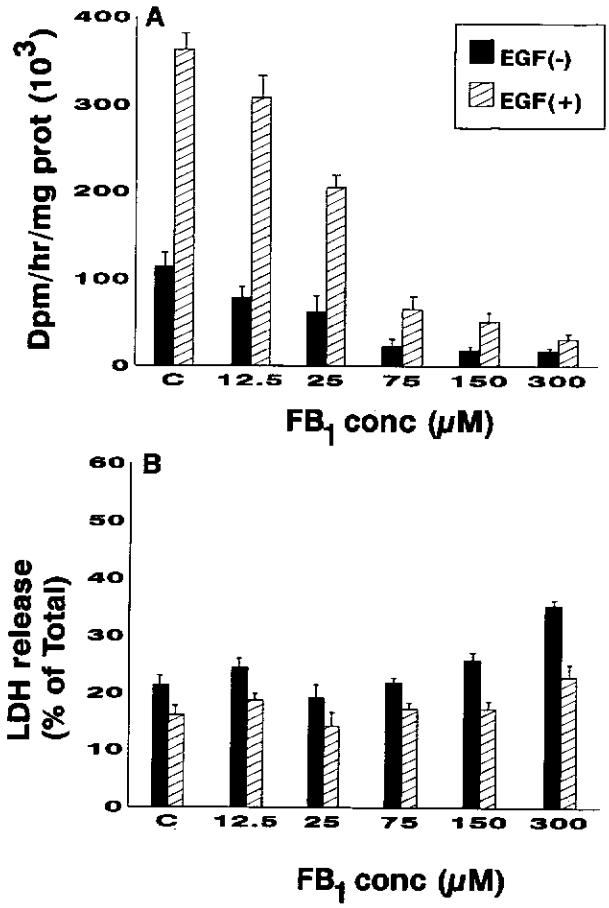

Fig. 1. (A) Inhibitory effect of different concentrations of FB_1 on the growth stimulatory response (DNA synthesis) of EGF in hepatocytes. (B) Cytotoxicity of the different concentrations of FB_1 to hepatocytes expressed as the amount of LDH released as a percentage of the control. Values represent means of triplicate determinations ± SE.

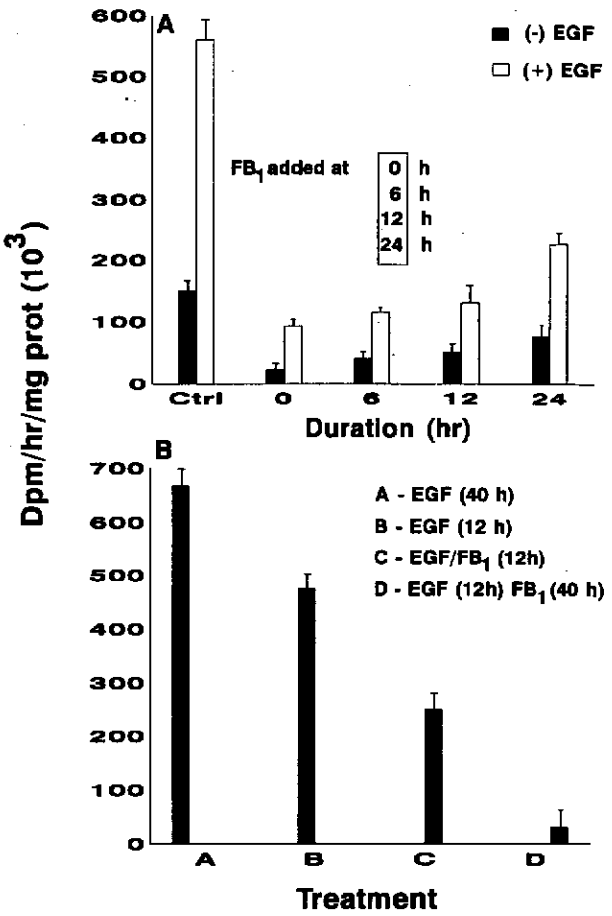

Fig. 2. Time-dependent effect and reversibility of FB_1 inhibition of the growth stimulatory response induced by EGF. (A) FB_1 (75 μM) added at various time intervals in relation to the initial addition of EGF. (B) Reversibility of the inhibitory effect of FB_1 (75 μM) on EGF response in hepatocytes exposed for 12 h to the growth factor. Values represent means of triplicate determinations ± SE.

when FB_1 and EGF were added simultaneously, i.e. 3 h after plating, while addition of FB_1 after 6, 12 and 24 h resulted in significantly ($P < 0.0001$) less inhibition (78%, 77% and 60% respectively). There was no significant difference ($P > 0.05$) between the 6 and 12 h treatments whilst both treatments showed a significantly higher ($P < 0.0004$) inhibition than when FB_1 was added after 24 h.

In a subsequent experiment the growth medium was removed 12 h after incubation with EGF in the presence and/or absence of FB_1 and replaced by the basal medium with or without FB_1. When EGF was removed after 12 h from the incubation medium, 70% of the total growth stimulatory response, i.e. when EGF is present for the entire incubation period, was obtained. These data imply that the majority of cells (±70%) have been programmed to enter the cell cycle after the first 12 h of exposure to EGF. The highest inhibition (80–85%) was (as described above) obtained when FB_1 (75 μM) was continuously present in the incubation medium after EGF was removed (Figure 2B). When FB_1 was also removed after 12 h, 50–53% of the maximum response, when EGF was removed after 12 h, was obtained. However, when FB_1 was present for the entire incubation period, only 6% of the cells entered the S-phase.

Pretreatment of hepatocytes

In a third series of experiments the 'memory' effect of hepatocytes exposed to FB_1 (75 μM) was monitored. As illustrated in Figure 3, pretreatment of the hepatocytes with FB_1 for 12 h slightly inhibited the EGF response (treatment B) although it was not significant ($P > 0.05$). When FB_1 was added for the entire duration of the experiment (treatment D) and/or 12 h after plating (treatment C), a significant ($P < 0.001$) inhibition of the EGF response was again obtained, which is in accordance with the above experiments. The continued presence of FB_1, i.e. 12 h pretreatment and the 44 h treatment in the presence of EGF, provided the highest inhibitory effect (72%), which was significantly ($P < 0.05$) higher than when the pretreatment was omitted, for which a 60% inhibition was obtained.

Effect of FB_1 on [^{125}I]EGF binding

Data obtained from the binding studies using [^{125}I]EGF (Table I) indicated that exposure (24 h) of hepatocytes to FB_1 did not significantly ($P > 0.05$) affect the interaction of the growth factor with its receptor.

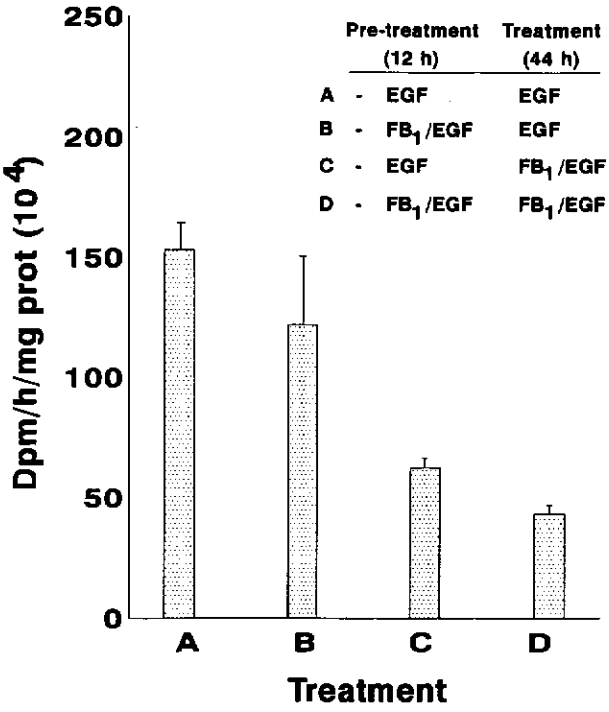

Fig. 3. Effect of a 12 h FB_1 (75 μM) pretreatment (memory effect) on the EGF-induced response. Pulse-labelling of the cultures was performed 40 h after the addition of EGF, i.e. 52 h after plating. Values represent means of triplicate determinations ± SE.

Table I. Saturation binding of EGF to primary rat hepatocytes treated with FB_1 for 24 h

Treatment	ng EGF/mg protein	Relative binding (% of control)
Control	28.40±4.96[a]	100
FB_1		
25 μM	23.93±1.67[a]	90.0
75 μM	25.97±2.38[a]	97.5
150 μM	23.00±3.63[a]	86.7
Non-specific binding*	4.50±1.46	24.5

*[^{125}I]EGF binding performed in the presence of a 500-fold excess unlabelled EGF. Values represent means of triplicate determinations ± standard error. The experiment was conducted twice with a similar pattern of results. Means (column) followed by the same letter do not differ significantly from each other.

Effects of sphinganine and sphingosine

Addition of the sphingoid bases to the culture dishes did not stimulate DNA synthesis in primary hepatocytes under the present conditions, nor did it enhance the growth stimulatory effect of EGF (Table III). However, FB_1 did significantly increase the level of sphinganine in the primary hepatocytes when exposed to different concentrations of the mycotoxin, while the sphingosine level was not altered (Table II). With respect to dose–response effects, no significant differences ($P > 0.1$) were obtained in the sphinganine concentrations in the hepatocytes exposed to either 1 or 300 μM FB_1, in one experiment, or between 5 and 500 μM in the other experiment (Table II). This implies that the sphinganine concentration already reached its maximum value in cultures exposed at 1 μM FB_1. A 5- to 8-fold increase in the level of sphinganine, which resulted in a 10- to 20-fold increase in the sphinganine/sphingosine ratio (Table II), was also noticed in the control dishes incubated for 44 h as compared to the zero time (3 h after plating) samples. The cytotoxicity monitored after 44 h in the control dishes was of the order of 20% (Figure 1B).

Table II. The effect of FB_1 on sphingolipid biosynthesis in primary hepatocytes

Sample	Concentration (pmol/mg protein)		Sa/So ratio
	Sphingosine (So)	Sphinganine (Sa)	
Control			
0 h	**22.3±3.5[a]**	**2.1±0.3[a]**	**0.1±0.02[a]**
	71.4±16.6[a]	9.1±3.4[a]	0.1±0.03[a]
44 h	**18.9±1.3[a]**	**16.4±2.4[b]**	**0.9±0.07[b]**
	18.0±3.6[b]	44.2±2.5[b]	2.6±0.7[b]
FB_1			
1 μM	**17.6±3.8[a]**	**379.0±5.4[a]**	**22.3±4.6[a]**
5 μM	23.6±7.0[a]	643.0±169[a]	28.1±6.4[a]
75 μM	25.3±4.8[a]	675.0±143[a]	26.6±0.7[a]
150 μM	19.71±6.2[a]	563.0±176[a]	28.9±3.5[a]
300 μM	**19.4±6.2[a]**	**455.0±74.4[a]**	**24.4±3.7[a]**
500 μM	18.5±5.4[a]	466.0±122.1[a]	25.3±2.2[a]

Values represent means ± SD of triplicate determinations. Means (columns) followed by the same letter do not differ significantly. If letter differs, then $P < 0.0001$. Data in bold type represent a different experiment. Stastistical comparisons were made within each experiment.

Discussion

The fumonisin B mycotoxins were characterized as cancer promoters utilizing a cancer initiation/promotion model (5) in rat liver with DEN as the cancer initiator and the formation of γ-glutamyltranspeptidase-positive foci as endpoint (9). The inhibitory effect of FB_1 on hepatocyte cell proliferation (4) has been suggested to be important during cancer promotion whereby the outgrowth of the DEN-initiated cells, resistant to this inhibitory effect, could be selectively stimulated. At present very little is known about the mechanism of the inhibitory effect of the fumonisins on hepatocyte cell proliferation and whether this could be responsible for the growth differential that selectively stimulates the outgrowth of initiated hepatocytes into hepatocyte nodules (3,4).

The pulse-labelling technique that was adapted and modified for the quantitative determination of the growth stimulatory response induced by EGF provided an ideal bioassay to study the *in vitro* mitoinhibitory effect of FB_1. The data indicated that FB_1 inhibited both the EGF-induced as well as the background (basal medium) DNA synthesis in a dose-dependent manner (Figure 1A). In this regard it has to be pointed out that the basal medium contains insulin (20 U/l), another growth stimulatory factor for hepatocytes. This inhibitory effect was not due to cell death as the effect was noticed at a concentration of 10 times less than the concentration that induces a cytotoxic effect. At present it is not known whether changes that would eventually precipitate the cytotoxic effect could be involved, as the highest inhibition was effected at a cytotoxic concentration of FB_1. In addition to FB_1, FB_2, the *N*-acetyl analogue (FA_1) and the hydrolysis product (AP_1) of FB_1 also inhibited EGF-stimulated DNA synthesis at concentration levels (25, 75 and 150 μM/dish) below those that induce cytotoxic effects (data not shown). At higher concentrations these compounds are, like FB_1, all cytotoxic to primary hepatocytes (18). However, FA_1 and AP_1 lack hepatotoxicity and cancer-initiating

Table III. Effect of sphinganine and sphingosine on the EGF-induced stimulatory effect in primary hepatocyte cultures

Sphingolipid	EGF	Sphingolipid concentration (μM/dish)			
		Control	1	2.5	5
Sphinganine [labelling	−	23.34±3.09	22.40±0.59	22.37±1.2	24.34±1.02
(d.p.m./h/mg protein)×10^4]	+	117.06±9.24	100.67±1.6	104.86±6.85	110.08±5.59
LDH release	−	**17.0±2.3**	**11.3±1.0**	**12.1±1.7**	**20.8±2.7**
(% of total)	+	**10.5±2.4**	**6.8±1.7**	**6.5±0.9**	**14.0±2.3**
Sphingosine [labelling	−	27.27±1.32	27.25±0.6	22.71±0.35	27.5±1.06
(d.p.m./h/mg protein)×10^4]	+	118.56±1.54	117.63±7.67	116.75±6.91	120.49±2.22
LDH release	−	**17.0±2.3**	**10.9±0.8**	**12.1±0.5**	**17.5±0.4**
(% of total)	+	**10.5±2.4**	**5.5±1.3**	**7.2±1.6**	**10.8±0.4**

Values represent means ± SD. The experiment was conducted three times with a similar pattern of results. Data in bold type represent the cytotoxicity measured as the release of LDH after 44 h. Data (rows) did not differ significantly from each other ($P > 0.05$) in any of the treatments.

activity *in vivo* when fed at specific dietary concentrations (18). The present data indicated that these compounds are likely to act as cancer promoters since they simulate the mitoinhibitory effect of FB_1 *in vitro*.

FB_1 seems to be an effective inhibitor of mainly the late events of the G_1 phase of the cell cycle although early events also appear to be slighty altered. Only 20% of the hepatocytes entered the S-phase when FB_1 was added 12 h after the addition of EGF and 40% when the compound was added after 24 h (Figure 2A). With respect to the first condition, i.e. when hepatocytes are exposed for 12 h to EGF, 70% of the cells are programmed to enter the S-phase of DNA synthesis (Figure 2B). When hepatocytes are exposed for 12 h to EGF, in the presence of FB_1, 50–53% of the cells (of a total of 70%) entered the S-phase (Figure 2B). In addition to this, a 12 h pretreatment of the cells with FB_1 only marginally ($0.05 < P < 0.1$) inhibited the EGF response (Figure 3), indicating that hepatocytes retained very little 'memory' of FB_1 exposure. In all the cases a continued presence of FB_1 resulted in the highest inhibition of the EGF response.

These data suggest that the EGF-induced DNA synthesis pathway is inhibited by FB_1 in a reversible manner. As FB_1 did not interfere with the binding of [^{125}I]EGF following an exposure period of 12 h, the EGF receptor is likely to stay intact in the presence of FB_1. The post-wash recovery of the hepatocytes after the simultaneous exposure to EGF and FB_1 seems to imply that the cells are arrested somewhere between the G_1- and S-phases. As 60% of the cells are inhibited from entering the S-phase when FB_1 is added 24 h after EGF (Figure 2A), maximum inhibition is likely to occur late during the G_1-phase and/or during the S-phase. However, the reversibility of the inhibitory effect on the EGF growth response complicated studies to determine when maximal inhibition occurred. These results imply that, for the fumonisins to exhibit their effects—i.e. cytotoxicity and inhibition of cell proliferation—continued exposure is required. This is in agreement with a previous study where cell proliferation was effectively delayed in partially hepatectomized rats fed FB_1 (4). A similar result was obtained with respect to the cytotoxicity of FB_1 to a renal epithelial cell line during which cells, exposed to a cytotoxic concentration, fully recovered after removal of the toxin (12).

The inhibitory effect of the fumonisins on sphingolipid biosynthesis, and more specifically the sphinganine and sphingosine *N*-acyltransferase (ceramide synthase), has been implicated in the toxicological and carcinogenic effects of the fumonisins (10,11,14) in experimental animals. Treatment of primary rat hepatocytes with FB_1 (1 μM) for 4 days inhibits sphingosine biosynthesis up to 95%, while the free sphinganine increased 110-fold (10). In a recent study, using a renal epithelial cell line (12), the cytotoxicity of FB_1 was correlated with the inhibition of sphingolipid biosynthesis and specifically with the accumulation of sphinganine. As high intracellular concentrations of this sphingoid base are cytotoxic to certain cell lines (25) the accumulation of sphinganine could be responsible for the cytotoxicity of FB_1 to cultured cells.

The free sphingosine concentration was also reported to decrease significantly (4-fold) after 4 days in the FB_1-treated cells. Sphingosine is known to be an endogenous inhibitor of PKC (15,25), which in turn is known to be one of the mechanisms involved in the regulation of the EGF receptor via the phosphorylation at threonine 654 (23). In addition, sphingosine has been shown to stimulate both the affinity and activity of the EGF receptor (16). The growth inhibitory effect and the cytotoxicity of sphinganine to hamster ovary cells are also of interest (25) as both these biological effects were correlated with PKC inhibition, and perhaps even more important is the fact that sphinganine blocks changes in protein phosphorylation patterns that occurred after phorbol 12-myristate 13-acetate (PMA) treatment. Both EGF and the insulin-induced growth stimulatory response are mediated via the activation of a receptor-associated tyrosine kinase (26,27), which in turn is regulated by both sphingosine and PKC (16). From these studies it would appear that the sphingoid bases are likely to be involved in the regulation of growth stimulatory responses of cells to growth factors.

The present data indicate that FB_1 marginally inhibited the EGF growth stimulatory response at a concentration of 12.5 μM, while lower concentrations (1 and 5 μM) had no effect. However, the sphinganine concentration was maximally increased (~20-fold above the control) at a FB_1 concentration of 1 μM (Table II). As there was no decrease in the sphingosine concentration after a period of 2 days the modulating role of sphingosine on the EGF-induced growth response has to be ruled out. In addition, when sphingosine and/or sphinganine was added to the culture medium no stimulation and/or inhibition of the EGF response was noticed (Table III). This again emphasizes that the activity and/or the affinity of the EGF receptor is not altered by the sphingoid bases in primary hepatocytes *in vitro*. When FB_1 was added to the hepatocytes at concentrations as low as 1 μM, in the absence of EGF, no stimulation of DNA synthesis occurred (Table III). This further supports the observation that the *de novo* disruption of the sphingolipid biosynthesis does not interfere with growth factor responses in primary hepatocytes. At higher FB_1

(5–500 μM) concentrations the sphinganine/sphingosine ratio was not significantly altered above that of the hepatocytes receiving 1 μM FB_1 (Table II), but the EGF-induced growth response was markedly affected (Figure 1A). The basal DNA synthesis was also inhibited by FB_1, suggesting that the stimulatory response induced by insulin, which is contained in the medium, was also inhibited. Both EGF and insulin exert their effect on growth through a tyrosine kinase mediated receptor (27). In summary, it would appear that the increased sphinganine concentration and hence the imbalance in the sphinganine/sphingosine ratio alone is not responsible for the mitoinhibitory effect of FB_1 in primary rat hepatocytes.

The accumulation of sphinganine also does not correlate with the cytotoxicity of FB_1 to the primary hepatocytes. No cell death occurred in primary hepatocytes at FB_1 concentrations up to 250 μM (18), and 300 μM caused a cytotoxic effect only in the absence of EGF. This is in agreement with previous observations (10) indicating that the disruption of sphingolipid biosynthesis in primary hepatocytes precedes any noticeable cytotoxic effects. In this regard primary hepatocytes appear to tolerate a considerably higher concentration of the sphingoid bases than other cell types in which an increased sphinganine concentration is correlated with cell death (12,25). Also of interest is the finding that the incubation of the hepatocyte cultures for 44 h was associated with a significant ($P < 0.0001$) increase in the sphinganine concentration and hence the sphinganine/sphingosine ratio (Table II). The background cell cytotoxicity increased between 15 and 20% during this incubation period. Therefore, the increase in the sphinganine concentration in primary hepatocytes can be ascribed to a FB_1-dependent inhibition of ceramide synthase and FB_1-independent effects, probably associated with events related to normal cell death and/or sphingolipid turnover. These findings have important implications in human and animal studies in which it has been suggested that the disruption of the sphinganine/sphingosine ratio in biological fluids could be used as a biomarker for FB_1 exposure (24).

With regard to the mitoinhibitory effect of FB_1 on hepatocyte cell proliferation, the role of other cancer promoters is also of interest. Treatment of hepatocytes with the cancer promoter orotic acid resulted in an imbalance in the nucleotide pools due to an increase in uridine and a decrease in adenine nucleotides which resulted in a decrease in the response of hepatocytes to EGF (28). Phenobarbital (PB) also inhibits the growth response of EGF *in vitro*, while *in vivo* studies showed that hepatocytes obtained from rats treated chronically with PB exhibited a lower labelling index (29). Studies have also indicated that the number of receptors for insulin and EGF (30,31) in rat hepatocytes decreased after exposure to PB. The present study indicated that the mitoinhibitory effect of FB_1 probably occurs beyond the growth factor receptor and receptor mediated events or even at the DNA synthesis level.

The mitoinhibitory effect of FB_1 on the growth stimulatory effects of growth factors could explain the inhibitory effect on cell proliferation *in vivo*, but the exact mechanism needs to be elucidated. The induction of 'resistant' hepatocytes by FB_1 *in vivo* (4) and their ability to escape the mitoinhibitory effect of the compound is likely to be a key component during cancer promotion by these non-genotoxic *Fusarium* mycotoxins.

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CANCER LETTERS

The cancer-promoting potential of fumonisin B_1 in rat liver using diethylnitrosamine as a cancer initiator

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Abstract

The cancer-promoting potential of fumonisin B_1 (FB_1) was investigated by feeding different dietary levels (10, 50, 100, 250, 500 mg FB_1/kg) to diethynitrosamine (DEN)-initiated rats for 21 days. Dietary levels containing 50 mg FB_1/kg and higher, markedly increased the number and size of the placental form of glutathione-*S*-transferase-positive ($GSTP^+$) foci in the liver of the rats. The cancer-promoting activity of FB_1 was associated with an inhibitory effect on partial hepatectomy (PH)-induced regenerative hepatocyte proliferation, as the incorporation of ^3H-labelled thymidine was significantly ($P < 0.05$) reduced by those FB_1-containing diets that exhibited cancer promotion. In vitro studies on the mitogenic activity of epidermal growth factor (EGF) in primary rat hepatocytes further supported the in vivo data in that FB_1, similar to other cancer promoters such as phenobarbital and 2-acetylaminofluorene (2-AAF), alters growth stimulatory responses in primary hepatocytes. No significant ($P > 0$ 05) changes in the sphinganine/sphingosine (Sa/So) ratio were observed in the liver of the rats fed the lowest FB_1-containing diet (50 mg FB_1/kg diet) that effected cancer promotion. The present study indicated that FB_1 exhibited cancer-promoting activity in the absence of adverse hepatotoxic effects and at dietary levels that failed to effect cancer initiation.

Keywords: Fumonisin B_1; Diethylnitrosamine; Rat liver; Cancer promotion

1. Introduction

Fumonisin B_1 (FB_1), a food-borne mycotoxin produced by the fungus *Fusarium moniliforme* on corn, is hepatocarcinogenic when fed chronically to rats over a period of 18-26 months [1,2]. Studies regarding the mechanism of cancer induction by these apparent non-genotoxic mycotoxins are complicated by the fact that cancer develops against a background of chronic active hepatitis which often masks the events related to cancer development [1]. However, short-term cancer studies, utilising different rat liver models, provided some insight into the basic mechanisms likely to be involved during cancer induction by many hepatocarcinogens. It is known that the induction of a resistant phenotype in initiated hepatocytes and their subsequent expansion into hepatocyte nodules is a common property induced by many genotoxic hepatocarcinogens [3]. These benign focal proliferations

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of hepatocytes have been shown to be important lesions for cancer development in the liver [4].

When utilising a short-term cancer initiating/promotion bioassay in rat liver [5,6], FB_1 closely mimics genotoxic carcinogens with respect to cancer initiation. FB_1 induces initiated hepatocytes that are resistant to the mitoinhibitory effects of 2-acetylaminofluorene (2-AAF), a carcinogen that selectively promotes the outgrowth of initiated cells by inhibiting the proliferation of normal hepatocytes in the presence of a stimulus of cell proliferation such as partial hepatectomy (PH) [7]. The only difference between FB_1 and the genotoxic carcinogens lies in the kinetics of the induction of the 'initiated' cells, as cancer initiation by the genotoxic carcinogens occurs very rapidly within a few hours or up to a few days depending on the initiator [4]. The fumonisins are slow initiators as prolonged exposure (at least 14 days) at relatively high dietary levels (250 mg FB_1/kg feed) is required [6]. Single or multiple gavage dosages, even in the presence of cell proliferation, a prerequisite for initiation [8], did not effect initiation [5].

The present study investigated the cancer-promoting potential of the fumonisins in order to obtain more information about the carcinogenic properties of these non-genotoxic hepatocarcinogens. As FB_1 inhibits the growth stimulatory effect of epidermal growth factor (EGF) in primary hepatocytes [9] and regenerative cell proliferation in vivo in partial hepatectomised rats [6], the role of the inhibitory effect of FB_1 on cell proliferation in the cancer-promoting activity of the compound was further evaluated.

2. Materials and methods

FB_1 was purified according to the method described by Cawood et al. [10] to a purity of 93-95%. [^{3}H]Thymidine (Amersham) was obtained from Weil organization (SA) while DEN and 2-AAF were ordered from Sigma Chemical Company (St. Louis, MO). Phenobarbital (PB) was purchased from MERCK, SA. DEN and 2-AAF were dissolved in dimethyl sulfoxide (DMSO) and PB in distilled water. The anti-GSTP antibody was a generous gift of Dr. E. Farber, Department of Pathology, University of Toronto, Canada, and C20-sphinganine was kindly supplied by Dr. A Merrill, Department of Biochemistry, Emory University, Atlanta, GA.

Male Fischer rats, weighing approximately 150 g, were fed a modified AIN 76 diet when weaned [6]. They were randomised (five animals/group) and housed separately prior to the start of the experiment. The different FB_1-containing diets, 10, 50, 100, 250, and 500 mg FB_1/kg, as well as the control, were prepared as described previously [6] and stored under nitrogen at −20°C for the duration of the experiment. Rats received a single initiating dose of DEN (200 mg/kg, i.p.) dissolved in DMSO (0.2 ml/100g b.w.). Promotion commenced 2 weeks after initiation by feeding different FB_1-containing diets to rats for 21 days, after which they were killed by decapitation and liver sections preserved in buffered formalin (pH 7.4) and ice-cold acetone for routine H and E and histochemical staining for the placental form of glutathione-*S*-transferase (GSTP) and gamma glutamyltranspeptidase (GGT) staining, respectively [11,12]. Initiated rats that received the normal AIN 76 diet served as the control. The number and size (area) of the enzyme altered foci were quantified (5xmagnification) on a Hewlett Packard image analyser with customised software. For more accurate measurements, the diameter (transverse and longitudinal) of the GSTP-positive ($GSTP^+$) foci in the liver sections of the rats fed the control, 10, 50 and 100 mg FB_1/kg diets was determined microscopically (10-40xmagnification).

In a separate experiment the different FB_1-containing diets were fed for 21 days to non-initiated rats under similar conditions. Following the feeding period, all the rats were subjected to partial hepatectomy (PH) and sacrificed after 24 h. One hour prior to the killing, each rat received a single dosage (20 μCi, i.p.) of [^{3}H]thymidine and DNA hydrolisates of the liver samples (1 g) prepared by perchloric acid digestion of the TCA perceptible material according to the method of Neal and Grabral [13]. The DNA content [14] and radioactivity were determined and the specific labelling of the DNA expressed as dpm/μg DNA. For sphingolipid analyses a part of the liver (300-500 mg), obtained during PH, was first ground to a fine powder in liquid nitrogen according to the method of Smuts et al. [15] and 100-150 mg suspended in 4 vols. (w/v) 0.5 M phosphate buffer (pH 7.0). Total lipids were extracted from a subsample (approximately 25 mg wet liver weight) of the buffered liver suspension using chloroform/methanol (1:2) and the sphinganine

Table 1

Cancer-promoting potential of different dietary levels of fumonisin B_1 (FB_1) fed to DEN-initiated rats for 21 days

Treatment (mg FB_1/kg diet)	Body wt. (g) (gain or loss)	GGT^+ foci			$GSTP^+$ foci		
		No./cm^2	Mean foci area	Mean % area/ liver section	No./cm^2	Mean foci area	Mean % area/ liver section
Control	33.17a	0.55a	0.002a	0.12a	3.59a	0.001a	0.29a
	(5.37)	(0.15)	(0.001)	(0.08)	(2.48)	(0.001)	(0.11)
10	31.40a	0.44a	0.002a	0.08a	6.50a	0.001a	0.31a
	(4.18)	(0.07)	(0.001)	(0.04)	(4.63)	(0.001)	(0.27)
50	29.00a	0.87a	0.002a	0.15a	12.76a	0.001a	0.97a
	(5.51)	(0.42)	(0.001)	(0.07)	(4.70)	(0.001)	(0.11)
100	21.50a	6.67a	0.001a	0.84a	47.83B	0.002a	13.73a
	(5.50)	(4.85)	(0.001)	(0.43)	(12.12)	(0.002)	(15.48)
250	9.83B	36.85B	0.002a	10.15B	43.61B	0.003a	13.43a
	(9.59)	(18.18)	(0.001)	(7.04)	(13.04)	(0.003)	(9.16)
500	–4.80B	66.00B	0.004B	23.59B	56.99B	0.005B	27.56B
	(7.92)	(17.01)	(0.001)	(4.59)	(24.37)	(0.005)	(10.37)

Values represent the mean ± SD of five rats per group. Means followed by the same letter do not differ significantly; when the letter and cases differ then $P < 0\ 01$.

(Sa) and sphingosine (So) concentrations quantified by HPLC using C20-sphinganine as internal standard [16]. Ten to 15 mg of the powdered liver was retained for protein determination [15].

Histological examination were performed on H and E sections of buffered formalin-fixed material of the liver lobes obtained during PH.

The comparative effect of different cancer promoters on the epidermal growth factor (EGF, 50 ng/dish) response were performed in primary hepatocytes. Hepatocytes were isolated according to the collagenase perfusion technique [17] and the primary cultures prepared and maintained as described previously [9]. The inhibitory effect on the EGF mitogenic response of different cancer promoters, 2-AAF (0.5 μM), PB (2 mM) was compared with FB_1 (150 μM). The mitogenic response, expressed as disintegrations per minute (dpm)/mg protein, was monitored by pulse labelling (4 h) the cultures with ^{3}H-labelled thymidine after 40 h of incubation during which most of the cells were in S-phase [9]. Cytotoxicity measurement was monitored by measuring the release of lactate dehydrogenase (LDH) as described elsewhere [17].

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.

3. Results

The body weight gain of the rats was markedly

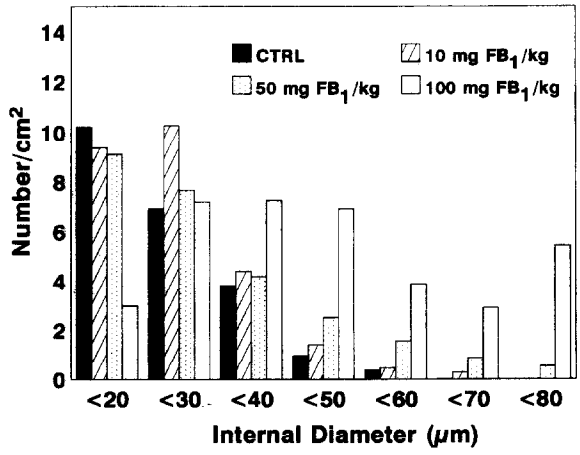

Fig. 1. The number of $PGST^+$ foci in tissue sections of rats fed different dietary levels of FB_1 (control, 10, 50 and 100 mg FB_1/kg diet) for 21 days. The foci were categorised according to their maximal internal diameter and expressed as number/cm^2. The values represent the means of five rats per group.

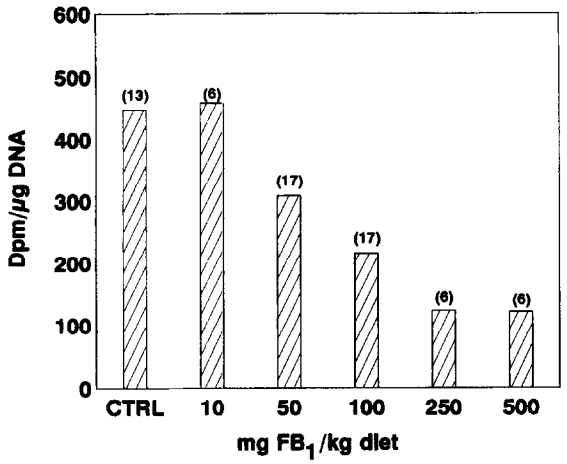

Fig. 2. Dose-response effect of FB_1-containing diets (control, 10, 50, 100, 250 and 500 mg FB_1/kg) on regenerative cell proliferation induced by partial hepatectomy. Rats were treated with ^{3}H-labelled thymidine (10 µCi/rat) 24 h after PH was performed. The values, expressed as dpm/µg DNA, are the means of the different number (value in parenthesis) of rats used per group.

reduced (non-significantly) in the 100 mg FB_1/kg diet group and significantly lowered (Table 1) in the rats fed the 250 ($P < 0.01$) and 500 ($P < 0.01$) mg FB_1/kg diets. A significant increase ($P < 0$ 05) in the number of GGT^+ foci was effected at the dietary levels of 250 and 500 mg FB_1/kg while it was markedly enhanced in the livers of the rats fed the 100 mg FB_1/kg diet (Table 1). When GSTP was used as histochemical marker, a marked increase in $GSTP^+$ foci was noticed in the livers of the rats fed the 50 mg FB_1/kg diet, although the increase was only significant ($P < 0$ 01) in the rats fed the 100 mg FB_1/kg diet and higher (Table 1). However, when the relative sizes (largest of the transverse or longitudinal diameter) of the $GSTP^+$ foci were monitored microscopically in the controls and rats fed 10, 50 and 100 mg FB_1/kg diets, a marked increase was noticed in the rats treated with the 50 mg FB_1/kg diet and above (Fig. 1). When considering the relative size of between 60 and 70 µm (i.d.), only 2/5, 3/5 and 5/5 of the rats treated with the 10, 50 and 100 mg contained liver foci while only rats fed the 50 (2/5) and 100 (5/5) mg FB_1/kg diet groups exhibited foci with an i.d. between 70 and 80 µm. None of the control rat livers contained foci with an i.d. between 60 and 80 µm. The feed intake profiles were not monitored, but the FB_1 intake obtained from a previous investigation [6] was used to estimate the apparent dosage of FB_1 to effect cancer promotion in rat liver.

FB_1 significantly ($P < 0$ 01) inhibits regenerative cell proliferation induced by PH in the livers of the rats fed the 50 mg FB_1/kg and higher dietary levels (Fig. 2). The Sa and So concentrations were significantly ($P < 0.01$) increased in the livers of the rats treated with the 250 and 500 mg FB_1/kg diet, while Sa was markedly (not significantly) increased in the livers of the rats fed the 50 and 100 mg FB_1/kg diet (Fig. 3). The Sa/So ratio was significantly increased ($P < 0$ 05) in the rats fed the 100 mg FB_1/kg and higher dietary levels (Fig. 3).

In primary hepatocyte cultures (viability between 90-95%) the addition of the cancer promoters, 2-AAF (0 5 µM), PB (2 mM) and FB_1 to the incubation medium significantly ($P < 0.05$ to $P < 0.01$) inhibited the mitogenic response of EGF (Fig. 4). No cytotoxic effects were noticed in the presence of the different cancer promoters.

Pathological changes caused by FB_1 have been described elsewhere [1,5,6] and were mainly observed in the livers of the rats treated with the 250 and 500 mg FB_1/kg diets. These include degenerative changes such as hydrophic swelling and hyaline droplet accumulation, single cell necrosis and lipid accumulation. Early signs of nodular regeneration, fibrosis and proliferation of duct epithelial cells (DEC, or 'oval' cells) were noticed which slightly distorted the fine structure

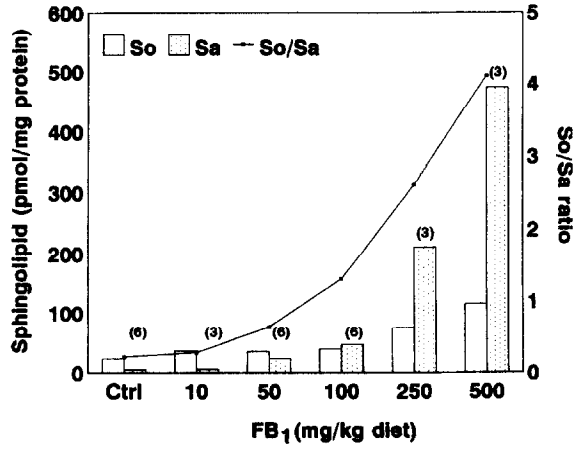

Fig. 3. The effect of different dietary levels of FB_1 on the sphingolipid concentrations and sphinganine to sphingosine (Sa/So) ratio. The data are the means of the number of rats (in parenthesis) used.

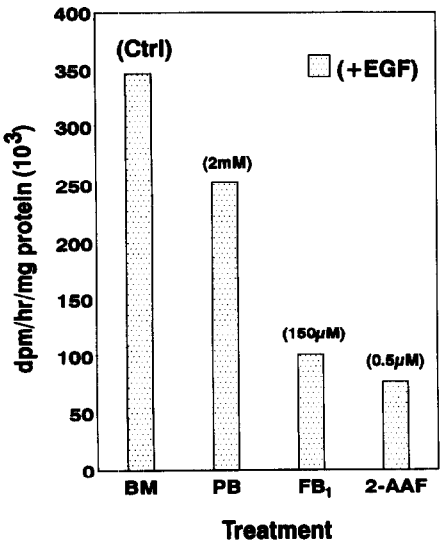

Fig. 4. The mitoinhibitory effect of different cancer promoters (FB_1, 2-AAF and PB) on the EGF-induced mitogenic response. Values are means of triplicate determinations. The experiment was repeated three times with a similar pattern of results.

of the liver acinus. The changes detected in the liver of the rats receiving the 100 mg FB_1/kg diet were far less severe and include early proliferation of DEC, single cell necrosis and the presence of mitotic figures in zone 1. In the 50 mg FB_1/kg-treated rats only a few necrotic cells were noticed in zone 1, but these lesions were very scant. No specific lesions could be detected in the 10 mg FB_1/kg and control groups.

4. Discussion

The present study indicated that FB_1 has the ability to increase the number and size of $GSTP^+$ foci in DEN-initiated rats from a dietary level of 50 mg FB_1/kg and higher over a period of 21 days. As FB_1-containing diets of 250 mg FB_1/kg and higher also exhibit cancer-initiating activity [6], the increase in the number of $GSTP^+$ foci could therefore be ascribed to a synergistic effect between DEN and FB_1, while the 50 and 100 mg FB_1/kg diets, which lack any cancer-initiating activity, solely effected cancer promotion. From the dietary intake profiles obtained from a previous study [6], the effective dosage level (EDL) for cancer initiation was calculated to be $14.2 < 30.8$ mg FB_1/100 g body weight over a 21 day period, which represents dietary levels between 100 and 250 mg FB_1/kg, respectively [6]. When a similar approach is followed, cancer promotion is effected at apparent dietary levels of between 10 and 50 mg FB_1/kg feed over the same period, giving an EDL value of $1.4 <$ 7.3 mg FB_1/100g body weight (Table 1). Marked hepatotoxic effects were induced in rat liver by FB_1 at the high dietary levels of 250 and 500 mg FB_1/kg that are know to initiate cancer. The toxicity was far less prominent in the 100 mg FB_1/kg dosage while only a few necrotic cells were noticed in some of the rats treated with the 50 mg FB_1/kg diet. Therefore, in contrast to cancer initiation, cancer promotion is effected at relatively low dietary levels of FB_1 where very little toxicity is observed.

As many hepatocarcinogens are know to be inhibitors of cell proliferation [4], their ability to induce a growth differential, whereby the initiated hepatocyte is allowed to proliferate while the normal hepatocyte is inhibited, has been proposed as a mechanism for cancer promotion for chemical carcinogens [7]. It has been suggested that the fumonisins effect cancer promotion by a similar mechanism as FB_1 inhibits regenerative cell proliferation induced by partial hepatectomy, either by a single dose applied 6 h after PH or when fed in the diet prior to performing PH [5,6]. The present study indicates that the regenerative proliferative response, induced by PH, is inhibited by feeding rats a FB_1-containing diet up to a level of 50 mg FB_1/kg over a period of 21 days. This inhibitory effect on hepatocyte proliferation is associated with an increase in the number and size of GSTP foci which reflects the cancer-promoting activity of the fumonisins. However, when considering a hypothesis for cancer promotion, other mechanisms that permit the outgrowth of initiated cells, such as promoters that selectively induce cell proliferation in initiated cells, also need to be considered [18]. In the latter mode the cancer promoter should be an inducer of cell proliferation. As FB_1 failed to induce cell proliferation under the present experimental conditions, such a mechanism for cancer promoting appears to be unlikely.

Several in vitro studies also reported on the antiproliferative effects of FB_1 in normal and transformed human cell types originated from epithelial, mesenchymal and parenchymal tissue, in rat hepatoma and different kidney epithelial cell lines [19–21]. The

induction of apoptosis in many of these cell culture systems has been proposed as a possible mechanism for the antiproliferative effects of FB_1 [19]. In this regard, the inhibition of sphingolipid metabolism, and more specifically, the enzyme ceramide synthase by FB_1 with the subsequent reduction in the level of ceramide and/or of complex sphingolipids, has been implicated in the development of the internal apototic signal. However, in contrast to this, FB_1 has been shown to inhibit apoptosis in murine lymphocytes and human monoblastic leukaemia cells which was ascribed to the inhibition of ceramide synthase [22]. It was suggested that regulation of ceramide synthase, that results in ceramide elevation, is required for the induction of apoptosis in eukaryotes. Apart from the antiproliferative effects, FB_1 acts as a mitogen in Swiss 3T3 cells which has also been correlated with the interruption of sphingolipid biosynthesis and the accumulation of the sphingoid bases. The latter effect has been suggested to be involved in the carcinogenicity of the fumonisins via the stimulation of cell proliferation [23]. It would appear that, from these studies, FB_1 effects cell proliferation differently in various cell culture systems and, except for Swiss 3T3 cells, mainly inhibits cell proliferation. Although ceramide synthase appears to be a common target enzyme for the fumonisins, the exact role of the altered complex sphingolipid levels in the induction of the apoptotic signal and hepatocarcinogenic effects of the compound still need to be elucidated.

Studies in primary rat hepatocytes indicated that FB_1 inhibits the EGF-induced mitogenic response in a reversible manner without an apparent involvement of the disruption of sphingolipid metabolism [9]. In the present study the Sa/So ratio was significantly ($P < 0\ 05$) increased at a dietary level of 100 mg FB_1/kg while it was only slightly (not significantly) increased at the lowest dietary level (50 mg FB_1/kg) that effected cancer promotion. Phospholipid analysis indicated that the complex sphingolipid, sphingomyelin, was only altered in rat liver of the animal fed the 250 mg FB_1/kg diet [24], while another study indicated that apoptosis is only induced in rats fed dietary levels from 234 mg/kg and higher [25] where adverse hepatotoxic effects were noticed. As cancer promotion is effected at a dietary level where little hepatotoxic effect is observed and below the level where sphingolipid metabolism and apoptosis is effected, other mechanisms also need to be considered to explain the cancer-promoting activity of FB_1 in vivo in rat liver.

The FB_1-induced mitoinhibitory effect on the EGF-induced mitogenic response in primary hepatocytes [9] is also known to be a property exhibited by many cancer promoters such as orotic acid [26], PB and ciprofibrate [27]. The present study indicated that 2-AAF, another cancer promoter, is far more effective in inducing a mitoinhibitory effect in primary hepatocytes than FB, with PB showing the least effective response (Fig. 4). In vivo studies regarding the cancer-promoting potency of AAF as compared to PB indicated that AAF creates a more intensive selection pressure with respect to the development of GGT^+ foci in the absence and/or presence of a stimulus for cell proliferation [8,28]. When using DEN-initiated rats, cancer promotion by PB is effected after 10-12 weeks when fed at a dietary level of 500 mg/kg diet [29]. FB_1 effects promotion only after 3 weeks at a dietary level of 50 mg FB_1/kg, implying that the compound is a more effective cancer promoter than PB. It is not know whether the inhibitory effect of PB on cell proliferation is required for its cancer-promoting activity, as PB does not create a strong differential mitoinhibitory effect between normal and nodular hepatocytes in vitro [30]. However, PB has very limited mitogenic activity for the liver and, after a brief period of hyperplastic cell proliferation, tends to inhibit cell proliferation [31].

The present study suggests that FB_1 is a more effective cancer promoter than initiator in rat liver and that the anti-proliferative properties are likely to be involved in creating a growth differential in order to stimulate the proliferation of initiated cells. Studies are in progress to monitor the effect of FB_1 on the proliferation of nodular hepatocytes to determine whether the induction of a growth differential could explain the cancer-promoting activity of FB_1. The exact role of other biological effects such as mitogenesis and the induction of apoptosis that are commonly seen in cell culture systems and known to be important determinants during the process of carcinogenesis, need to be elucidated under in vivo studies to clarify their role in FB_1-induced carcinogenesis in rat liver. Other biological effects such as the FB_1-induced disruption of fatty acid metabolism in

hepatocytes in vitro [32] and in rat liver in vivo [24], that could play an important part in the regulation of cell proliferation in the liver, also need to be considered.

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Effect of Fumonisin B_1 on Protein and Lipid Synthesis in Primary Rat Hepatocytes

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Abstract—The effect of fumonisin B_1 (FB_1) on protein and lipid synthesis was evaluated in primary rat hepatocytes. FB_1 did not affect incorporation of [^{3}H]leucine into hepatocytes at either non-toxic (150 μM) or cytotoxic (500 μM) concentrations indicating that protein synthesis was not affected. However, FB_1 significantly ($P < 0.01$ to $P < 0.0001$) inhibited incorporation of [^{14}C]palmitic acid into hepatocyte cultures implying that lipid synthesis was altered. Incorporation of the radiolabel was significantly ($P < 0.05$ to $P < 0.0001$) lowered in triacylglycerol (TAG) and sphingomyelin fractions and increased in phosphatidylcholine (PC) and phosphatidylethanolamine (PEA) in both FB_1 concentrations. The incorporation pattern of [^{14}C]palmitic acid closely resembles the changes in phospholipid levels in the treated cells. The sphingolipid, sphinganine (Sa), was significantly ($P < 0.0001$) increased in treated cells but there was no significant difference between the toxic and non-toxic dose levels implying that the increased Sa level alone is not responsible for the *in vitro* toxicity. FB_1 significantly ($P < 0.01$ to $P < 0.001$) decreased the level of free cholesterol within the cell, resulting in an increased PC:cholesterol ratio suggesting a more rigid membrane structure. Subsequent studies on the fatty acid (FA) profiles in PC and the neutral lipid, TAG, indicated that FB_1 significantly ($P < 0.05$ to $P < 0.0001$) increased the levels of the polyunsaturated FAs C18:2n-6 and C20:4n-6 at both concentrations. The FB_1-induced changes to cellular membranes, specifically those related to FA changes in the major membrane phospholipids, and the altered FA content of the hepatocytes are likely to be key events in explaining the cytotoxic effects and altered growth responses induced by fumonisins in primary hepatocytes.

INTRODUCTION

Studies regarding cancer induction with chemicals mainly focus on the interaction of the carcinogen with cellular DNA (Bishop, 1991; Weinstein, 1988). However, when a cell is exposed to a foreign compound numerous reactions could occur apart from those changes at the genetic level (Farber, 1987 and 1991) and could have important implications in the slow evolvement of potentially preneoplastic cells into cancer. The role of more subtle changes in the cell, especially changes involving the plasma membrane, has become more prominent in studies regarding cancer development and forms part of the epigenetic mechanism of cancer induction in certain organs (Weinstein, 1991).

The fumonisin B (FB) mycotoxins are structural analogues produced by the fungus *Fusarium moniliforme* in corn (Cawood *et al.*, 1991; Gelderblom *et al.*, 1988). Even though the compounds lack any genotoxic effects in short-term mutagenesis (Gelderblom and Snyman, 1991) and in DNA repair assays (Gelderblom *et al.*, 1992; Norred *et al.*, 1992a), FB_1 is hepatocarcinogenic in rats (Gelderblom *et al.*, 1991). Subsequent studies on the mechanism of cancer induction by fumonisins indicated that they are slow cancer initiators and that prolonged exposure at high dietary levels is required (Gelderblom *et al.*, 1992, 1993 and 1994). Cancer initiation is effected by the induction of 'resistant' hepatocytes, the outgrowth of which can be stimulated selectively by a stimulus of cell proliferation in the presence of a mitoinhibitory effect induced by 2-acetylaminofluorene (Gelderblom *et al.*, 1992 and 1993). When diethylnitrosamine is used as a cancer initiator FB_1 acts as a cancer promoter, as indicated by the formation of γ-glutamyltranspeptidase positive foci (Gelderblom *et al.*, 1988). Despite the fact that fumonisins are non-genotoxic, FB_1 can effect both the early events of cancer induction, namely cancer initiation and promotion, similarly to that of genotoxic

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Abbreviations: CE = cholesterol ester(s); CM = chloroform/methanol; DMSO = dimethyl sulfoxide; EGF = epidermal growth factor; FA = fatty acid(s); FB = fumonisin(s); LDH = lactate dehydro-genase; PC = phosphatidylcholine; PEA = phos-phatidylethanolamine; PUFA = polyunsaturated fatty acid(s); Sa = sphinganine; SM = sphingomyelin; So = sphingosine; TAG = triacylglycerol; WE = Williams' E; P/S = polyunsaturated/saturated fatty acid.

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