

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

**Wentzel Christoffel Andreas Gelderblom**

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In the Faculty of Natural Sciences  
University of Stellenbosch

**Promoter** Prof PS Swart  
Department Chair: Biochemistry  
University of Stellenbosch

**Co–Promoter** Prof WFO Marasas  
Extra-ordinary Professor  
Department of Plant Pathology  
University of Stellenbosch

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carcinogens. The non-genotoxic peroxisome proliferator, clofibrate, was also found to induce 'resistant' hepatocytes after prolonged exposure to high dietary levels (Nagai *et al.*, 1993). At present very little is known about the mechanisms involved during induction of initiated cells by non-genotoxic carcinogens in the liver and whether the characteristic 'resistant' phenotype (Roomi *et al.*, 1985) is induced similarly to that of genotoxic chemicals. It is suggested that the inhibitory effect of FB<sub>1</sub> on hepatocyte cell proliferation, as for many genotoxic carcinogens (Farber *et al.*, 1989), is a critical factor determining the cancer promoting activity of fumonisins (Gelderblom *et al.*, 1993 and 1994). A recent study indicated that FB<sub>1</sub> inhibits the mitogenic response of epidermal growth factor (EGF) in primary hepatocytes (Gelderblom *et al.*, 1995). However, at present very little is known about the possible mechanism involved in the inhibition of growth-related responses in hepatocytes.

Recent studies regarding the biological effects of fumonisins indicated that they selectively inhibit ceramide synthase, a key enzyme in the sphingolipid biosynthetic pathway (Wang *et al.*, 1991). It was suggested that the subsequent accumulation of the sphingoid bases, sphinganine (Sa) and sphingosine (So), could have an important role in the toxicological effects of fumonisins in the kidney and the liver of rats (Norred *et al.*, 1992b; Yoo *et al.*, 1992). In addition, as the sphingoid bases are important bioactive regulators of cellular growth and differentiation (Merrill, 1991) the continued disruption of sphingolipid biosynthesis has been implicated in the hepatocarcinogenicity of fumonisins (Schroeder *et al.*, 1994).

The present study evaluated aspects regarding lipid biosynthesis in primary hepatocytes to obtain more information about changes in cellular lipids that could provide more insight regarding cytotoxicity as well as in the alteration of growth-related responses induced by fumonisins.

#### MATERIALS AND METHODS

##### *Mycotoxin standards*

FB<sub>1</sub> and FB<sub>2</sub> were extracted and purified as described by Cawood *et al.* (1991) to a purity of 98%. The toxins were dissolved in saline (FB<sub>1</sub>) and dimethyl sulfoxide (DMSO)/saline (1:1) (FB<sub>2</sub>) and added in 50- $\mu$ l quantities to hepatocyte cultures. The respective solvents were also added to the control dishes.

##### *Preparation of hepatocyte cultures*

Rat hepatocytes 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  $2 \times 10^5$  (3-cm dishes) and  $6 \times 10^5$  (5-cm dishes) viable cells per dish for 3 hr in 2-ml modified Williams' E (WE) medium, containing foetal bovine serum (10%), insulin (20 U/litre), L-glutamine

(2 mM), HEPES (10 mM) and penicillin (100 U/ml). After plating, the cells were washed with Hanks' buffer solution and supplemented with 2 ml serum-free modified WE medium containing L-proline (2 mM) and sodium pyruvate (10 mM) in addition to the components described above. Cytotoxicity was measured at 24 and 48 hr by monitoring the release of lactate dehydrogenase (LDH) in the medium (Hayes *et al.*, 1984).

##### *Incubations*

**Leucine incorporation.** Protein synthesis was measured as the incorporation of L-[4,5-<sup>3</sup>H]leucine (82 Ci/mmol; Amersham, Bucks., UK) in acid-precipitable material by a modification of the procedure of Garberg and Högberg (1991). Hepatocytes (3-cm dishes) were incubated for 2 and 24 hr with FB<sub>1</sub> (150 and 500  $\mu$ M) and FB<sub>2</sub> (50 and 150  $\mu$ M). Without changing the incubation media, 10  $\mu$ Ci [<sup>3</sup>H]leucine was added to the hepatocytes and incubated for another hour. The incubation media were removed and the plated cells washed with saline (2  $\times$  2 ml), 5% trichloroacetic acid (3  $\times$  2 ml) and absolute ethanol (3  $\times$  2 ml). The precipitated cells were dissolved in

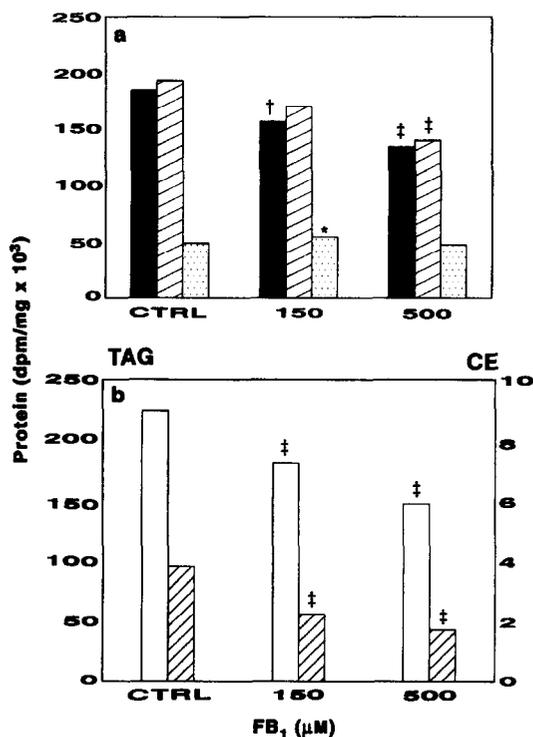


Fig. 1. (a) Radiolabelling of hepatocytes (■), total lipids (▨) and phospholipid (▩) fractions and (b) neutral lipids (□, TAG and ▨, CE) after exposure of hepatocytes to <sup>14</sup>C labelled palmitic acid for 1 hr. Hepatocytes were exposed for 24 hr to different FB<sub>1</sub> concentrations before addition of the radioactive FA. Values represent means of four different experiments with triplicate determinations. Values differ significantly from the control (ctrl) treatment: \**P* < 0.05; †*P* < 0.01; ‡*P* < 0.0001 (Proc Mixed statistical procedure).

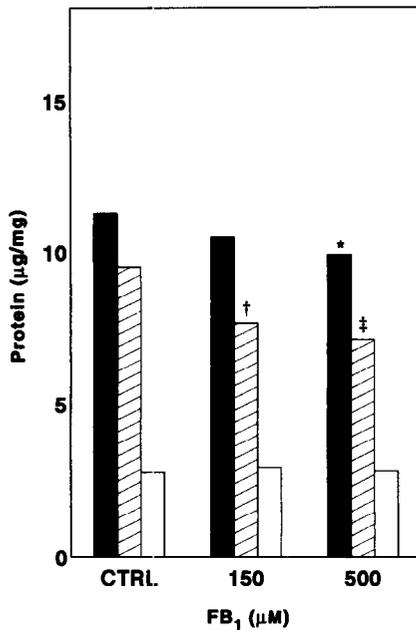


Fig. 2. Cholesterol (■), total cholesterol, □, cholesterol ester and ▨, free cholesterol) concentrations in primary hepatocytes exposed to FB<sub>1</sub> for 24 hr. Values represent means of three different experiments with triplicate determinations. Values differ significantly from the control (ctrl) treatment. \* $P < 0.05$ ; † $P < 0.01$ ; ‡ $P < 0.001$  (Proc Mixed statistical procedure).

1 ml 2% sodium dodecylsulfate containing 2 mM ethylenediamine tetraacetic acid and 20 mM sodium bicarbonate for the determination of radioactivity and protein concentration. Cytotoxicity of FB<sub>1</sub> to the primary hepatocytes was monitored at 24 and 48 hr.

**Palmitic acid incorporation.** [1-<sup>14</sup>C]Palmitic acid (55.6 mCi/mmol; Amersham) used in the incubations was complexed with delipidated albumin (Sigma Chemical Co., St Louis, MO, USA), as described by the method of Ellsworth *et al.* (1986). 38 µCi [<sup>14</sup>C]palmitic acid and 3.5 mg unlabelled palmitic acid were mixed in 1 ml 95% ethanol containing 1 mg phenolphthalein. The mixture was titrated to a pH of 9.5 with 1 M sodium hydroxide. After evaporation of the solvent, the residue was suspended in 1 ml 0.9% saline at 70°C for 20 min. To prepare the albumin complex, 1 ml 0.9% saline containing 200 mg albumin (pH 7.4) was mixed overnight with 1 ml of the fatty acid (FA) solution. Hepatocytes were plated (5-cm dishes) and incubated for 24 hr with 150 and 500 µM FB<sub>1</sub>, whereafter the 50 µl (1 µCi) of the albumin-FA complex was added for 60 min to determine [<sup>14</sup>C]palmitate uptake by the hepatocytes. The plated cells were washed three times with saline after incubation and harvested by scraping with a rubber policeman into 1.5 ml saline. An aliquot (100 µl) was removed for determination of cellular protein and radioactivity, while the remainder was retained for lipid extractions.

**Effect on cholesterol synthesis.** A similar study was performed as described earlier but using unlabelled palmitic acid instead. Total cholesterol and unesterified cholesterol present in chloroform/methanol (CM) extracts were determined by an enzymatic iodide method using cholesterol oxidase and esterase enzymatic preparations (Smuts *et al.*, 1992). The concentration of cholesterol ester (CE) was obtained by subtraction.

**Effect on Sa and So synthesis.** The hepatocytes were plated as described earlier and the medium supplemented with 150 and 500 µM FB<sub>1</sub>. After the incubation period of 48 hr, the cells were washed with ice-cold saline and collected in a final volume of 1.5 ml saline. The sphingolipids were extracted in CM (1:2, v/v) with a solvent to sample ratio of 1:8 and quantified by HPLC as described elsewhere (Riley *et al.*, 1994) using C20 Sa as an internal standard.

#### Determination of radioactivity

Radioactivity was determined by liquid scintillation counting in Ready Value scintillation cocktail (Beckman, Capetown, S. Africa) by a Packard (Downers Grove, IL, USA) Tricarb 460 CD instrument.

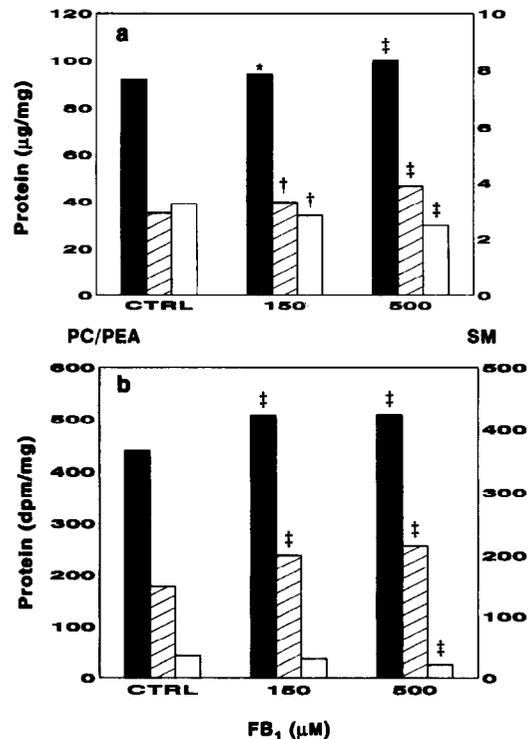


Fig. 3. (a) Concentrations and (b) radiolabelling of PC (■), PEA (▨) and SM (□) fractions purified from lipid extracts of primary hepatocytes exposed to FB<sub>1</sub> for 24 hr. Values represent means of three different experiments with triplicate determinations. Values differ significantly from the control (ctrl) treatment. \* $P < 0.05$ ; † $P < 0.01$ ; ‡ $P < 0.0001$  (Proc Mixed statistical procedure).

Table 1. FA profiles of major membrane phospholipid, PC and PEA, and the neutral lipid, TAG, fractions of primary hepatocytes exposed to toxic and non-toxic levels of FB<sub>1</sub>

FA profile	Control			FB <sub>1</sub> (150 µM)			FB <sub>1</sub> (500 µM)		
	PC	PEA	TAG	PC	PEA	TAG	PC	PEA	TAG
<b>Saturates</b>									
16:0	26.1 ± 0.4	18.1 ± 0.2	39.8 ± 0.6	27.2 ± 1.2	18.1 ± 0.2	37.4 ± 0.6†	27.8 ± 0.8†	19.0 ± 0.8	30.7 ± 0.1*
18:0	16.9 ± 0.5	23.6 ± 0.7	2.5 ± 0.2	15.5 ± 0.7	23.6 ± 0.7	2.4 ± 0.2	16.1 ± 0.7	25.3 ± 1.2	2.8 ± 0.4
Total	43.0 ± 0.6	41.6 ± 0.8	42.3 ± 0.4	42.7 ± 0.9	41.6 ± 0.8	39.7 ± 0.6†	43.8 ± 1.2	44.3 ± 1.9	33.5 ± 0.3*
<b>Monounsaturates</b>									
16:1	2.0 ± 0.1	0.5 ± 0.1	8.0 ± 0.2	1.9 ± 0.1	0.5 ± 0.1	7.5 ± 0.1	1.4 ± 0.1§	0.5 ± 0.1	7.7 ± 0.5
18:1	17.0 ± 0.4	10.2 ± 0.3	38.0 ± 0.2	16.9 ± 0.5	10.2 ± 0.3	38.7 ± 0.7	13.6 ± 0.5‡	9.5 ± 0.6	39.3 ± 0.7
Total	19.0 ± 0.3	10.7 ± 0.4	46.0 ± 0.2	18.7 ± 0.4	10.7 ± 0.6	46.2 ± 0.7	15.1 ± 0.6§	10.0 ± 0.9	46.9 ± 0.7
<b>N6 FA</b>									
18:2n-6	6.6 ± 0.2	3.4 ± 0.3	7.8 ± 0.1	7.1 ± 0.3‡	3.5 ± 0.3	10.3 ± 0.2*	7.4 ± 0.6§	3.6 ± 0.2	13.5 ± 0.2*
18:3n-6	0.1 ± 0.1	0.1 ± 0.1	0.0	0.0	0.2 ± 0.1	0.1 ± 0.1	0.0	0.0	0.3 ± 0.0
20:3n-6	0.7 ± 0.1	0.1 ± 0.1	0.2 ± 0.01	0.6 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.4 ± 0.3	0.0	0.2 ± 0.1
20:4n-6	25.3 ± 0.6	31.5 ± 0.7	1.5 ± 0.1	26.3 ± 0.8†	30.8 ± 1.1	1.8 ± 0.2	28.3 ± 0.1‡	30.3 ± 0.3	3.0 ± 0.5‡
22:4n-6	0.2 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.0	0.5 ± 0.4	0.2 ± 0.1	0.0	0.4 ± 0.3	0.3 ± 0.1
22:5n-6	1.6 ± 0.3	3.6 ± 0.2	0.6 ± 0.1	1.3 ± 0.2	3.6 ± 0.1	0.6 ± 0.1	1.4 ± 0.3	3.3 ± 0.5	0.8 ± 0.1
Total	34.5 ± 0.4	38.8 ± 0.3	10.4 ± 0.3	35.3 ± 0.9	37.7 ± 1.2	13.1 ± 0.5‡	37.4 ± 0.7‡	37.6 ± 1.2	18.1 ± 0.5*
<b>N3 FA</b>									
18:3n-3	0.4 ± 0.1	1.1 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	1.2 ± 0.1	0.4 ± 0.0	0.6 ± 0.1	1.2 ± 0.1	0.4 ± 0.0
22:6n-3	3.2 ± 0.5	7.6 ± 0.5	0.6 ± 0.1	2.9 ± 0.4	6.8 ± 1.0	0.6 ± 0.3	3.1 ± 0.4	6.8 ± 1.0	1.0 ± 0.3
Total	3.6 ± 0.5	8.9 ± 0.7	1.4 ± 0.3	3.3 ± 0.4	8.1 ± 1.2	1.0 ± 0.3	3.7 ± 0.6	8.1 ± 1.2	1.5 ± 0.3
Total PUFA	38.0 ± 0.7	46.9 ± 0.4	11.8 ± 0.6	38.6 ± 1.3	45.7 ± 2.3	14.1 ± 0.6‡	41.1 ± 1.0§	45.7 ± 2.3	19.6 ± 0.6†
P/S ratio	0.88 ± 0.03	1.2 ± 0.1	0.28 ± 0.02	0.90 ± 0.05	1.2 ± 0.03	0.35 ± 0.02	0.94 ± 0.05	1.03 ± 0.1	0.59 ± 0.02

Values represent means ± SD of triplicate analyses. The experiment was repeated twice with a similar pattern of results. †P < 0.05; ‡P < 0.01; §P < 0.001; \*P < 0.0001.

#### Lipid extraction and isolation

Lipids of the cell suspension (1.0–1.2 ml containing 1–1.5 mg protein) were extracted with 24 ml CM (2:1, v/v) containing 0.01% butylated hydroxytoluene as antioxidant, as described previously (Smuts *et al.*, 1992). After evaporation of the solvents the lipid extract was dissolved in certain volume (100–150 µl) of CM (saturated with saline) and an aliquot (10 µl) taken for determination of radioactivity of the total lipid. The neutral lipid fractions were separated by thin-layer chromatography as described by Skipski *et al.* (1965) using diethyl ether (filtered through an aluminium oxide 90 column)–petroleum ether–acetic acid (30:90:1, by vol) as mobile phase. The phospholipids were finally separated by the method of Gilfillan *et al.* (1983) using chloroform–methanol–petroleum ether–acetic acid–boric acid (40:20:30:10:1.8, v/w) as the mobile phase. The plates were sprayed with CM (1:1, v/v) containing 2,5-bis-(5'-*tert*-butylbenzoxazolyl)-[2]thiophene and the spots visualized under UV light. The spots of lipids corresponding with triacylglycerol (TAG), CE and total phospholipids were marked, scraped and quantitatively transferred into counting vials and the radioactivity determined. In the case of the individual phospholipids, namely sphingomyelin (SM), phosphatidylcholine (PC) and phosphatidylethanolamine (PEA), both the radioactivity and the respective concentrations were determined.

#### Phospholipid and FA analyses

Phospholipid concentrations were determined as inorganic phosphate by means of a colorimetric

method with malachite green dye after digestion in saturated perchloric acid at 170°C for 1 hr (Itaya and Ui, 1966). FA analyses of the major phospholipids (PC and PEA) and the neutral lipid, TAG, were performed by gas–liquid chromatography as described by Smuts *et al.* (1992). Areas corresponding with the aforementioned components were scraped into glass-stoppered tubes and transmethylated with 2.5 ml methanol–18 M sulfuric acid (95:5, v/v) at 70°C for 2 hr. The resultant FA methyl esters were analysed on a Varian model 3700 Gas Chromatograph using fused silica megabore DB-225 columns (J&W Scientific, Folsom, CA, USA, cat. no. 125-2232). The individual FA methyl esters were identified by comparison of the retention times with those of a standard mixture of free FA C14:0 to C22:6.

#### Statistical analysis

All analyses were performed using the statistical analysis system program package. In order to correct for variations in the repeated measurements obtained from different hepatocytes isolation batches log transformation was performed to normalize the measurement data. The Proc Mixed procedure, which fits a wider class of mixed linear models, was used to test for significant differences between the means. The Proc Mixed procedure is a generalization of the GLM ANOVA procedure with the advantage that data with several sources of variation, that is, repeated measurement situations using different hepatocyte primary cultures as in the present study, can be analysed statistically.

## RESULTS

*Incorporation of <sup>14</sup>C- and <sup>3</sup>H-labelled precursors*

The incorporation of [<sup>3</sup>H]leucine was not affected in the fumonisin (FB<sub>1</sub> and FB<sub>2</sub>)-treated hepatocytes. There was no significant difference in the extent of incorporation of radiolabelled leucine in the control dishes after 2 (40,712 ± 1868 dpm/hr/mg protein ± SD) and 24 (42,519 ± 9645 dpm/hr/mg protein ± SD) hr. Treatment of hepatocytes with FB<sub>1</sub> for 2 hr (150 μM, 40,969 ± 2850 dpm/hr/mg protein ± SD; 500 μM, 39,952 dpm/hr/mg protein ± SD) or 24 hr (150 μM, 41,691 ± 7650 dpm/hr/mg protein ± SD; 500 μM, 40,806 ± 2461 dpm/hr/mg protein ± SD) did not significantly alter incorporation of [<sup>3</sup>H]leucine. FB<sub>2</sub> which was incorporated at concentrations of 50 and 150 μM, showed a similar pattern of results. A significant reduction was, however, noticed in the incorporation of [<sup>14</sup>C]palmitic acid in primary hepatocytes in the presence of 150 ( $P < 0.01$ ) and 500 μM FB<sub>1</sub> ( $P < 0.0001$ ) (Fig. 1a). Incorporation of radiolabel in the total lipid fraction (phospholipid and neutral lipids) tended to decrease with increasing FB<sub>1</sub> concentration (Fig. 1a) and was significantly ( $P < 0.0001$ ) lowered in hepatocytes treated with 500 μM FB<sub>1</sub>. The total phospholipid fraction

showed a significant ( $P < 0.05$ ) increase in incorporation of radiolabel hepatocytes treated with 150 μM FB<sub>1</sub>, while at 500 μM FB<sub>1</sub>, no increase was observed compared with that of the controls. With respect to the neutral lipids a significant ( $P < 0.0001$ ) decrease in the incorporation of label in both TAG and CE was noticed at both fumonisin concentrations (Fig. 1b). Quantification of the total and free cholesterol levels indicated that FB<sub>1</sub> (500 μM) significantly ( $P < 0.02$ ) lowered total cholesterol by reducing the level of free cholesterol ( $P < 0.001$ ) in hepatocytes, while the CE level remained constant (Fig. 2). At a level of 150 μM FB<sub>1</sub> total cholesterol was only slightly decreased (not statistically significant) while free cholesterol was decreased significantly ( $P < 0.01$ ).

*Synthesis and radiolabelling of phospholipids*

Synthesis of the main membrane phospholipids was significantly altered as indicated by the respective increase in the levels of PC ( $P < 0.0001$ ) and PEA ( $P < 0.0001$ ) and the decrease in the level of SM ( $P < 0.0001$ ) in hepatocytes exposed to a cytotoxic dose (500 μM) of FB<sub>1</sub> (Fig. 3a). At the lower dose of FB<sub>1</sub> (150 μM) the levels of PC ( $P < 0.05$ ) and PEA ( $P < 0.01$ ) were increased and that of SM ( $P < 0.001$ ) decreased significantly as described for hepatocytes treated with 500 μM FB<sub>1</sub>. The incorporation of radiolabel into the respective phospholipids closely resembled the increase and/or decrease in their respective levels (Fig. 3b). The radiolabelling of SM was not significantly decreased in hepatocytes treated with 150-μM FB<sub>1</sub> but was significantly reduced ( $P < 0.0001$ ) in the 500 μM FB<sub>1</sub>-treated hepatocytes. With respect to PC ( $P < 0.0001$  for 150 and 500 μM FB<sub>1</sub>/dish) and PEA ( $P < 0.0001$  for 150 and 500 μM FB<sub>1</sub>/dish) the radiolabelling was increased as a result of FB<sub>1</sub> treatment.

*Effect on FA composition of PC, PEA and TAG*

**Saturated FA: C16:0 and C18:0.** A significant ( $P < 0.05$ ) increase in C16:0 occurred in PC in hepatocytes treated with 500 μM for 24 hr while no effect was observed in PEA (Table 1). In contrast, there was a significant decrease in the incorporation of the FA in TAG in the presence of 150 μM ( $P < 0.0001$ ) and 500 μM ( $P < 0.0001$ ) FB<sub>1</sub>. No significant difference was obtained in the level of C18:0 in the phospholipids and/or TAG of hepatocytes treated with fumonisins. The total saturated FAs were significantly ( $P < 0.0001$ ) lower in TAG of hepatocytes treated with both fumonisin concentrations.

**Monounsaturated FA: C16:1 and C18:1.** Both FA were significantly decreased in PC ( $P < 0.001$ ) in hepatocytes treated with 500 μM FB<sub>1</sub> (Table 1). No effect was noticed in PEA and TAG in hepatocytes treated with 150 or 500 μM FB<sub>1</sub>.

**Polyunsaturated FA: C18:2n-6.** This FA was significantly ( $P < 0.01$  to  $P < 0.001$ ) increased in PC in the presence of both FB<sub>1</sub> concentrations. The increase of the FA was markedly enhanced ( $P < 0.0001$ ) in

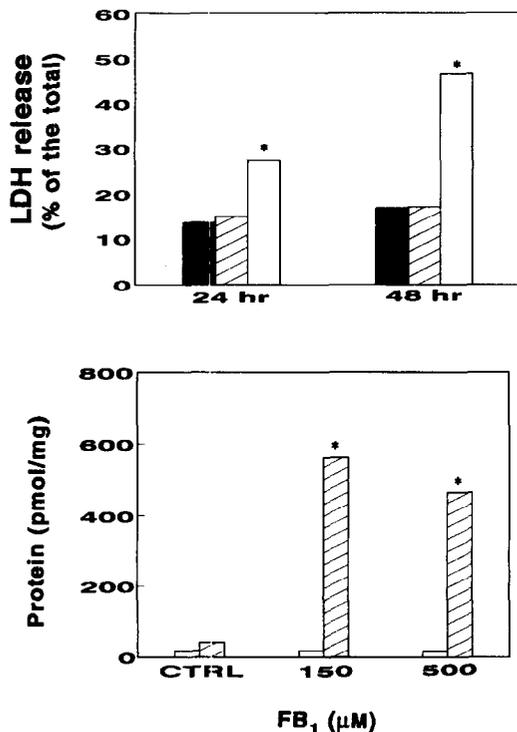


Fig. 4. (a) Effect of FB<sub>1</sub> (▨, 150 μM and □, 500 μM) on the release of LDH from primary hepatocytes exposed for various time intervals. (b) Sa (▨) and So (□) concentrations in primary hepatocytes exposed for 48 hr to different FB<sub>1</sub> concentrations. Values represent means of three different experiments with triplicate determinations. \*Values differ significantly from the control (ctrl) treatment;  $P < 0.0001$  (Tukey's Studentized Range method). ■, control.

TAG after both treatments while no effect was observed in PEA.

*C20:4n-6*. A significant increase was noticed in the PC ( $P < 0.001$ ) and TAG ( $P < 0.001$ ) fractions of hepatocytes exposed to  $500 \mu\text{M}$   $\text{FB}_1$ . A significant increase ( $P < 0.05$ ) was noticed in PC in hepatocytes exposed to  $150 \mu\text{M}$ , while it was markedly (not significantly) higher in TAG. No significant effect was noticed in the PEA fractions at both concentrations.

As a result of the described changes the total polyunsaturated FA (PUFA) increased significantly in PC ( $P < 0.001$ ) and TAG ( $P < 0.0001$ ) in hepatocytes exposed to a cytotoxic dose of  $\text{FB}_1$  ( $500 \mu\text{M}$ ), while it was significantly ( $P < 0.0001$ ) increased in TAG at a non-toxic dose ( $150 \mu\text{M}$ ).

#### *FB<sub>1</sub> cytotoxicity and alterations in sphingolipid biosynthesis*

No cytotoxicity was noticed when  $\text{FB}_1$  was incorporated at a concentration of  $150 \mu\text{M}$  while at the higher concentration ( $500 \mu\text{M}$ ) a cytotoxic effect was noticed at both 24 ( $P < 0.0001$ ) and 48 ( $P < 0.0001$ ) hr (Fig. 4a). Treatment of hepatocytes with fumonisins ( $150$  and  $500 \mu\text{M}$ ) resulted in a seven- to 12-fold increase in the concentration of Sa while So was not significantly altered (Fig. 4b). There was no significant difference between the Sa levels in hepatocytes treated with the different fumonisin concentrations. The respective baseline values of So and Sa after 48 hr were  $17.9 \pm 3.6$  and  $44.2 \pm 2.5$  pmol/mg protein  $\pm$  SD, resulting in a Sa/So ratio of  $2.6 \pm 0.7$ . In the presence of  $\text{FB}_1$  ( $150$  and  $500 \mu\text{M}$ ) the Sa/So ratio was increased 25–28-fold above that of the controls.

#### *Effects on parameters related to membrane fluidity and metabolism of FA*

The polyunsaturated/saturated FA ratio (P/S ratio) and the N-6/N-3 ratio were not altered in the respective lipid fractions (Table 1). The PC/free cholesterol ratio increased markedly, that is 12.0 and 14.1 in hepatocytes treated with  $150$  and  $500 \mu\text{M}$ , respectively, compared with the control value of 9.2. The C20:3/C18:2 ratio was significantly decreased ( $P < 0.001$ ) at  $150$  ( $0.06 \pm 0.01$ , mean  $\pm$  SD) and  $500 \mu\text{M}$  ( $0.07 \pm 0.01$ , mean  $\pm$  SD) compared with that of the control ( $0.10 \pm 0.01$ ). In the case of the C20:4/C20:3 ratio both concentrations ( $150 \mu\text{M}$   $67.5 \pm 23.5$ ; and  $500 \mu\text{M}$   $66.7 \pm 17.9$ , means  $\pm$  SD) showed a significant ( $P < 0.0001$ ) increase compared with the control value ( $44.5 \pm 11.5$ ).

#### DISCUSSION

The hepatotoxicity of fumonisins in rats has been implicated to have an important role during cancer induction by these mycotoxins (Gelderblom *et al.*, 1992 and 1994). In the present study, primary hepatocytes were exposed to a cytotoxic and non-cytotoxic dose of  $\text{FB}_1$  and  $\text{FB}_2$  to investigate whether certain

biological processes within the cell are affected by  $\text{FB}_1$ . This could provide better insight into the mechanisms likely to be involved in cytotoxicity in primary hepatocytes.

$\text{FB}_1$  and  $\text{FB}_2$  did not affect protein synthesis in primary rat hepatocytes, even when exposed for 24 hr to a cytotoxic concentration of  $500 \mu\text{M}$ . The present data indicate that inhibition of protein synthesis by culture extracts of *F. moniliforme*-contaminated corn (Norred *et al.*, 1990) cannot be ascribed to the presence of FB mycotoxins. In contrast to this,  $\text{FB}_1$  treatment resulted in a significant reduction in the incorporation of  $^{14}\text{C}$ -labelled palmitic acid in primary hepatocytes (Fig. 1a). Radiolabelling of total lipids (phospholipids and neutral lipids) decreased significantly in the presence of increasing  $\text{FB}_1$  concentration. A prominent aspect concerning the decrease in radiolabelling was the significant reduction in radioactivity incorporated in neutral lipids, namely CE ( $P < 0.0001$ ) and TAG ( $P < 0.0001$ ) at  $150$  and  $500 \mu\text{M}$  (Fig. 1b). As the level of CE remained constant it would appear that palmitic acid is competitively incorporated in membraneous compartments other than this neutral lipid.  $\text{FB}_1$  also significantly reduced the free cholesterol level (Fig. 2) at  $150$  ( $P < 0.01$ ) and  $500 \mu\text{M}$  ( $P = 0.001$ ), implying impaired synthesis. As free cholesterol occurs mainly as an integral component of cellular membranes, it is likely that a decrease in cholesterol will affect the fluidity of hepatocyte membranes. The increased PC/free cholesterol ratio in the  $\text{FB}_1$  treated cells may therefore be indicative of a shift to a more rigid membrane structure.

As stimulation of PC and PEA synthesis is not dependent on protein synthesis,  $\text{FB}_1$  could activate either the microsomal enzymes which regulate *de novo* synthesis or the turnover of these phospholipids. With respect to the latter, the decrease in SM level is of relevance. It is known that  $\text{FB}_1$  inhibits ceramide synthase (Wang *et al.*, 1991), a key enzyme in sphingolipid synthesis, which results in the accumulation of Sa and thus in a decrease in ceramide. The synthesis of SM in the liver is catalysed by two enzymes, namely phosphatidylcholine-ceramide-phosphocholine transferase and phosphatidylethanolamine-ceramide-phosphoethanolamine transferase (Nikolova-Karakashian *et al.*, 1991). Therefore, the decrease in SM synthesis could result in decreased utilization of PC and PEA, which could explain the increase in their respective levels. The low cholesterol levels in hepatocytes are likely to decrease the activities of these enzymes as cholesterol-enriched plasma membranes enhance their activities (Nikolova-Karakashian *et al.*, 1991). In addition, the decrease in the levels of free cholesterol could be the result of the decreased level of SM in cellular membranes which influences cholesterol synthesis and/or metabolism (Gupta and Rudney, 1991; Merrill and Jones, 1990). A recent investigation concerning the phospholipid profiles of J774 macrophages (Smith and Merrill,

1993) and primary hepatocytes (Wang *et al.*, 1991) indicated that, as presented in this study, FB<sub>1</sub> enhances incorporation of [<sup>14</sup>C]palmitate in PC and PEA.

The decrease in the radiolabelling of TAG could be related to an inhibitory effect of FB<sub>1</sub> on *de novo* synthesis. In this regard the inhibitory effect of FB<sub>1</sub> on Sa acyltransferase in the sphingolipid biosynthetic pathway (Wang *et al.*, 1991) could be of relevance, since TAG synthesis is also catalysed by diacylglycerol acyltransferase. The latter enzyme is regulated by FA as indicated by the fact that C16:0 and C18:2 stimulate while C20:5 decreases the activity of the enzyme. *In vivo* studies showed that conversion of C18:2 to C20:4 is required for suppression of TAG synthesis (Strum-Odin *et al.*, 1987). Therefore, alteration of FA profiles within the cell could alter the activity of diacylglycerol acyltransferase. Conversely, the decrease in the incorporation of [<sup>14</sup>C]palmitic acid could have resulted from the selective increase in the incorporation of C20:4 and C18:2 rather than a decrease in the synthesis of TAG. The enhanced incorporation of the latter is likely to result from an increase in the cellular content of these FA (see later).

Incorporation of [<sup>14</sup>C]palmitic acid into PC, PEA and SM reflects changes in phospholipid level (Fig. 3a,b). However, the FA profiles of the membrane phospholipid, PC, as well as the neutral lipid, TAG, were altered in the presence of FB<sub>1</sub>. At a non-toxic dose of FB<sub>1</sub> (150 μM) changes in FA composition were noticed in PC with C18:2 ( $P < 0.01$ ) and C20:4 ( $P < 0.05$ ) that were significantly increased (Table 1). TAG showed reduced ( $P < 0.001$ ) incorporation of C16:0, while C18:2 was significantly ( $P < 0.0001$ ) increased. However, most of the FA changes occurred in hepatocytes treated with 500-μM FB<sub>1</sub>—that is, when a slight cytotoxic effect was obtained after 24 hr of exposure (Fig. 4a). These changes included significant ( $P < 0.01$  to  $P < 0.0001$ ) increases in C18:2 and C20:4 in both PC and TAG (Table 1). When the C20:3/C18:2 and C20:4/C20:3 ratios of PC are considered the former was significantly ( $P < 0.001$ ) decreased at both concentrations of FB<sub>1</sub>, implying that the Δ6 desaturase, a rate-limiting enzyme in FA metabolism (Ulman *et al.*, 1991), is inhibited. In contrast to this, the C20:4/C20:3 ratio was significantly ( $P < 0.0001$ ) enhanced in the presence of 150 and 500 μM-FB<sub>1</sub>, respectively. This implies that Δ5 desaturases are activated and/or the level of C20:4 is increased due to an inhibitory effect of FB<sub>1</sub> on the prostanoid synthetic pathway.

The increased incorporation of long-chain unsaturated FA in PC could be regarded as a compensatory effect to counteract the increase in rigidity due to decreased free cholesterol. However, apart from the effect on membrane fluidity, changes in the content of certain FA in PC, especially C18:2 and C20:4, could have several important implications for hepatocytes. A change in unsaturation could modify the responsiveness of cells to transformation, expression of a

specific phenotype or clonal selection which are important events during chemical hepatocarcinogenesis (Baldwin and Parker, 1985). Furthermore, alterations in membrane structure, as indicated by the increase in PC and PEA and the decrease in SM and cholesterol levels as well as changes in the FA composition of PC, could have altered the responsiveness of cells by the flux of nutrients, release of growth substances and/or growth inhibitors and normal cell-to-cell interactions. In this regard, FB<sub>1</sub> inhibited hepatocyte cell proliferation *in vivo* (Gelderblom *et al.*, 1994) and EGF-induced DNA synthesis in primary hepatocytes (Gelderblom *et al.*, 1995). It has been suggested that the inhibitory effect of cell proliferation is a key event during cancer promotion induced by fumonisins (Gelderblom *et al.*, 1992, 1994 and 1995). The increase in FA unsaturation in FB<sub>1</sub>-treated hepatocytes could have important implications regarding the control of cell proliferation, especially prostaglandin synthesis (Cornwell and Morisaki, 1984). Prostaglandins or oxygen-centred radicals produced during their biosynthesis may either inhibit or stimulate cell proliferation depending on the cell type and prostaglandin concentration. At present it is unclear whether FB<sub>1</sub> inhibits cell proliferation indirectly by disruption of prostaglandin synthesis. However, the inhibitory effect of the non-steroidal anti-inflammatory drug ibuprofen on the EGF-induced DNA synthesis stimulatory response (W. C. A. Gelderblom, unpublished data, 1996) suggests that disruption of prostanoid biosynthesis could explain the inhibitory effect of FB<sub>1</sub> on cell proliferation.

It has been suggested that the accumulation of Sa in cells could be responsible for the toxicological effects of fumonisins in animals (Merrill *et al.*, 1993). As there is no significant difference in the Sa levels of hepatocytes exposed to toxic and non-toxic concentrations of FB<sub>1</sub> (Fig. 4b), it appears that inhibition of sphingolipid biosynthesis and specifically, the subsequent accumulation of Sa, is not solely responsible for the cytotoxicity of fumonisins in hepatocyte cultures. This was confirmed by a recent study indicating that the accumulation of Sa was effected maximally at a FB<sub>1</sub> concentration of 1 μM in primary hepatocytes (Gelderblom *et al.*, 1995). At this FB<sub>1</sub> concentration no cytotoxicity was observed (Gelderblom *et al.*, 1995), even after an incubation period of 4 days (Norred *et al.*, 1992b). The disruption of sphingolipid biosynthesis and the subsequent accumulation of Sa is also not associated with the mitoinhibitory effect of FB<sub>1</sub> on the EGF mitogenic response in primary hepatocytes (Gelderblom *et al.*, 1995). Therefore, *de novo* interruption of lipid and FA biosynthesis at different levels, which are likely to affect the structure of cellular membranes, appears to be an important event determining alterations in growth-related responses induced by fumonisins in primary rat hepatocyte cultures. In addition, the accumulation of PUFA in hepatocytes treated with high doses of FB<sub>1</sub> could

also be an important determinant in the induction of cell death.

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## Effect of Fumonisin B<sub>1</sub> on the Levels and Fatty Acid Composition of Selected Lipids in Rat Liver *In Vivo*

W. C. A. GELDERBLOM\*†, C. M. SMUTS‡, S. ABEL†, S. D. SNYMAN†, L. VAN DER WESTHUIZEN†, W. W. HUBER§ and S. SWANEVELDER||

†Programme on Mycotoxins and Experimental Carcinogenesis and ‡National Research Programme for Nutritional Intervention, Medical Research Council, PO Box 19070, Tygerberg 7505, South Africa, and §Institut für Tumorbologie & Krebsforschung, Der Universität Wien, Borschkegasse 8a, A-1090 Vienna, Austria and ||Centre for Epidemiological Research in South Africa, Medical Research Council, PO Box 19070, Tygerberg 7505, South Africa

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**Abstract**—The modulating role of fumonisin B<sub>1</sub> (FB<sub>1</sub>) on lipid biosynthesis was evaluated in a short-term (21 day) experiment using male Fischer rats fed high dietary levels (50, 100 and 250 mg FB<sub>1</sub>/kg) and in a long-term (2 yr) experiment using male BD IX rats fed low dietary levels (1, 10 and 25 mg FB<sub>1</sub>/kg) of FB<sub>1</sub>. The total serum and liver cholesterol was significantly ( $P < 0.01$ ) increased in the rats fed 250 mg FB<sub>1</sub>/kg diet for 21 days, while the liver phospholipids, sphingomyelin and phosphatidylethanolamine (PE) were significantly decreased ( $P < 0.01$ ) and increased ( $P < 0.05$ ), respectively. In the long-term study, only PE was significantly ( $P < 0.05$ ) increased in all the FB<sub>1</sub>-treated animals. Fatty acid (FA) analysis of PE indicated that C18:2n-6 was significantly increased ( $P < 0.05$  to  $P < 0.01$ ) in the FB<sub>1</sub>-treated rats of the short-term study, while it was markedly (not significantly) increased in phosphatidylcholine (PC). The same pattern was observed in the PC and PE fractions of the liver of the FB<sub>1</sub>-treated rats from the long-term studies, but the changes were not significant due to the small number (three rats per group) of rats analysed. The levels of C22:5n-6 and C22:6n-3 were also markedly decreased and increased respectively in the 10 and 25 mg FB<sub>1</sub>/kg-treated groups. When the FAs were determined in the total lipids in a larger number of rats (four to six animals per group) the level of C18:2n-6 was significantly increased in the 10 ( $P < 0.01$ ) and 25 ( $P < 0.05$ ) mg FB<sub>1</sub>/kg-treated groups. Similar effects were noticed in plasma PC with respect to the C18:2n-6 and C22:5n-6 in both the long- and short-term treated groups, except that C20:4n-6 was also lower in both cases. The total n-6 FAs and polyunsaturated FAs were significantly ( $P < 0.01$ ) and markedly reduced in PC and PE, respectively, of the rats fed the 250 mg FB<sub>1</sub>/kg diet. In the long-term experiment the n-6/n-3 ratio was significantly ( $P < 0.01$ ) decreased in PE and markedly lowered in PC due to a significant ( $P < 0.05$ ) increase in the n-3 FAs of both phospholipid fractions. The sphinganine/sphingosine ratio was significantly ( $P < 0.05$ ) altered in the liver of the rats fed the 100 and 250 mg FB<sub>1</sub>/kg diets for 21 days, while in the long-term study no significant changes were noticed in either the liver or sera. The present data indicate that FB<sub>1</sub> affects lipid biosynthesis in rat liver and plasma differently, depending on the dietary level and duration of treatment. Alterations to the n-3 and n-6 FA biosynthetic pathways, detected in rats fed relatively low dietary levels of FB<sub>1</sub>, are likely to be important mediators for FB<sub>1</sub>-induced effects on hepatocyte cell proliferation. © 1997 Elsevier Science Ltd

**Abbreviations:** ANOVA = analysis of variance; CE = cholesterol ester; CM = chloroform/methanol; EGF = epidermal growth factor; FAs = fatty acids; FB<sub>1</sub> = fumonisin B<sub>1</sub>; GSTP = glutathione-S-transferase placental form; PC = phosphatidylcholine; PE = phosphatidylethanolamine; P/S = polyunsaturated/saturated; PUFAs = polyunsaturated fatty acids; Sa = sphinganine; SM = sphingomyelin; So = sphingosine.

### INTRODUCTION

The toxicological effects of the fumonisins, mycotoxins produced by *Fusarium moniliforme*, have been investigated in different animal species and cell culture systems (Abbas *et al.*, 1993; Colvin and Harrison, 1992; Gelderblom *et al.*, 1991; Kellerman

*et al.*, 1990; Shier *et al.*, 1991; Voss *et al.*, 1995; Yoo *et al.*, 1992). Fumonisin B<sub>1</sub> (FB<sub>1</sub>) exhibits moderate toxic effects in both *in vivo* experiments in rat liver (Gelderblom *et al.*, 1988 and 1994) and *in vitro* to primary hepatocyte cultures (Cawood *et al.*, 1994). The carcinogenicity of the fumonisins has been associated with a hepatotoxic effect, since studies have indicated that the toxicity is a prerequisite for cancer initiation (Gelderblom *et al.*, 1994) and a

\*Author for correspondence.

chronic toxic hepatitis for the development of liver cancer (Gelderblom *et al.*, 1991 and 1996a).

As the hepatotoxicity of the fumonisins appears to be important for cancer development, the biochemical changes associated with the FB<sub>1</sub>-induced hepatotoxic effects could, indirectly, also facilitate the induction of events required for cancer development. Of importance are changes in the lipid composition of cellular membranes that are likely to alter the responsiveness of cells to external stimuli as well as growth-related responses within the cell (Spector and Burns, 1987). Such changes are associated with cellular adaptive responses that eventually could lead to the induction of the cancer phenotype and/or its subsequent modulation (Farber, 1993; Farber and Rubin, 1991).

Detailed studies on lipid biosynthesis in primary rat hepatocytes (Gelderblom *et al.*, 1996b) indicated that FB<sub>1</sub> affects the level and fatty acid (FA) composition of the major phospholipids and thus the structural components of cellular membranes. These include the accumulation of the membrane phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the sphingolipid sphinganine (Sa) and a decrease in sphingomyelin (SM) and free cholesterol. Studies regarding the FA composition of the phospholipids PC and PE and the neutral lipid triacylglycerol showed an increase in the levels of the FAs C18:2n-6 and C20:4n-6 suggesting that FB<sub>1</sub> alters lipid biosynthesis at different levels in primary hepatocytes. The decreased SM levels resulted from the FB<sub>1</sub>-induced inhibition of ceramide synthase, a key enzyme in the synthesis of sphingolipids (Wang *et al.*, 1991). The latter effect of the fumonisins have been implicated in the toxicological and carcinogenic properties of the fungus *F. moniliforme* in different animal species (Schroeder *et al.*, 1994; Wang *et al.*, 1991).

The present study further evaluates the effect of FB<sub>1</sub> on the levels of selected lipids in the liver of rats fed different dietary levels of FB<sub>1</sub> for different periods of time. Detailed analyses of the fatty acid composition of important membrane phospholipids will also be presented.

## MATERIALS AND METHODS

### Chemicals

FB<sub>1</sub> was purified to a purity of 90–95% according to the method of Cawood *et al.* (1991). C<sub>20</sub>-sphinganine was a gift from Dr A. H. Merrill, Atlanta, Georgia, USA, while free FA analytical standards (C16:0 to C22:6), used for calibration and identification, were obtained from Nu-Chek-Prep, Inc (Efamol Special Preparation no. 455). All the chemical solvents, such as chloroform and methanol, were of analytical grade and glass distilled prior to use.

### Animals and diets

Male F344 rats (150 g) fed the modified AIN-76 diet (Gelderblom *et al.*, 1994) were used in short-term feeding studies, while in the long-term studies male BD IX rats fed a semi-purified cereal-based diet (Jaskiewicz *et al.*, 1987) were used. Sunflower oil was used as the source of dietary fat and incorporated in the different diets at levels of 5% (AIN-76) and 3% (semipurified diet), respectively. Gas chromatographic analyses of sunflower oil indicated that it consists mainly of C18:2n-6 (65%), while C18:3n-3 (0.9%) was the only source for the n-3 FAs (data not shown).

The FB<sub>1</sub>-containing diets were prepared by evaporating the mycotoxin, dissolved in methanol, on a small subsample (200 g) of the diet to allow for even distribution. After the subsamples were dried in a fume cupboard for 4 hr, they were mixed with the control diets to obtain the desired FB<sub>1</sub> levels and stored under nitrogen at 4°C. The FB<sub>1</sub> diets for the long-term experiment were prepared on a monthly basis and stored under similar conditions. All the rats were caged individually in a controlled environment (23–25°C) with a 12-hr light/dark cycle. They had free access to feed and water and were weighed three times per week.

### Treatments

*Long-term study.* The BD IX rats (20 rats per group) were fed three different diets containing 1, 10 and 25 mg FB<sub>1</sub>/kg, in addition to the control diet, for a period of 2 yr. The animals were killed with sodium pentobarbitone anaesthesia, at which time part of the liver was stored in saline at –80°C for biochemical analyses.

*Short-term study.* Different diets containing 50, 100 and 250 mg FB<sub>1</sub>/kg were fed to F344 rats (five rats per group) over a period of 21 days. Rats were killed by decapitation, and their livers removed, weighed and stored at –80°C in saline.

### Lipid analyses

The liver samples obtained from the short-term (five rats per group) and long-term experiments (three rats per group selected randomly) were subjected to detailed lipid analysis. These included the quantification of the individual phospholipids PC, PE, SM and total cholesterol, while the FA composition of PC and PE were determined. Extraction of the liver tissue was performed as described by Smuts *et al.* (1992). In short, part of the liver was ground to a fine powder in liquid nitrogen and weighed (approx. 100–150 mg) in glass-stoppered tubes. The tissue was suspended in 0.5 ml saline and the lipids extracted with 24 ml chloroform–methanol (C:M; 2:1, v/v) containing 0.01% butylated hydroxytoluene as antioxidant. The mixture was filtered (sinterglass filters using Whatman glass microfibre filters) and the filtrate evaporated to dryness *in vacuo* at 40°C, transferred to glass stoppered tubes and stored at 4°C under nitrogen until analysed.

### Fatty acid analyses

Lipid extracts of the different samples were fractionated by thin-layer chromatography using chloroform-methanol-petroleum ether-acetic acid-boric acid (40/20/30/10/1.8, by vol) as the mobile phase (Gilfillan *et al.*, 1983). After the plates were sprayed with CM (1:1) containing 2,5-bis-(5'-*tert*-butylbenzoxazolyl-[2'])thiophene, the visualized spots corresponding to the individual phospholipids (SM, PC and PE) were scraped and quantitatively transferred to glass-stoppered tubes and the respective levels determined. Duplicate plates were run under similar conditions to determine the FA profiles of PC and PE. The phospholipid fractions were transmethylated with 2.5 ml methanol-11.6 M sulfuric acid (95:5, v/v) at 70°C for 2 hr in glass-stoppered tubes as described by Smuts *et al.* (1992). The resultant FA methyl esters were analysed on a Varian model 3700 Gas Chromatograph using fused silica megabore DB-225 columns (J & W Scientific, Cat. No. 125-2232) and subsequently identified by comparison of retention times to those of a standard mixture of free FAs C14:0 to C22:6. FA profiles of the total liver lipids were also determined on C:M (2:1) extracts (Folch *et al.*, 1957) of liver homogenates of the rats (four to six rats per group selected randomly) fed low dietary levels of FB<sub>1</sub> (long-term study). The FA profiles of plasma PC of the rats from the short-term (five rats per group) and long-term studies (four rats per group selected randomly) were determined according to the method of Smuts *et al.* (1992).

### Phospholipid determination

Phospholipid levels were determined colorimetrically using a malachite green dye (Itaya and Ui, 1966) after digestion with perchloric acid (16 N) at 170°C for approximately 1 hr.

### Effect on cholesterol content

Total cholesterol and unesterified cholesterol on the CM lipid extracts (long-term study) were determined by an enzymatic iodide method using cholesterol oxidase and esterase enzymatic preparations (Smuts *et al.*, 1992). The concentration of cholesterol ester (CE) was obtained by subtraction. For the short-term feeding experiments the total cholesterol was determined by gas chromatography as by the method of Ishikawa *et al.* (1974) using stigmasterol as the internal standard.

### Effect on Sa and sphingosine (So) synthesis

The liver of the rats were ground to a fine powder as described earlier and subsamples subjected to sphingolipid (100-150 mg) and protein (10-15 mg) determinations. The powdered samples were suspended in 4 vol (w/v) 0.05 M phosphate buffer (pH 7.0) and a subsample (approx. 25 mg) extracted in CM (1:2). Sa and So were quantified by HPLC as described elsewhere (Riley *et al.*, 1994) using C20-sphinganine as the internal standard. Serum ana-

lyses of Sa and So of randomly selected rats (seven to nine rats per group) from the long-term study were performed as described by Riley *et al.* (1994).

### Protein determination

The powdered liver preparations (10-15 mg) were solubilized in 5% sodium dodecyl sulfate in 0.5 N NaOH at 37°C and the protein content determined using a modified method of Lowry with bovine serum albumin as the protein standard (Markwell *et al.*, 1978).

### Statistical analyses

Data, regarding the body weight gain, relative liver weight of the rats, FA profiles of the different phospholipid fractions and serum PC, and liver sphingolipid analyses from the short-term experiments (five rats per group), were analysed by two-way analyses of variance (ANOVA) followed by the Tukey T-test to determine whether the means differed statistically. The non-parametric Kruskal-Wallis statistical method was used to determine whether changes concerning the FA profiles of the liver phospholipid fractions (three rats per group) and serum PC (four rats per group) and sphingolipid analyses of the liver (three rats per group) of the long-term study differed significantly within the treatment groups. Statistical differences between the serum Sa/So ratios of the different treatment groups (seven to nine rats per group) and the total liver FA profiles (four to six animals per group) of the long-term study were determined by ANOVA as already described.

## RESULTS

### Effect on body weight gains and relative liver weights

*Short-term feeding study (F344 rats).* The body weight gain and relative liver weight were significantly decreased in the rats fed a dietary level of 250 mg FB<sub>1</sub>/kg over a period of 21 days (Table 1). When compared with the control and 50 mg FB<sub>1</sub>/kg diet groups significant reductions in the feed intake profiles ( $P < 0.05$  to  $P < 0.01$ ) were noticed in the rats fed the 100 and 250 mg FB<sub>1</sub>/kg diets.

*Long-term feeding study (BD IX rats).* The mean feed consumption profiles over the 24-month treatment period of the control, 1, 10 and 25 mg FB<sub>1</sub>/kg diet groups were  $3.2 \pm 0.8$ ;  $3.4 \pm 0.9$ ;  $3.4 \pm 0.7$ ;  $3.5 \pm 0.6$  g/day/100 g body weight, respectively. On the basis of these values, the estimated mean daily FB<sub>1</sub> intake profiles were 0.0005,  $0.003 \pm 0.001$ ,  $0.03 \pm 0.01$  and  $0.08 \pm 0.01$  mg FB<sub>1</sub>/100g body weight for the respective groups. Detailed information concerning the feed consumption, body weight gains and relative liver weight will be published elsewhere. Except for the relative liver weight, which was significantly ( $P < 0.05$ ) reduced in the FB<sub>1</sub>-treated rats, there was no significant difference due to FB<sub>1</sub> treatment in any of these parameters over the experimental period

Table 1. Effect of FB<sub>1</sub> on feed intake profiles and rat growth parameters

Treatment (mg FB <sub>1</sub> /kg diet)	Feed intake g/day/100 g body weight	Apparent FB <sub>1</sub> intake mg/day/100 g	Body weight gain (g)	Liver weight (% of body weight)
Control	8.1 ± 0.3a	—	78.6 ± 7.1a	4.5 ± 0.1a
50	8.4 ± 0.4aA	0.4 ± 0.0	81.4 ± 4.3a	4.4 ± 0.2a
100	7.9 ± 0.3b	0.8 ± 0.0	72.4 ± 8.6a	4.3 ± 0.2a
250	7.6 ± 0.3b	1.9 ± 0.1	54.6 ± 8.8b	3.1 ± 0.1b

Values are the means ± SD of five rats per group. Means in a column followed by the same letter do not differ significantly. If the letters differ (lower case) then  $P < 0.05$ ; if the cases and letters differ then  $P < 0.01$ .

### Histopathological changes

**Short-term studies.** The major toxicological effects induced by the fumonisins over the 21-day treatment period in F344 rats have been described previously (Gelderblom *et al.*, 1994 and 1996c) while studying the cancer initiating and promoting potential of the same dietary levels used in the present study. Similar pathological changes were observed in the present study, which include single cell necrosis in the periportal areas of the rats receiving 50 mg FB<sub>1</sub>/kg diet. These lesions became more prominent in the 100 and 250 mg FB<sub>1</sub>/kg groups and tended to extend into the liver lobules. There was also a moderate increase in the proliferation of duct epithelial cells ('oval cells') in the liver of the rats treated with the 100 and 250 mg FB<sub>1</sub>/kg diets. The livers of the 250 mg FB<sub>1</sub>/kg-treated rats showed signs of nodular regeneration and fibrosis which, together with the proliferation of duct epithelial cells, slightly distorted the architecture of the liver.

**Long-term studies.** A description of the detailed histopathological changes will be published elsewhere. As compared to the aforementioned toxicological effects, rats treated with the 1 mg FB<sub>1</sub>/kg dietary level showed no toxic effects. The rats treated with the 10 mg FB<sub>1</sub>/kg diet showed only mild toxic effects, including atrophy of hepatocytes, mild to prominent anisonucleosis, foci of lipid accumulation, single cell necrosis, proliferation of duct epithelial cells and hepatocyte nodules (one to two per liver) in a few rats. In the high-dose group (25 mg FB<sub>1</sub>/kg diet) these changes occurred more frequently, but were still mild. In some rats of the high-dose treatment group, hepatocyte nodules (one to five per liver) were noticed macroscopically, while basophilic and eosinophilic foci were noticed microscopically. In one rat early signs of fibrosis was noticed with a slight distortion of the lobular structure due to focal fibrosis and proliferation of the bile ductules. There was a single area of cholangiofibrosis in one of the rats treated with the high dietary level of FB<sub>1</sub>. An increase in the number and size of foci that stained positively for the placental form of glutathione-S-transferase (GSTP), a marker for preneoplasia in rat liver (Rushmore *et al.*, 1987), was noticed in the livers of the rats that received the 10 and 25 mg FB<sub>1</sub>/kg diets.

### Lipid analyses

**Phospho- and sphingolipids levels.** In the short-term experiments FB<sub>1</sub> significantly decreased the levels of SM only in the rats treated with the 250 mg FB<sub>1</sub>/kg diet (Table 2). The level of PC was not altered, while PE was increased significantly ( $P < 0.01$ ) in the 250 mg FB<sub>1</sub>/kg group. No effect was noticed on the level of the total phospholipids (data not shown). The Sa concentration and the Sa/So ratio were significantly increased ( $P < 0.05$ ) in the rats fed dietary levels of 100 and 250 mg FB<sub>1</sub>/kg, while the level of So was unaffected. The Sa concentration was slightly increased (not significantly) in the 50 mg FB<sub>1</sub>/kg diet-treated rats, but due to variations in both the Sa and So levels the Sa/So ratio was not affected.

In the long-term experiments there were no changes in the concentrations of PC and SM, while PE was increased significantly ( $P < 0.05$ ) in all the treated groups. The Sa/So ratio was only slightly increased in one of the rats (1/3) that received the 25 mg FB<sub>1</sub>/kg diet due to an increase in the Sa concentration. However, serum analyses of Sa and So (seven to nine animals per group) indicated no significant differences ( $P > 0.05$ ) in the Sa/So ratio (Table 2) between the different treatment groups.

### Cholesterol levels

The total serum and liver cholesterol levels were significantly increased only in the rats treated with the high FB<sub>1</sub>-containing diet (250 mg/kg) over a period of 21 days. No effect was noticed in the long-term experiments in the FB<sub>1</sub> groups. The PC/free cholesterol ratio was markedly decreased (3.7) in the 250 mg FB<sub>1</sub>/kg-treated group as compared with that of the control and 50 and 100 mg FB<sub>1</sub>/kg-treated groups (6.7).

### FA profiles of plasma, total liver and selected phospholipid fractions

**Short-term experiments.** The level of C18:2n-6 increased significantly in the PE phospholipid fraction (Table 3) of the rats fed the 50 ( $P < 0.05$ ), 100 and 250 ( $P < 0.01$ ) mg FB<sub>1</sub>/kg diets. A marked increase was also noticed in the PC fraction in the rats fed the 250 and 100 mg FB<sub>1</sub>/kg diets but the difference was not significant. The level of C22:5n-6 was significantly decreased in PE of the rats fed diets containing 100 ( $P < 0.01$ ) and 250 mg ( $P < 0.05$ ) FB<sub>1</sub>/kg. As described previously this FA

Table 2. Phospholipid, sphingolipid and cholesterol levels of plasma and liver samples of rats treated with different dietary levels of FB<sub>1</sub> for 21 days (short-term) and 2 yr (long-term)

Treatment (mg FB <sub>1</sub> /kg diet)	Sphingolipid (nmol/mg protein)					Phospholipid (µg/mg protein)					Cholesterol (total)	
	Sa	So	Sa/So ratio	SM	PE	PC	Serum	Liver	(mmol/litre)	(mg/100 mg)		
<b>Short-term</b>												
Control	6.9 ± 2.3a	54.8 ± 23.5a	0.2 ± 0.1a	9.8 ± 2.3a	44.2 ± 3.4a	131.3 ± 17.7	1.9 ± 0.1a	19.6 ± 1.0a				
50	16.7 ± 3.9ab	73.6 ± 4.9a	0.2 ± 0.1a	7.6 ± 0.5a	49.1 ± 3.6a	143.9 ± 9.5	nd	21.2 ± 3.2a				
100	36.6 ± 13.1b	79.3 ± 14.7a	0.5 ± 0.2b	7.6 ± 1.7a	51.3 ± 2.9a	149.5 ± 9.1	nd	22.2 ± 3.5a				
250	130.3 ± 52.0b	65.1 ± 25.3a	2.1 ± 0.8b	5.5 ± 0.9A	56.5 ± 3.1A	144.3 ± 12.4	2.8 ± 0.3A	38.6 ± 6.7A				
<b>Long-term</b>												
Control	10.1 ± 8.4	51.9 ± 11.2	0.2 ± 0.1 (0.8 ± 0.8)	7.1 ± 1.7	33.5 ± 4.0a	197.3 ± 13.2	1.9 ± 0.3	16.8 ± 1.3				
1	14.7 ± 11.6	86.4 ± 60.2	0.2 ± 0.1 (0.6 ± 0.6)	9.2 ± 0.9	55.9 ± 2.7b	197.0 ± 5.4	1.7 ± 0.3	19.3 ± 1.23				
10	12.3 ± 5.0	68.4 ± 10.7	0.2 ± 0.1 (0.7 ± 0.9)	9.1 ± 3.8	69.5 ± 4.2b	188.2 ± 10.4	1.5 ± 0.4	18.7 ± 1.2				
25	82.4 ± 96.8	148.0 ± 113.0	0.5 ± 0.2 (1.7 ± 1.5)	7.4 ± 3.0	55.9 ± 9.4b	190.7 ± 40.9	1.9 ± 0.3	18.8 ± 1.3				

Values represent means ± SD of five rats per group (short-term) and three rats per group (long-term). Means in a column followed by the same letter do not differ significantly, when letters differ then  $P < 0.05$ , if the cases differ then  $P < 0.01$ . Values in parentheses represent the Sa/So ratio of the corresponding serum samples of seven to nine rats per treatment group. BD IX and F344 male rats were used in the 2-yr and 21-day studies, respectively.

Table 3. Fatty acid profiles of liver phospholipids (PC and PE) of rats treated with different dietary levels of FB<sub>1</sub> for various time intervals

Treatment	Phospholipid	C16:0	C16:1	C18:0	C18:1	C18:2 (n-6)	C18:3 (n-6)	C20:4 (n-6)	C22:4 (n-6)	C22:5 (n-6)	C22:5 (n-3)	C22:6 (n-3)
<b>Short-term</b>												
Control	PC	17.7 ± 0.5	1.0 ± 0.3	24.9 ± 1.1	6.8 ± 0.6	7.7 ± 0.1	0.1 ± 0.0	33.6 ± 0.8	0.6 ± 0.2	5.9 ± 0.4	0.1 ± 0.1	1.0 ± 0.2
50	PE	18.2 ± 1.5	0.6 ± 0.1	22.8 ± 0.7	6.3 ± 0.5	4.5 ± 0.8a	0.2 ± 0.1	29.1 ± 0.7	2.8 ± 0.5	12.8 ± 1.6a	0.1 ± 0.1	2.3 ± 0.4
100	PC	17.4 ± 0.9	1.1 ± 0.3	24.9 ± 2.7	7.4 ± 1.2	7.7 ± 0.8	0.1 ± 0.0	33.8 ± 1.2	0.5 ± 0.1	5.6 ± 0.4	0.1 ± 0.0	1.0 ± 0.2
250	PE	20.0 ± 0.4	0.7 ± 0.2	21.8 ± 1.9	6.8 ± 1.3	4.8 ± 0.9b	0.2 ± 0.1	28.9 ± 1.2	2.5 ± 0.4	11.4 ± 0.9a	0.1 ± 0.1	2.5 ± 0.5
Control	PC	18.2 ± 1.1	1.3 ± 0.3	23.3 ± 1.2	7.4 ± 0.5	9.1 ± 0.2	0.1 ± 0.0	33.4 ± 1.0	0.7 ± 0.1	4.5 ± 0.8	0.1 ± 0.1	1.1 ± 0.2
50	PE	18.6 ± 1.3	0.7 ± 0.1	21.4 ± 0.9	7.2 ± 0.7	5.8 ± 0.6B	0.2 ± 0.1	30.3 ± 1.6	3.1 ± 0.2	9.4 ± 1.4bB	0.3 ± 0.1	2.3 ± 1.0
100	PC	19.2 ± 1.2	1.0 ± 0.2	26.0 ± 0.7	7.8 ± 0.7	9.5 ± 0.7	0.1 ± 0.0	29.5 ± 0.9	0.9 ± 0.6	4.6 ± 1.2	0.1 ± 0.1	1.0 ± 0.3
250	PE	18.5 ± 0.9	0.8 ± 0.3	24.0 ± 1.5	7.2 ± 0.9	7.9 ± 1.8B	0.1 ± 1.2	28.9 ± 1.2	2.1 ± 1.2	7.7 ± 2.8b	0.2 ± 0.2	2.0 ± 1.1
<b>Long-term</b>												
Control	Total FAs*	27.6 ± 5.0	4.4 ± 1.5	21.9 ± 1.5	18.6 ± 4.8	13.1 ± 4.7a	—	24.3 ± 2.5	—	—	—	—
1	PC	20.2 ± 3.1	1.0 ± 0.3	24.3 ± 2.8	6.8 ± 0.4	9.3 ± 1.2	0.6 ± 0.1	31.9 ± 1.2	0.7 ± 0.1	3.6 ± 0.2	0.1 ± 0.1	1.3 ± 0.3
10	PE	18.2 ± 1.3	0.8 ± 0.3	23.9 ± 1.2	5.9 ± 0.3	5.1 ± 0.9	0.4 ± 0.1	30.4 ± 0.7	2.4 ± 0.2	8.9 ± 2.3	0.2 ± 0.3	3.8 ± 0.5
25	Total FAs	25.6 ± 3.2	4.6 ± 1.1	18.6 ± 3.6	20.5 ± 4.5	14.6 ± 2.9a	—	20.7 ± 3.5	—	—	—	—
Control	PC	23.8 ± 3.2	0.7 ± 0.6	21.6 ± 0.6	7.2 ± 1.5	15.2 ± 4.7	0.3 ± 0.3	26.1 ± 4.5	0.6 ± 0.1	2.6 ± 0.6	0.2 ± 0.1	1.8 ± 0.8
10	PE	17.2 ± 1.2	0.5 ± 0.4	26.8 ± 3.8	4.7 ± 2.3	6.8 ± 1.5	0.2 ± 0.1	28.7 ± 1.3	2.4 ± 0.2	7.2 ± 1.8	0.3 ± 0.2	5.1 ± 1.9
25	Total FAs	22.4 ± 2.5	2.5 ± 0.8	16.6 ± 3.5	18.2 ± 2.9	23.3 ± 3.3bB	—	20.6 ± 2.7	—	—	—	—
Control	PC	15.5 ± 2.1	0.6 ± 0.2	22.9 ± 2.9	7.0 ± 0.9	13.4 ± 1.6	0.4 ± 0.1	29.1 ± 1.2	0.6 ± 1.8	2.3 ± 0.8	0.2 ± 0.1	1.6 ± 0.2
10	PE	15.3 ± 1.2	0.2 ± 0.0	26.0 ± 1.8	5.5 ± 1.8	7.0 ± 1.4	0.3 ± 0.0	31.0 ± 0.6	2.5 ± 0.5	6.4 ± 1.9	0.8 ± 0.1	4.7 ± 0.6
25	Total FAs	22.3 ± 1.9	2.5 ± 0.2	18.0 ± 3.4	16.8 ± 2.4	20.8 ± 3.5b	—	22.1 ± 4.5	—	—	—	—
Control	PC	27.5 ± 3.0	0.8 ± 0.6	15.8 ± 3.1	9.3 ± 0.5	17.5 ± 5.9	0.3 ± 0.4	22.5 ± 9.3	1.0 ± 0.5	2.3 ± 0.1	0.2 ± 0.1	2.6 ± 0.8
10	PE	13.7 ± 1.9	0.2 ± 0.2	24.0 ± 0.8	7.1 ± 1.0	7.9 ± 1.7	0.2 ± 0.2	29.5 ± 5.5	3.2 ± 1.0	6.5 ± 0.4	0.7 ± 0.1	7.0 ± 1.6

Values represent the means ± SD of five (short-term) and three (long-term) determinations per experiment. Means followed by the same letter do not differ significantly. If the letters differ then  $P < 0.05$ , if the letters and cases differ then  $P < 0.01$ . F344 and BD IX male rats were used for the short- (21 days) and long-term (2 yr) experiments, respectively. \*The FA profiles of the total liver (long-term study) represent the means ± SD of four to six animals per treatment group.

was also marginally ( $P < 0.1$ ) lower in the PC phospholipid fraction, while no changes were observed in the C20:4n-6 level in either of the phospholipid fractions. A similar response with respect to C18:2n-6 ( $P < 0.01$ ) and C22:5n-6 ( $P < 0.01$ ) was noticed in the FA profiles of plasma PC (Fig. 1) but, in contrast to the liver, the level of C20:4n-6 was also decreased significantly ( $P < 0.01$ ) in the rats fed the 100 mg FB<sub>1</sub>/kg diets. No analysis was performed on the sera of the rats fed the 250 mg FB<sub>1</sub>/kg diet.

**Long-term experiment.** When the total FA profiles of the rats were monitored (Table 3) using four to six rats per group the C18:2n-6 level was significantly increased ( $P < 0.01$  and  $P < 0.05$ ) in the rats that received the 10 and 25 mg FB<sub>1</sub>/kg diets, respectively, as compared with the control and the 1 mg FB<sub>1</sub>/kg-treated groups. As for the short-term experiments a similar response with respect to the n-6 FAs C18:2 and C22:5n-6 was noticed in the plasma, while C20:4n-6 differed significantly ( $P < 0.05$ ) between the groups as it was markedly lower in the 10 and 25 mg FB<sub>1</sub>-treated groups (Fig. 1). With respect to the n-3 FAs, C22:5n-3 was increased in the 10 ( $0.22 \pm 0.08$ ) and 25 ( $0.25 \pm 0.04$ ) mg FB<sub>1</sub>/kg-treated groups as compared with the control group ( $0.15 \pm 0.02$ ). In contrast to the plasma, C22:6n-3 was mainly increased

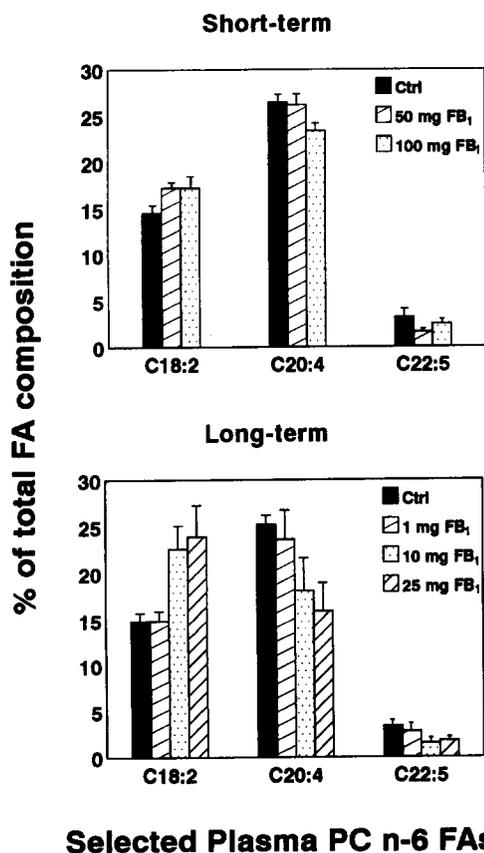


Fig. 1. Profiles of specific n-6 FAs in plasma PC of rats fed different dietary levels of FB<sub>1</sub> over 21 days (short-term) using male F344 rats (five rats per group) and 2 yr (long-term) using male BD IX rats (four rats per group). The values represent the means  $\pm$  SD.

Table 4. The effect of FB<sub>1</sub> on the FA and lipid parameters of the major membrane phospholipids in the liver of rats fed FB<sub>1</sub>-containing diets for different time periods

Treatment	Phospholipid	Saturated FAs	Mono-unsaturated FAs	n-6 FAs	n-3 FAs	n-6/n-3 ratio	PUFAs (Tot)	P/S ratio
Short-term Control	PC	42.6 $\pm$ 1.0	7.8 $\pm$ 0.9	48.5 $\pm$ 0.3a	1.1 $\pm$ 0.2	47.8 $\pm$ 11.5	49.6 $\pm$ 0.6a	1.2 $\pm$ 0.04
	PE	41.0 $\pm$ 1.7	6.9 $\pm$ 0.6	49.7 $\pm$ 1.2	2.4 $\pm$ 0.4	20.9 $\pm$ 3.8	52.1 $\pm$ 1.4	1.3 $\pm$ 0.09
	PC	42.3 $\pm$ 2.6	8.5 $\pm$ 1.5	48.2 $\pm$ 1.2a	1.1 $\pm$ 0.2	45.5 $\pm$ 7.5	49.3 $\pm$ 1.4a	1.2 $\pm$ 0.10
	PE	41.9 $\pm$ 2.0	7.5 $\pm$ 1.4	48.1 $\pm$ 0.4	2.6 $\pm$ 0.6	19.3 $\pm$ 3.7	50.7 $\pm$ 0.8	1.2 $\pm$ 0.08
	PC	41.4 $\pm$ 1.0	8.7 $\pm$ 0.5	48.7 $\pm$ 0.9a	1.2 $\pm$ 0.3	43.1 $\pm$ 8.8	49.8 $\pm$ 1.0a	1.2 $\pm$ 0.05
	PE	40.1 $\pm$ 0.9	8.0 $\pm$ 0.7	49.4 $\pm$ 1.0	2.6 $\pm$ 0.8	20.2 $\pm$ 5.2	52.0 $\pm$ 1.3	1.3 $\pm$ 0.06
Short-term 50	PC	45.3 $\pm$ 2.0	8.8 $\pm$ 0.7	45.0 $\pm$ 1.7A	1.0 $\pm$ 0.2	46.3 $\pm$ 8.1	46.0 $\pm$ 1.9A	1.0 $\pm$ 0.01
	PE	42.6 $\pm$ 2.1	8.0 $\pm$ 1.2	47.2 $\pm$ 1.1	2.3 $\pm$ 1.2	19.2 $\pm$ 2.2	49.4 $\pm$ 2.0	1.2 $\pm$ 0.10
	PC	44.5 $\pm$ 0.9	7.8 $\pm$ 0.7	46.3 $\pm$ 0.3	1.4 $\pm$ 0.3a	35.1 $\pm$ 8.3	47.7 $\pm$ 0.5	1.1 $\pm$ 0.03
	PE	42.0 $\pm$ 0.9	6.7 $\pm$ 0.5	47.3 $\pm$ 0.6	3.9 $\pm$ 0.4a	12.1 $\pm$ 1.0a	51.3 $\pm$ 0.8	1.2 $\pm$ 0.04
	PC	45.4 $\pm$ 3.0	7.8 $\pm$ 2.1	45.0 $\pm$ 1.2	1.8 $\pm$ 0.8a	28.3 $\pm$ 9.8	46.8 $\pm$ 1.3	1.0 $\pm$ 0.10
	PE	44.0 $\pm$ 3.8	5.2 $\pm$ 2.7	45.5 $\pm$ 1.9	5.3 $\pm$ 1.6a	9.1 $\pm$ 2.6a	50.9 $\pm$ 1.4	1.2 $\pm$ 0.13
Short-term 100	PC	44.4 $\pm$ 1.5	7.6 $\pm$ 0.9	46.2 $\pm$ 1.9	1.8 $\pm$ 0.4a	26.7 $\pm$ 5.8	48.0 $\pm$ 1.5	1.1 $\pm$ 0.06
	PE	41.4 $\pm$ 0.7	5.7 $\pm$ 1.8	47.4 $\pm$ 0.5	5.3 $\pm$ 0.6a	8.6 $\pm$ 1.0a	52.9 $\pm$ 1.1	1.3 $\pm$ 0.01
	PC	43.4 $\pm$ 3.4	10.1 $\pm$ 0.1	43.9 $\pm$ 3.9	2.6 $\pm$ 0.7b	17.4 $\pm$ 5.8	46.5 $\pm$ 3.2	1.1 $\pm$ 0.16
	PE	38.9 $\pm$ 4.1	7.1 $\pm$ 1.5	46.5 $\pm$ 3.9	7.6 $\pm$ 1.5b	6.3 $\pm$ 1.7A	54.0 $\pm$ 2.5	1.4 $\pm$ 0.21
	PC	44.5 $\pm$ 0.9	7.8 $\pm$ 0.7	46.3 $\pm$ 0.3	1.4 $\pm$ 0.3a	35.1 $\pm$ 8.3	47.7 $\pm$ 0.5	1.1 $\pm$ 0.03
	PE	42.0 $\pm$ 0.9	6.7 $\pm$ 0.5	47.3 $\pm$ 0.6	3.9 $\pm$ 0.4a	12.1 $\pm$ 1.0a	51.3 $\pm$ 0.8	1.2 $\pm$ 0.04
Long-term Control	PC	42.6 $\pm$ 1.0	7.8 $\pm$ 0.9	48.5 $\pm$ 0.3a	1.1 $\pm$ 0.2	47.8 $\pm$ 11.5	49.6 $\pm$ 0.6a	1.2 $\pm$ 0.04
	PE	41.0 $\pm$ 1.7	6.9 $\pm$ 0.6	49.7 $\pm$ 1.2	2.4 $\pm$ 0.4	20.9 $\pm$ 3.8	52.1 $\pm$ 1.4	1.3 $\pm$ 0.09
	PC	42.3 $\pm$ 2.6	8.5 $\pm$ 1.5	48.2 $\pm$ 1.2a	1.1 $\pm$ 0.2	45.5 $\pm$ 7.5	49.3 $\pm$ 1.4a	1.2 $\pm$ 0.10
	PE	41.9 $\pm$ 2.0	7.5 $\pm$ 1.4	48.1 $\pm$ 0.4	2.6 $\pm$ 0.6	19.3 $\pm$ 3.7	50.7 $\pm$ 0.8	1.2 $\pm$ 0.08
	PC	41.4 $\pm$ 1.0	8.7 $\pm$ 0.5	48.7 $\pm$ 0.9a	1.2 $\pm$ 0.3	43.1 $\pm$ 8.8	49.8 $\pm$ 1.0a	1.2 $\pm$ 0.05
	PE	40.1 $\pm$ 0.9	8.0 $\pm$ 0.7	49.4 $\pm$ 1.0	2.6 $\pm$ 0.8	20.2 $\pm$ 5.2	52.0 $\pm$ 1.3	1.3 $\pm$ 0.06
Long-term 1	PC	45.3 $\pm$ 2.0	8.8 $\pm$ 0.7	45.0 $\pm$ 1.7A	1.0 $\pm$ 0.2	46.3 $\pm$ 8.1	46.0 $\pm$ 1.9A	1.0 $\pm$ 0.01
	PE	42.6 $\pm$ 2.1	8.0 $\pm$ 1.2	47.2 $\pm$ 1.1	2.3 $\pm$ 1.2	19.2 $\pm$ 2.2	49.4 $\pm$ 2.0	1.2 $\pm$ 0.10
	PC	44.5 $\pm$ 0.9	7.8 $\pm$ 0.7	46.3 $\pm$ 0.3	1.4 $\pm$ 0.3a	35.1 $\pm$ 8.3	47.7 $\pm$ 0.5	1.1 $\pm$ 0.03
	PE	42.0 $\pm$ 0.9	6.7 $\pm$ 0.5	47.3 $\pm$ 0.6	3.9 $\pm$ 0.4a	12.1 $\pm$ 1.0a	51.3 $\pm$ 0.8	1.2 $\pm$ 0.04
	PC	45.4 $\pm$ 3.0	7.8 $\pm$ 2.1	45.0 $\pm$ 1.2	1.8 $\pm$ 0.8a	28.3 $\pm$ 9.8	46.8 $\pm$ 1.3	1.0 $\pm$ 0.10
	PE	44.0 $\pm$ 3.8	5.2 $\pm$ 2.7	45.5 $\pm$ 1.9	5.3 $\pm$ 1.6a	9.1 $\pm$ 2.6a	50.9 $\pm$ 1.4	1.2 $\pm$ 0.13
Long-term 10	PC	44.4 $\pm$ 1.5	7.6 $\pm$ 0.9	46.2 $\pm$ 1.9	1.8 $\pm$ 0.4a	26.7 $\pm$ 5.8	48.0 $\pm$ 1.5	1.1 $\pm$ 0.06
	PE	41.4 $\pm$ 0.7	5.7 $\pm$ 1.8	47.4 $\pm$ 0.5	5.3 $\pm$ 0.6a	8.6 $\pm$ 1.0a	52.9 $\pm$ 1.1	1.3 $\pm$ 0.01
	PC	43.4 $\pm$ 3.4	10.1 $\pm$ 0.1	43.9 $\pm$ 3.9	2.6 $\pm$ 0.7b	17.4 $\pm$ 5.8	46.5 $\pm$ 3.2	1.1 $\pm$ 0.16
	PE	38.9 $\pm$ 4.1	7.1 $\pm$ 1.5	46.5 $\pm$ 3.9	7.6 $\pm$ 1.5b	6.3 $\pm$ 1.7A	54.0 $\pm$ 2.5	1.4 $\pm$ 0.21
	PC	44.5 $\pm$ 0.9	7.8 $\pm$ 0.7	46.3 $\pm$ 0.3	1.4 $\pm$ 0.3a	35.1 $\pm$ 8.3	47.7 $\pm$ 0.5	1.1 $\pm$ 0.03
	PE	42.0 $\pm$ 0.9	6.7 $\pm$ 0.5	47.3 $\pm$ 0.6	3.9 $\pm$ 0.4a	12.1 $\pm$ 1.0a	51.3 $\pm$ 0.8	1.2 $\pm$ 0.04
Long-term 25	PC	45.4 $\pm$ 3.0	7.8 $\pm$ 2.1	45.0 $\pm$ 1.2	1.8 $\pm$ 0.8a	28.3 $\pm$ 9.8	46.8 $\pm$ 1.3	1.0 $\pm$ 0.10
	PE	44.0 $\pm$ 3.8	5.2 $\pm$ 2.7	45.5 $\pm$ 1.9	5.3 $\pm$ 1.6a	9.1 $\pm$ 2.6a	50.9 $\pm$ 1.4	1.2 $\pm$ 0.13
	PC	44.4 $\pm$ 1.5	7.6 $\pm$ 0.9	46.2 $\pm$ 1.9	1.8 $\pm$ 0.4a	26.7 $\pm$ 5.8	48.0 $\pm$ 1.5	1.1 $\pm$ 0.06
	PE	41.4 $\pm$ 0.7	5.7 $\pm$ 1.8	47.4 $\pm$ 0.5	5.3 $\pm$ 0.6a	8.6 $\pm$ 1.0a	52.9 $\pm$ 1.1	1.3 $\pm$ 0.01
	PC	43.4 $\pm$ 3.4	10.1 $\pm$ 0.1	43.9 $\pm$ 3.9	2.6 $\pm$ 0.7b	17.4 $\pm$ 5.8	46.5 $\pm$ 3.2	1.1 $\pm$ 0.16
	PE	38.9 $\pm$ 4.1	7.1 $\pm$ 1.5	46.5 $\pm$ 3.9	7.6 $\pm$ 1.5b	6.3 $\pm$ 1.7A	54.0 $\pm$ 2.5	1.4 $\pm$ 0.21

Values represent the means  $\pm$  SD of five (short-term) and three (long-term) determinations per experiment. Means in the columns followed by the same letter do not differ significantly. If the letters differ then  $P < 0.05$ , if the letters and cases differ then  $P < 0.01$ . F344 and BD IX male rats were used for the short- (21 days) and long-term (2 yr) experiments respectively.

in the liver PE and PC fractions, while the level of C22:5n-3 was unaffected. When compared with the total FA, the levels of C18:2n-6 and C22:5n-6 were also increased and decreased respectively in both phospholipids but the changes were only marginally significant ( $P < 0.1$ ) due to the small number of rats (three per group) used in the analyses (Table 3).

The FB<sub>1</sub>-induced changes to the n-6 and n-3 FA profiles and the different lipid parameters of the major phospholipids are summarized in Table 4. The polyunsaturated FAs (PUFAs) were significantly decreased ( $P < 0.01$ ) in the liver PC fraction of the 250 mg/kg-treated animals, resulting in a marked decrease in the polyunsaturated/saturated FA (P/S) ratio.

In the long-term experiments the n-3 FAs were significantly ( $P < 0.01$ ) increased in the PE fractions of the rats treated with the high FB<sub>1</sub>-containing diet. This resulted in a significant decrease ( $P < 0.01$ ) in the n-6/n-3 ratio in the PE fraction. The ratio in PC was also markedly lower, but the difference was not significant.

#### DISCUSSION

Although the fumonisins lack genotoxicity, studies regarding the carcinogenic potential indicate that FB<sub>1</sub> closely mimics the properties of genotoxic carcinogens with respect to the cancer initiation and promotion phases of cancer induction in rat liver (Gelderblom *et al.*, 1988 and 1994). At present very little is known about the mechanism(s) by which FB<sub>1</sub> affects the different stages of cancer development in rat liver. As the hepatotoxicity of the fumonisins appears to be a key determinant during FB<sub>1</sub>-induced hepatocarcinogenesis (Gelderblom *et al.*, 1991, 1994 and 1996a), cancer induction by a cytotoxic mechanism, as suggested for non-genotoxins by Cohen and Ellwein (1990), needs to be considered.

Several presumably cytotoxic FB<sub>1</sub>-induced mechanisms were found to interfere with membrane lipids. Of these the inhibition of the enzyme ceramide synthase, a key enzyme in sphingolipid biosynthesis, has been investigated extensively (Wang *et al.*, 1991; Yoo *et al.*, 1992) and the FB<sub>1</sub>-related accumulation of Sa was found to be associated with the cytotoxicity of the fumonisins in renal epithelial cells (LLC-PK<sub>1</sub>) of the pig *in vitro* (Yoo *et al.*, 1992). However, a recent study indicated that the accumulation of Sa was not associated with FB<sub>1</sub>-induced cytotoxicity in primary rat hepatocytes (Gelderblom *et al.*, 1995a). As PUFAs accumulate in hepatocytes treated with cytotoxic levels of FB<sub>1</sub>, it was suggested that they could be a key factor determining the cytotoxic effects of the compound (Gelderblom *et al.*, 1996a).

The disruption of sphingolipid biosynthesis has also been implicated as a possible mechanism for cancer induction by the fumonisins (Schroeder *et al.*, 1994). Studies regarding the carcinogenic potential of the fumonisins, utilizing a short-term cancer in-

itiation/promotion bioassay in rat liver, showed that cancer initiation is effected at relative high dietary level of the compound (250 mg FB<sub>1</sub>/kg; Gelderblom *et al.*, 1994) while cancer promotion is obtained at a dietary level of 50 mg FB<sub>1</sub>/kg (Gelderblom *et al.*, 1996c) over a period of 21 days. In the present study the Sa levels and Sa/So ratio were significantly increased in the livers of the rats (male F344) fed the high FB<sub>1</sub> dietary levels (100 and 250 mg FB<sub>1</sub>/kg) over a similar period of time. No significant changes were observed in the rats receiving the 50 mg FB<sub>1</sub>/kg diet concerning the So and the complex sphingolipid SM, while the Sa concentration was slightly increased. Even when the Sa/So ratio was significantly altered, for instance in the rats treated with 100 mg FB<sub>1</sub>/kg diet due to the increase in Sa concentration, it did not necessarily affect the biosynthesis of SM. In the long-term study (male BD IX rats) the Sa/So ratio was not significantly altered above the ratio obtained in the F344 rats treated with the low FB<sub>1</sub>-containing diet (50 mg FB<sub>1</sub>/kg) for 21 days (Table 2). Serum analyses of the rats (long-term treatment) also did not reveal any significant changes in the Sa/So ratio (Table 2). Preneoplastic changes associated with fumonisin B<sub>1</sub> treatment were noticed in the livers of the rats that received the 10 and 25 mg FB<sub>1</sub>/kg dietary levels. These include the presence of basophilic and eosinophilic foci and an increase in the number and size of foci that stained positively for GSTP. These data suggested that altered sphingolipid metabolism is not associated with the induction of these preneoplastic changes induced by FB<sub>1</sub> in rat liver. This is in agreement with a recent study indicating that altered sphingolipid is not a prerequisite for the cancer-promoting potential of FB<sub>1</sub> in a short-term cancer initiating/promoting bioassay (Gelderblom *et al.*, 1996c). *In vitro* studies in primary hepatocytes also show that the disruption of sphingolipid biosynthesis and the subsequent accumulation of sphinganine are not associated with the mitoinhibitory effect of FB<sub>1</sub> on the epidermal growth factor (EGF) mitogenic response, a common property exhibited by many cancer promoters (Gelderblom *et al.*, 1995a). The present data do not imply that alterations in sphingolipid metabolism are not involved in toxicological and/or carcinogenic properties of FB<sub>1</sub> in rat liver but merely suggest that other biological events (as discussed here) also need to be considered as possible mediators for the hepatotoxicity and carcinogenic effects of the fumonisins in rat liver. It is well accepted that sphingolipids are potent modulators of cellular growth and differentiation (Merrill, 1991) and therefore could still play an important modulating role in the processes underlying the slow evolution of preneoplastic hepatocyte populations, induced by the fumonisins, into cancer.

The modulating role of FB<sub>1</sub> on the levels and FA patterns of selected membrane phospholipids is also of interest with respect to explaining some of the biological effects of the fumonisins. When the

present *in vivo* data are compared with the earlier results in primary hepatocyte cultures similarities as well as differences are revealed. In the short-term (21 days) study a significant decrease in SM and an increase in the level of PE was noticed only at the high-dose level that exhibited advanced toxicological lesions. Likewise, a cytotoxic dose was required to alter the level of phospholipids *in vitro* with the exception that the PC level remained unchanged *in vivo*. In the long-term feeding study (2 yr) involving lower doses inducing only mild cytotoxicity, no changes were noticed in the levels of SM and PC, while PE was again markedly increased in all the FB<sub>1</sub>-treated groups. The increased level of PE in the short-term study could be explained by the altered SM synthesis (Gelderblom *et al.*, 1996a) or as a result of an increased breakdown of Sa into ethanolamine 1-phosphate, a precursor for PE synthesis (Smith and Merrill, 1995). However, the latter arguments cannot explain the increase in the PE level in the liver of the rats treated with low dietary levels of FB<sub>1</sub> for 2 yr (Table 2) that failed to alter sphingolipid biosynthesis. The cholesterol level, which was reduced in primary hepatocytes, was increased in the liver and serum of the rats (short-term study) treated with the 250 mg FB<sub>1</sub>/kg diet. This would imply that *in vivo* the uptake of cholesterol by the liver and/or the metabolism thereof seems to be affected. This is in agreement with a previous study in vervet monkeys where the plasma cholesterol was also increased after consumption of low dietary levels of fungal culture material containing FB<sub>1</sub> (Fincham *et al.*, 1992). In contrast to the *in vitro* data, the PC:total cholesterol ratio decreased (Gelderblom *et al.*, 1996b). This could be due to a compensatory effect to counteract the reduction in the PUFA levels (see later) in PC and PE in an attempt to retain membrane fluidity. No analyses were performed to determine the concentrations of CEs, as it is known that free cholesterol constitutes the major fraction of the total cholesterol.

Regarding the FA profiles, the C18:2n-6 level was significantly ( $P < 0.05$ ) increased in PE and increased markedly in PC in the liver of the rats fed the 50 mg FB<sub>1</sub>/kg diet and more in the short-term study. In the long-term study, C18:2n-6 was also elevated in the phospholipids, PC and PE and the total lipids. As for the latter compartment, this increase was significant in the 10 ( $P < 0.01$ ) and 25 ( $P < 0.05$ ) mg FB<sub>1</sub>/kg diet groups. In the primary hepatocytes FB<sub>1</sub> effected the accumulation of C18:2n-6 in PC but not in PE (Gelderblom *et al.*, 1996b). Therefore, *in vitro* exposure of primary hepatocytes for a relative short period of 24 hr mainly affected the FA profiles of PC, while PE appeared to be more susceptible to changes after *in vivo* exposure over longer periods. This could be related to the relative position of the phospholipid in the lipid bilayer, where PE tends to occur in the inside of the membrane relative to PC and, therefore, is not

easily accessible to changes in FA content (Devaux and Zachowski, 1994).

The accumulation of C18:2n-6 with a concomitant decrease ( $P < 0.05$  to  $P < 0.01$ ) in C22:5n-6 *in vivo* suggests that FB<sub>1</sub> alters the n-6 FA metabolic pathway presumably by affecting the activity of the delta 6 desaturase enzyme. This is supported by a decrease in the C18:3/C18:2 ratio, which is an indirect measure of the activity of the enzyme. A similar effect was noticed in the FA composition of plasma PC of the rats from both the short- and long-term feeding studies. In contrast to the *in vitro* study, where C20:4n-6 was significantly increased in the hepatocytes, the level was not affected in PC or PE in the liver while it was decreased in plasma PC. The latter findings further support the described phenomenon that FB<sub>1</sub> affected the n-6 FA metabolic pathway, although it is manifested differently in the various cellular environments with respect to the dosage used and the duration of the treatment. In the short-term studies the disruption of the n-6 FA metabolism resulted in a decrease in the total n-6 FAs and PUFAs at the high dose (250 mg FB<sub>1</sub>/kg) where enhanced hepatotoxic effects were observed. As PUFAs are known to be particularly good targets for oxidative damage, it has been suggested (Gelderblom *et al.*, 1996a,b) that their reduction could have been caused by oxidative damage presumably by lipid peroxidation, thereby facilitating the FB<sub>1</sub>-associated damage to membrane integrity. However, in the long-term studies where only mild hepatotoxic effects were noticed, the total PUFA levels were unaffected. The n-6/n-3 FA ratio of PE was significantly lower in the high-dose group (long-term study) due to an increase in the n-3 FA content of both phospholipid fractions. The n-3 FAs are known to be better substrates for the delta desaturase enzymes as compared with the n-6 FAs (Horrobin, 1992). The resultant production of different series of eicosanoids and their varying effects on the control of cell growth (Cornwell and Morisaki, 1984) could have important implications on the inhibitory effect of the fumonisins on hepatocyte cell proliferation *in vivo* (Gelderblom *et al.*, 1994 and 1996c) and *in vitro* (Gelderblom *et al.*, 1995a).

Although the exact mechanism for the inhibition of cell proliferation is not known at present it has been proposed as a possible mechanism by which FB<sub>1</sub> promotes the outgrowth of initiated hepatocytes in the liver of rats (Gelderblom *et al.*, 1996c). *In vitro* studies in primary hepatocytes indicated that FB<sub>1</sub> inhibits the EGF-induced mitogen response (Gelderblom *et al.*, 1995a) and the n-6 FA synthetic pathway (Gelderblom *et al.*, 1996a). Preliminary investigations showed that the regulation of the synthesis of prostaglandin E<sub>2</sub> appear to be involved in the mitoinhibitory effect of FB<sub>1</sub> (Gelderblom *et al.*, 1995b) which is in accordance with the involvement of prostaglandin E<sub>2</sub> in the EGF-induced mitogenic response in BALB/c3T3 fibroblasts (Nolan *et al.*, 1988). The present study

supports further the described hypothesis as FB<sub>1</sub>-induced changes to the n-3 and n-6 FA metabolic pathways that are likely to affect the potential of hepatocytes to proliferate *in vivo* by regulating the synthesis of different prostaglandins.

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# Regulation of fatty acid biosynthesis as a possible mechanism for the mitoinhibitory effect of fumonisin B<sub>1</sub> in primary rat hepatocytes

W. C. A. Gelderblom,<sup>1</sup> S. Abel,<sup>1</sup> C. M. Smuts,<sup>2</sup> S. Swanevelder,<sup>3</sup> S. D. Snyman<sup>1</sup>

<sup>1</sup>Programme on Mycotoxins and Experimental Carcinogenesis

<sup>2</sup>National Research Programme for Nutritional Intervention

<sup>3</sup>Center for Epidemiology Research in South Africa, P.O. Box 19070, Tygerberg, 7505, South Africa

**Summary** The mitoinhibitory effect of fumonisin B<sub>1</sub> (FB<sub>1</sub>) on the mitogenic response of epidermal growth factor (EGF) was investigated in primary hepatocyte cultures with respect to the alterations in the  $\omega$ 6 fatty acid metabolic pathway. Fatty acid analyses of hepatocytes showed that EGF treatment resulted in a significant decrease in the relative levels of 20:4 $\omega$ 6 (arachidonic acid) and an increase in 18:2 $\omega$ 6 (linoleic acid). Supplementation of the hepatocyte cultures with 20:4 $\omega$ 6 in the absence of EGF resulted in an increase in the total  $\omega$ 6 and  $\omega$ 6/ $\omega$ 3 fatty acid ratio. Addition of 20:5 $\omega$ 3 (eicosapentaenoic acid) resulted in an increase of the relative levels of the long chain  $\omega$ 3 fatty acids at the expense of the  $\omega$ 6 fatty acids. When 20:4 $\omega$ 6 and 20:5 $\omega$ 3 was added in the presence of EGF, the mitogenic response of EGF was increased and decreased respectively. When compared to the fatty acid profiles in the absence of EGF, the decreased mitogenic response coincided with a decrease of total  $\omega$ 6 fatty acids and total polyunsaturated fatty acids (PUFA). In addition, the saturated and mono-unsaturated fatty acids increased and the polyunsaturated/saturated (P/S) fatty acid ratio decreased which implied a more rigid membrane structure. Addition of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and prostaglandin E<sub>1</sub> (PGE<sub>1</sub>) stimulated and inhibited the mitogenic response respectively. Ibuprofen, a known cyclooxygenase inhibitor, and FB<sub>1</sub> inhibited the EGF-induced mitogenic response in a dose-dependent manner. The mitoinhibitory effect of FB<sub>1</sub> on the EGF response was counteracted by the addition of PGE<sub>2</sub>. FB<sub>1</sub> also disrupts the  $\omega$ 6 fatty acid metabolic pathway in primary hepatocytes, resulting in the accumulation of C18:2 $\omega$ 6 in phosphatidylcholine and triacylglycerol. The disruption of the  $\omega$ 6 fatty acid metabolic pathway and/or prostaglandin synthesis is likely to be an important event in the mitoinhibitory effect of FB<sub>1</sub> on growth factor responses.

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## INTRODUCTION

The control of cell proliferation is known to play an important role in the process of cancer development,<sup>1</sup> but it would appear that the relative contribution to carcinogenesis is both tissue and time specific.<sup>2</sup> In rat liver, cell proliferation is involved during cancer initiation, the process of cancer promotion whereby the growth of initi-

ated hepatocytes is selectively stimulated to develop into nodules and the subsequent cancer progression stage.<sup>3–5</sup> As most hepatocarcinogens inhibit cell proliferation, the initiated cell has the ability to proliferate in an environment where the proliferation of normal cells is inhibited. The latter is regarded as one of the major mechanisms of cancer promotion in rat liver.<sup>5</sup> Apart from increased cell proliferation in hepatocyte nodules and/or foci, cancer promoters can also affect the cell death rates by inhibiting apoptosis in these lesions leading to the accumulation of focal and nodular cells and hence their subsequent growth.<sup>6</sup> Studies in the colon suggest that changes in the apoptotic rate, rather than cell proliferation, could be a better determinant for the development of cancer.<sup>7,8</sup>

The inhibitory effect of FB<sub>1</sub>, a hepatocarcinogen pro-

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Correspondence to: W. C. A. Gelderblom; Fax: 0027 21 938 0260

**Abbreviations:** FB<sub>1</sub>, fumonisin B<sub>1</sub>; EGF, epidermal growth factor; PUFA, polyunsaturated fatty acid(s); P/S fatty acid ratio; polyunsaturated/saturated fatty acid ratio; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; WE, Williams E medium

duced by the fungus *Fusarium moniliforme*, on hepatocyte cell proliferation has been implicated in the cancer-promoting activity of the compound. FB<sub>1</sub> selectively stimulated the outgrowth of diethylnitrosamine (DEN)-initiated cells in rat liver, presumably via the process of differential inhibition, as it inhibited regenerative cell proliferation in the liver induced by partial hepatectomy.<sup>9</sup> Studies in primary hepatocytes indicated that FB<sub>1</sub> closely mimics the mitoinhibitory effect of many cancer promoters on the epidermal growth factor (EGF)-induced mitogenic response.<sup>10</sup> The EGF-induced mitogenic response was reversibly inhibited at concentrations that were<sup>15-20</sup> times below the cytotoxic dosage. Recent studies indicate that FB<sub>1</sub> also inhibits cell proliferation<sup>11,12</sup> and induced apoptosis in a variety of cell culture systems.<sup>13</sup> The mechanisms involved in the inhibitory effect on cell proliferation and the induction of apoptosis are not known at present, but the disruption of these processes are likely to play a major role in the cancer promoting activity of the fumonisins.

Different mechanisms have been postulated which attempted to explain the toxicity and carcinogenicity of the fumonisins. Studies in various cell culture systems and in vivo in ponies and pigs indicated that FB<sub>1</sub> inhibits the de novo synthesis of sphingolipids by inhibiting ceramide synthase, the key enzyme in the biosynthetic pathway.<sup>14,15</sup> Ceramide and sphingosine are known to be important sphingolipid signalling molecules that ensure normal cell processes by inter- and intracellular communication.<sup>16,17</sup> Although it has been suggested that the interruption of sphingolipid biosynthesis could play a role in FB<sub>1</sub>-induced toxicological effects in different biological systems, their exact role is unknown. The modulating effect of FB<sub>1</sub> on phospholipid and fatty acid synthesis was recently implicated in the toxicity and carcinogenicity of the fumonisins.<sup>18,19,20</sup> Studies in primary hepatocytes indicated that FB<sub>1</sub> decreased the concentration of the complex sphingolipid, sphingomyelin (SM), due to the inhibition of ceramide synthase, while levels of sphinganine and the phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were increased.<sup>10,18</sup> Fatty acid analyses of the major membrane phospholipid, PC, and the neutral lipid, triacylglycerol, indicated that, depending on the concentration, FB<sub>1</sub> increased the relative levels of 18:2 $\omega$ 6 and 20:4 $\omega$ 6 in the hepatocytes, which results in a significant increase in the total PUFA.<sup>18</sup> In vivo studies indicated that the relative level of 18:2 $\omega$ 6 was increased in the PC and PE in the liver, while 20:4 $\omega$ 6 was significantly lowered in serum PC.<sup>19</sup> The total  $\omega$ 6 fatty acids and the total PUFA were decreased in both PE and PC with a concomitant increase of  $\omega$ 3 fatty acids in both phospholipid fractions resulting in changes in the  $\omega$ 6/ $\omega$ 3 fatty acid ratio. It was suggested that FB<sub>1</sub>-induced changes to the fatty acid metabolic

pathways could play an important role in the altered growth responses induced by the fumonisins in primary rat hepatocytes and in the liver in vivo.<sup>20</sup> Recent studies indicated that FB<sub>1</sub> alters signal transduction pathways and cell cycle progression by repressing the expression of protein kinase C and AP-1-dependant transcription in CV-1 (of the African green monkey kidney) cells,<sup>21</sup> inhibiting protein serine/threonine phosphatases in vitro,<sup>22</sup> activated mitogen-activated protein kinase in Swiss 3T3 fibroblasts<sup>23</sup> and resulted in the overexpression of cyclin D1 in hepatic rat tumors.<sup>24</sup>

The present study investigates the mechanisms involved in the mitoinhibitory effect of FB<sub>1</sub> on the EGF mitogenic response in primary hepatocytes. These include investigations regarding the modulating role of selected fatty acids and prostaglandins on the EGF response. The alterations in the fatty acid metabolic pathways induced by FB<sub>1</sub> will be discussed in relation to the cancer promoting and other adverse biological effects of the mycotoxin in rat liver.

## MATERIALS AND METHODS

### Chemicals

EGF, prostaglandins E<sub>1</sub> and E<sub>2</sub> (PGE<sub>1</sub> and PGE<sub>2</sub>) and the 20:4 $\omega$ 6 and 20:5 $\omega$ 3 fatty acids were obtained from Sigma Chemical Corporation, and [<sup>3</sup>H] thymidine from Amersham, London, UK. FB<sub>1</sub>, with a purity of 98%, was dissolved in saline to obtain a specific concentration and added in 50  $\mu$ l quantities to each culture dish.

### Primary hepatocyte cultures

Primary hepatocytes were prepared from male Fischer 344 rats (160–200 g in body weight) by the collagenase perfusion technique as described previously.<sup>10</sup> Cells were washed and harvested at low speed centrifugation (50  $\times$  g) to minimise contamination with smaller non-parenchymal cells. The viability of the isolated hepatocytes from repeated isolation batches varied from 90 to 95%. Viable hepatocytes (2  $\times$  10<sup>5</sup> per culture dish) were plated on collagen coated dishes (35 mm) in modified Williams E medium containing fetal bovine serum (10%) for 3 h at 37°C in air/carbon dioxide (95:5). After plating, the cells were washed with Hanks buffer solution and supplemented with serum-free, modified Williams E medium and the plates incubated for 44 h. EGF (50 ng/dish) was added to the incubating medium after the cells were plated for the whole incubation period. The pulse labelling technique, using [<sup>3</sup>H] thymidine (50  $\mu$ Ci/plate), was used to determine the labelling index (incorporation of [<sup>3</sup>H] thymidine into the DNA) of the hepatocytes exposed to EGF, quantitatively.<sup>8</sup> In short, the radiolabel (50  $\mu$ Ci/dish)

was added to the dishes for a period of 4 h (between 40–44 h). The cells were washed three times with saline and the specific labelling (dpm/h/mg protein) was determined in 5% trichloroacetic acid-precipitable material dissolved in 0.5% sodiumdodecylsulfate (SDS). Protein determinations were performed according to the method of Kaushal and Barnes.<sup>25</sup>

#### Mitogenic response of EGF: fatty acids changes and effect of 20:4 $\omega$ 6 and 20:5 $\omega$ 3 supplementation

Hepatocyte cultures (4 × 5 cm dishes per treatment) were incubated for 44 h in the absence or presence of EGF. Following incubation, the cells were first washed with ice-cold saline (3 × 2 ml) and then harvested by scraping in saline (1.5 ml). An aliquot (0.2 ml) was retained for protein determination<sup>25</sup> and 1 ml, containing at least 1 mg protein/ml, was subjected to fatty acid analyses (see below) of the major phospholipids (PC and PE).

In a separate experiment, the effect of 20:4 $\omega$ 6 (10 and 25  $\mu$ M/dish) and 20:5 $\omega$ 3 (10, 25 and 50  $\mu$ M/dish) on the mitogenic response of EGF was determined by the addition of these fatty acids, complexed to delipidated albumin (fatty acid:albumin ratio of 1:4) to the hepatocyte cultures and the incorporation of [<sup>3</sup>H] thymidine determined as described above. A similar study was performed whereby the hepatocytes were incubated with the individual fatty acids (25  $\mu$ M/dish) and the PC and PE phospholipid fractions subjected to fatty acid analyses. All the experiments were repeated three times.

#### Effects of PGE<sub>1</sub>, PGE<sub>2</sub> and ibuprofen on the EGF mitogenic response

The modulation of the EGF mitogenic response by PGE<sub>1</sub> and PGE<sub>2</sub> was monitored by incubating increasing concentrations of each prostaglandin (2.5, 5.0, 10  $\mu$ M/dish) in the presence or absence of EGF over a period of 44 h. The modulating role of ibuprofen (2, 5 and 10  $\mu$ M/dish), a non-steroidal anti-inflammatory drug that blocks PGE<sub>2</sub> synthesis,<sup>26</sup> and FB<sub>1</sub> (75  $\mu$ M) were monitored in a similar way. Ibuprofen and the different prostaglandins, were dissolved in ethanol (95%) and diluted with the growth medium such that the final ethanol concentration was 0.1%.

#### FB<sub>1</sub>-induced mitoinhibitory effect on the EGF response: effect of PGE<sub>2</sub>

The mitoinhibitory effect of FB<sub>1</sub> on the EGF response was determined by the addition of different concentrations (25, 75, 150 and 300  $\mu$ M) of the compound to the cultures for the whole incubation period. The modulating role of PGE<sub>2</sub> on the FB<sub>1</sub>-induced response was studied by the

addition of 12.5  $\mu$ M at the same time as FB<sub>1</sub> and EGF. The specific labelling of the hepatocytes was monitored as described above.

#### Dose-response effects of FB<sub>1</sub> on hepatocyte fatty acid profiles

Hepatocyte cultures were incubated with different concentrations of FB<sub>1</sub> (10, 25, 50 and 75  $\mu$ M/cultured dish) for 44 h. Following the incubation period, the cells were washed with ice-cold saline and the cells of four dishes (6 cm i.d.) were scraped into a total volume of 1.5 ml saline, which yielded approximately 1–1.5 mg protein. The hepatocyte/saline mixture was extracted with chloroform/methanol (2:1; 20 times the volume of the aqueous phase) and the extract evaporated to dryness under nitrogen at 37°C. The dried extract was further fractionated by thin layer chromatography (TLC) and PC, PE and the neutral lipid, triacylglycerol (TAG) were subjected to detailed fatty acid analyses as described previously.<sup>18</sup>

#### Cytotoxicity measurements

The cytotoxicity of the different prostaglandins, ibuprofen, 20:4 $\omega$ 6 and 20:5 $\omega$ 3 were measured by monitoring release of lactate dehydrogenase in the incubation medium after the 44 h incubation period. The fatty acids were first complexed with delipidated bovine serum albumin as described above. Cytotoxicity was expressed as a percentage of the total amount of lactate dehydrogenase (LDH) released from control dishes after treatment with 1% Triton X100.<sup>27</sup>

#### Statistical analyses

The nonparametric Kruskal–Wallis statistical test and the standard analysis of variance with the Tukey test were used to analyse the data ( $n = 3$ ) in order to determine the significant differences between the groups. The fatty acid profiles of the different treatment groups ( $n > 3$ ) were also analysed by performing a standard analysis of variance and the Tukey test, using the Tukey Standardized Range method to determine whether the differences between the groups were significant.

## RESULTS

### Modification of fatty acid profiles of hepatocytes

#### (i) In the absence of EGF

The most prominent changes in the fatty acid profiles were noticed in the PC fraction (Table 1) with only marginal ( $P > 0.1$ ) changes (data not shown) in the PE fraction. Supplementation of the cultured hepatocytes with

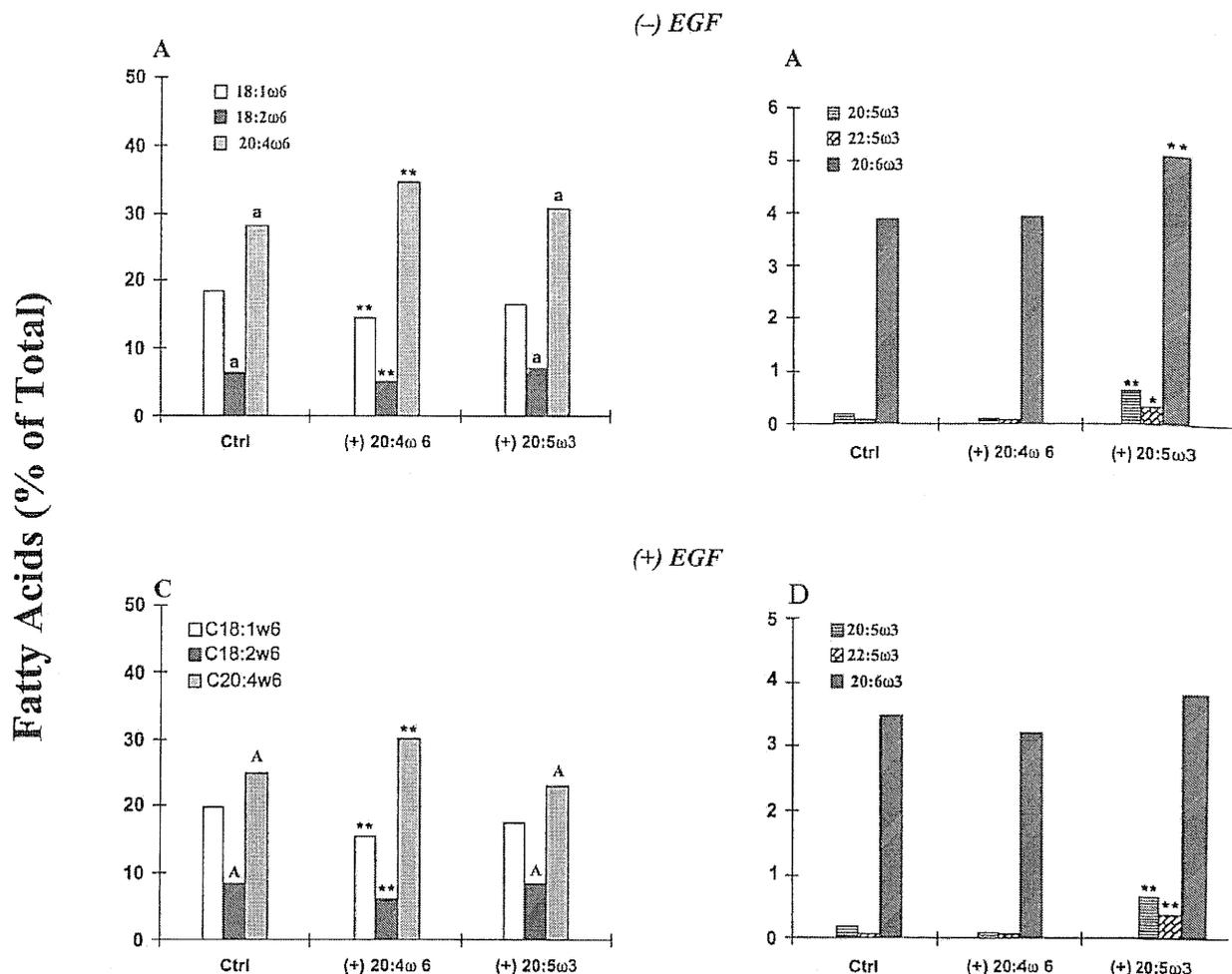


Fig. 1 Relative levels of selected fatty acids of primary hepatocyte cultures incubated with 20:4 $\omega$ 6 and 20:5 $\omega$ 3 incorporated in the medium for a period of 44 h (A and B) in the absence (A & B) or presence (C & D) of EGF. Both fatty acids were added at a concentration of 25  $\mu$ M/cultured dish and the lipids extracted as described under Materials and Methods. Statistical comparisons were made within a treatment group; \* $P < 0.05$ , \*\* $P < 0.01$ ) and between the (-) and (+) EGF treatments; a vs A,  $P < 0.01$ . Values represent the means of triplicate determinations and the experiment was repeated three times. The SDs of the mean fatty acid values are presented in Table 1.

20:4 $\omega$ 6 increased 20:4 $\omega$ 6 ( $P < 0.01$ ), while 18:1 $\omega$ 9 and 18:2 $\omega$ 6 were decreased ( $P < 0.01$ ) as compared to the BSA treated controls (Fig. 1A). When 20:5 $\omega$ 3 was added, the levels of 20:4 $\omega$ 6, 18:2 $\omega$ 6 and 18:1 $\omega$ 9 were not affected (Fig. 1A) while the levels of 20:5 $\omega$ 3 ( $P < 0.05$ ), C22:5 $\omega$ 3 ( $P < 0.01$ ) and 22:6 $\omega$ 3 ( $P < 0.05$ ), were increased significantly (Fig. 1B). Addition of C20:4 $\omega$ 6 and C20:5 $\omega$ 3 also significantly reduced ( $P < 0.05$  to  $P < 0.01$ ) and increased ( $P < 0.01$ ) the relative levels of C16:0 and C18:0, respectively (Table 1). When the total fatty acid parameters are considered the mono-unsaturated fatty acids were decreased ( $P < 0.01$ ) in the presence of 20:4 $\omega$ 6 and 20:5 $\omega$ 3 (Table 2). Addition of 20:4 $\omega$ 6 resulted in an increase in the total  $\omega$ 6 fatty acids ( $P < 0.01$ ),  $\omega$ 6/ $\omega$ 3 ratio ( $P < 0.05$ ) and total PUFA ( $P < 0.01$ ). Addition of 20:5 $\omega$ 3 resulted in

an increase in the total  $\omega$ 3 fatty acids ( $P < 0.05$ ), PUFA ( $P < 0.05$ ) and P/S ratio ( $P < 0.1$ ) while the  $\omega$ 6/ $\omega$ 3 ratio ( $P < 0.01$ ) decreased.

#### (ii) In the presence of EGF

The most prominent changes in the EGF treated cells, as compared to the control cells, were an increase ( $P < 0.01$ ) in 18:2 $\omega$ 6 with a concomitant decrease ( $P < 0.01$ ) in 20:4 $\omega$ 6 (Fig. 1A vs 1C – a vs A). The saturated fatty acids C16:0 ( $P < 0.05$ ) and C18:0 ( $P < 0.05$ ) decreased and increased respectively in the EGF-treated hepatocytes (Table 1; indicated with asterisks). Addition of 20:4 $\omega$ 6 in the presence of EGF counteracted the above mentioned decrease of 20:4 $\omega$ 6, as it was significantly ( $P < 0.01$ ) increased, with a concomitant reduction ( $P < 0.01$ ) of

Table 1 Fatty acid parameters of phosphatidylcholine (PC) of hepatocytes supplemented with C20:4 $\omega$ 6 (23  $\mu$ M) and C20:5 $\omega$ 3 (25  $\mu$ M) in the growth medium in the presence and absence of epidermal growth factor (EGF)

Treatment	C16:0	C16:1	C18:0	C18:1	C18:2 ( $\omega$ 6)	C20:3 ( $\omega$ 6)	C20:4 ( $\omega$ 6)	C22:4 ( $\omega$ 6)	C22:5 ( $\omega$ 6)	C20:5 ( $\omega$ 3)	C22:5 ( $\omega$ 3)	C22:6 ( $\omega$ 3)
<b>(-) EGF</b>												
Control	18.55a	1.52	21.02a	18.50a	6.58a	0.71	28.17a	0.09a	1.31	0.16a	—	3.89a
(BSA)	(2.75)	(0.56)	(1.93)	(1.34)	(0.89)	(0.36)	(1.80)	(0.10)	(0.60)	(0.15)	—	(1.34)
C20:4 $\omega$ 6	13.82b	1.17	25.12A	14.60A	5.37A	0.51	34.41A	0.36A	1.29	0.03a	0.07a	3.93a
	(4.36)	(1.09)	(1.08)	(1.82)	(0.75)	(0.43)	(4.57)	(0.04)	(0.17)	(0.07)	(0.10)	(0.72)
C20:5 $\omega$ 3	11.78C	1.18	25.15A	16.40a	6.93a	0.90	30.95a	0.19a	1.17	0.62A	0.33b	5.11A
	(3.20)	(0.65)	(2.54)	(1.35)	(0.62)	(0.18)	(1.26)	(0.01)	(0.31)	(0.24)	(0.19)	(1.33)
<b>(+) EGF</b>												
Control	15.72*	1.13	23.31*	19.88	8.48a**	1.01a	25.59a**	0.13a	1.35	0.18a	0.05a	3.02
(BSA)	(2.36)	(0.47)	(1.88)	(2.12)	(1.07)	(0.24)	(1.09)	(0.13)	(0.40)	(0.22)	(0.09)	(0.43)
C20:4 $\omega$ 6	15.43	0.94	25.81	15.72	6.23A	0.65b	30.69A	0.42A	1.26	0.08a	0.08a	3.38
	(3.77)	(0.48)	(1.73)	(2.09)	(0.99)	(0.31)	(5.10)	(0.02)	(0.13)	(0.13)	(0.10)	(0.57)
C20:5 $\omega$ 3	16.37**	2.02	25.40	17.90(*)	8.45a**	0.81a	23.70a**	0.17a	1.03	0.65A	0.39A	3.84*
	(3.39)	(1.80)	(3.74)	(1.88)	(0.73)	(0.35)	(1.86)	(0.02)	(0.17)	(0.29)	(0.05)	(0.62)

Data are means (S.D.). Statistical analyses (in a column) within the different treatment groups were performed in either the presence or absence of EGF: a vs A,  $P < 0.01$ ; a vs b,  $P < 0.05$ . Comparisons between the different treatment groups of the (-) and (+) EGF groups: (\*)  $P < 0.05$  and \*\* $P < 0.01$ .

18:2 $\omega$ 6 (Fig. 1C). Treatment of hepatocytes with 20:5 $\omega$ 3 in the presence of EGF did not significantly alter the 20:4 $\omega$ 6 and 18:2 $\omega$ 6 levels (Fig. 1D), while 20:5 $\omega$ 3 and 22:5 $\omega$ 3 were increased ( $P < 0.01$ ) as described above (Fig. 1D). However, no significant increase was noticed for 22:6 $\omega$ 3 as described in the absence of EGF, presumably due to the significant ( $P < 0.05$ ) decrease in C22:6  $\omega$ 3 that is noticed in the presence of EGF (Table 1). When considering the fatty acid parameters the total  $\omega$ 6,  $\omega$ 6/ $\omega$ 3, and P/S ratios, were not altered significantly in response to 20:4 $\omega$ 6 (Table 2). Similarly, 20:5 $\omega$ 3 only resulted in a marginal increase in the total  $\omega$ 3 ( $P < 0.1$ ), while no significant effect was

noticed with respect to PUFA and the P/S ratio. When comparing incubations with 20:5 $\omega$ 3 in the absence or presence of EGF (Table 2; changes indicated with asterisks) the total saturated fatty acids increased ( $P < 0.01$ ) due to an increase in C16:0. The  $\omega$ 6 fatty acids and PUFA decreased ( $P < 0.01$ ), resulting in a decrease ( $P < 0.01$ ) in the P/S fatty acid ratio. The total monounsaturated fatty acids increased ( $P < 0.01$ ) due to marginal increases in C16:1 and C18:1 (Table 1). No significant changes were noticed when comparing the total fatty acid parameters of cultures treated with 20:4 $\omega$ 6 in the presence or absence of EGF.

Table 2 Total fatty acid parameters of phosphatidylcholine (PC) of hepatocytes supplemented with C20:4 $\omega$ 6 (25  $\mu$ M) and C20:5 $\omega$ 3 (25  $\mu$ M) in the growth medium in the presence and absence of epidermal growth factor (EGF)

Treatment	Total saturates	Total mono-unsat	Total $\omega$ 6 FA	Total $\omega$ 3 FA	$\omega$ 6/ $\omega$ 3 ratio	Total PUFA	P/S ratio
<b>(-) EGF</b>							
Control	39.58	20.01A	36.36a	4.05a	9.66a	40.41a	1.03a
(BSA)	(2.99)	(1.63)	(2.00)	(1.33)	(2.50)	(2.55)	(0.13)
C20:4 $\omega$ 6	38.94	15.77B	41.27A	4.03a	10.48aA	45.3b	1.20a
	(5.16)	(1.89)	(4.81)	(0.78)	(1.70)	(5.22)	(0.28)
C20:5 $\omega$ 3	36.94	17.58B	39.63(b)	5.85b	7.16bB	45.48b	1.24(b)
	(1.87)	(1.11)	(1.16)	(1.49)	(1.76)	(1.77)	(0.11)
<b>(+) EGF</b>							
Control	39.02	21.01bA	36.02	3.94	10.52a	39.96	1.03
(BSA)	(2.79)	(2.54)	(2.00)	(2.09)	(3.30)	(3.70)	(0.17)
C20:4 $\omega$ 6	41.24	16.66aB	38.55A	3.55	11.28aA	42.10	1.04
	(4.04)	(2.40)	(5.06)	(0.68)	(2.77)	(4.83)	(0.22)
C20:5 $\omega$ 3	41.77**	19.92b**	33.83B**	4.72(b)	7.31bB	38.31**	0.92**
	(1.49)	(1.61)	(2.26)	(0.76)	(1.49)	(1.80)	(0.10)

Data are means (S.D.). Statistical analyses (in a column) within the different treatment groups were performed in either the presence or absence of EGF: A vs B,  $P < 0.01$ ; a vs b,  $P < 0.05$ ; and a vs (b),  $P < 0.1$ . Comparisons between the different treatment groups of the (-) and (+) EGF groups: \*\*  $P < 0.01$ .

#### Effect of ibuprofen, PGE<sub>1</sub>, PGE<sub>2</sub> and fatty acids on the EGF-induced mitogenic response

With the addition of ibuprofen, a known PGE<sub>2</sub> synthesis inhibitor,<sup>26</sup> the EGF-induced synthesis of DNA was inhibited (Fig. 2A) in a dose-dependent manner. Supplementation of FB<sub>1</sub> (75  $\mu$ M) to the incubation medium inhibited ( $P < 0.01$ ) the mitogenic response. To further investigate the involvement of prostaglandins as mediators of the EGF-induced mitogenic response different concentrations of PGE<sub>1</sub> and PGE<sub>2</sub> were added to the incubation medium (Fig. 2B). An increase ( $P < 0.05$ ) in the EGF-induced response was noticed at the higher concentrations of PGE<sub>2</sub> that were tested. In contrast PGE<sub>1</sub> inhibited ( $P < 0.05$  to  $P < 0.01$ ) the mitogenic response in a dose-dependent manner. Arachidonic acid (10  $\mu$ M/dish) enhanced ( $P < 0.05$ ), while 20:5 $\omega$ 3 decreased ( $P < 0.05$ ) the mitogenic response at 25 and 50  $\mu$ M/dish (Fig. 3A).

#### Role of PGE<sub>2</sub> on the FB<sub>1</sub>-induced mitoinhibitory effect

FB<sub>1</sub> inhibited the mitogenic response of EGF in a dose-

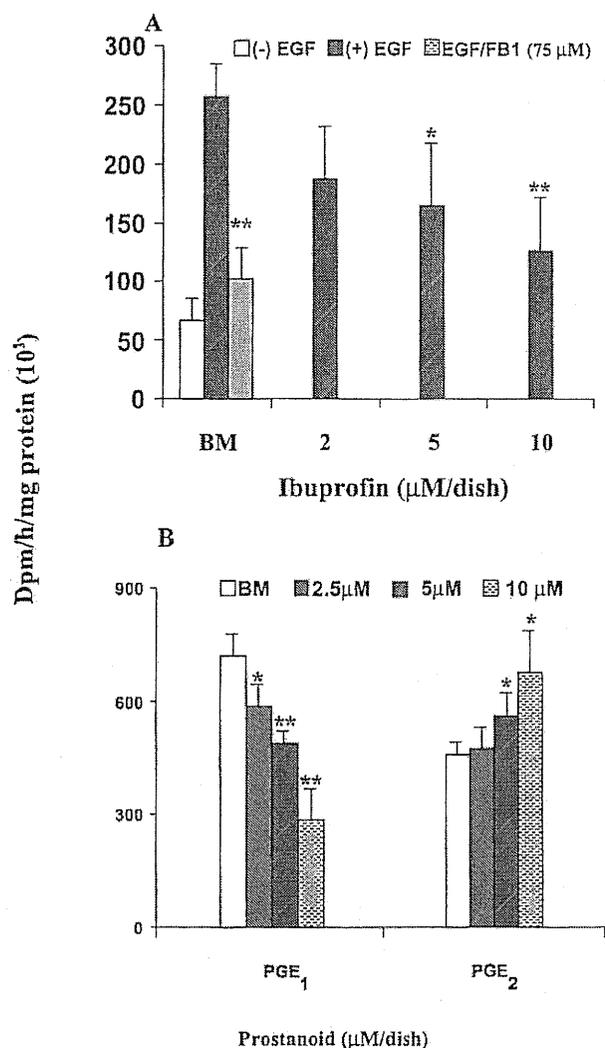


Fig. 2 The effect of FB<sub>1</sub> and different concentrations of ibuprofen (A) and prostaglandins E<sub>1</sub> and E<sub>2</sub> (B) on the mitogenic response of EGF in primary hepatocytes (\* $P < 0.05$ ; \*\* $P < 0.01$ ). Values are means of triplicate determinations of two different experiments.

dependent manner (Fig. 4A). The inhibitory response was effected ( $P < 0.01$ ) from 75 μM FB<sub>1</sub>/dish and higher. Addition of PGE<sub>2</sub> (12.5 μM) counteracted the inhibitory effect obtained at all the concentrations of FB<sub>1</sub> used.

Fatty acid analyses of hepatocytes exposed to different concentrations of FB<sub>1</sub> (10, 25, 50 and 75 μM/dish), increased ( $P < 0.01$ ) the level of 18:2ω6 in PC from 10 μM FB<sub>1</sub>/dish and triacylglycerol (TAG) from 50 μM (Fig. 4B).

#### Cytotoxicity

Dose-response studies regarding ibuprofen, PGE<sub>1</sub> and PGE<sub>2</sub> showed no cytotoxic effects at any of the treatments

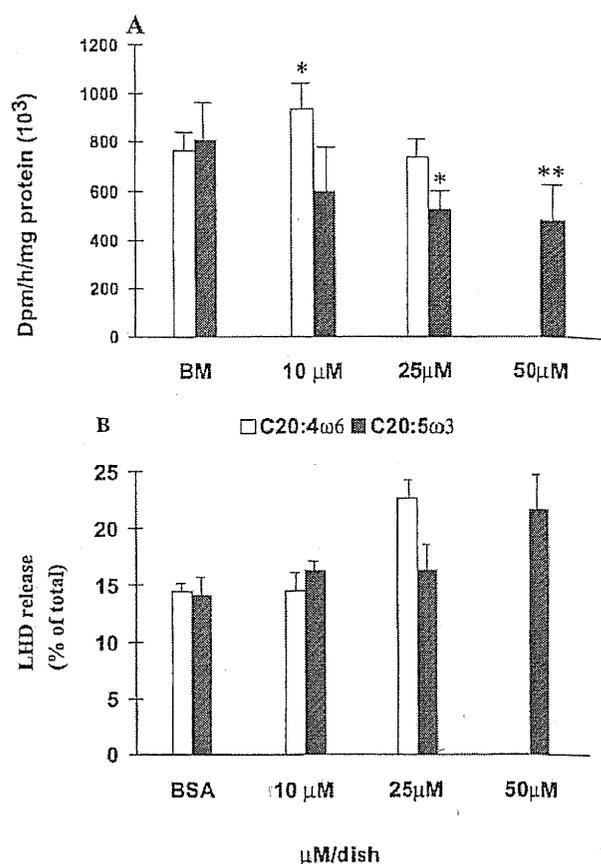


Fig. 3 Modulating role of different concentrations of 20:4ω6 and 20:5ω3 on the EGF mitogenic response in primary hepatocytes (A). The respective cytotoxicities, expressed as LDH release, of the fatty acids are illustrated (B). Values are means of triplicate determinations and experiments were repeated three times (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

used in the present study (data not shown). An increase in the cytotoxicity of 20:4ω6 was noticed ( $P < 0.05$ ) at 25 μM, while 20:5ω3 was found to be less toxic with no significant increase in the LDH release at 50 μM (Fig. 3B). FB<sub>1</sub> exhibited a low cytotoxic effect and concentrations of 250–300 μM significantly enhanced the release of LDH in cultures (data not shown) which is in accordance with the findings of a previous report.<sup>10</sup>

#### DISCUSSION

PUFA and their eicosanoid metabolites, the prostaglandins are known to modulate cell proliferation in many in vivo studies in experimental animals and in vitro studies utilising different cell culture systems.<sup>28</sup> The release of 20:4ω6 (arachidonic acid) and its subsequent metabolism into prostaglandins of the 2-series is a prerequisite for the EGF-induced mitogenic effect in BALB/c 3T3

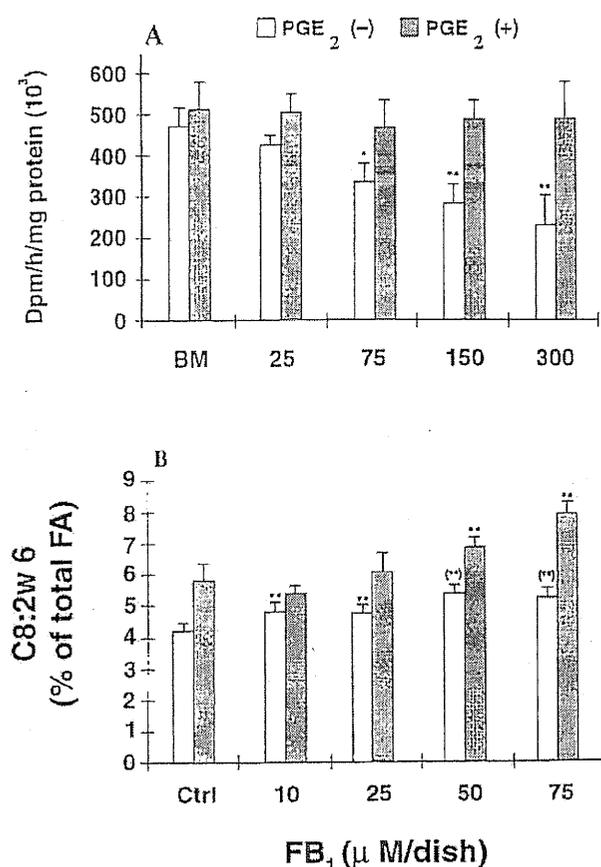


Fig. 4 Role of PGE<sub>2</sub> (12.5 μM/dish) on the mitoinhibitory response of FB<sub>1</sub> in primary hepatocytes (A). The dose-response effect of FB<sub>1</sub> on the accumulation of 18:2ω6 in the PC fraction and in TAG is illustrated in (B). Values are means of triplicate determinations and are the means of two or three experiments. \**P* < 0.05; \*\**P* < 0.01. (\*\*\*) differs significantly (*P* < 0.01) from \*\* in the PC group.

fibroblasts.<sup>29,30</sup> In primary hepatocytes, hepatocyte growth factor (HGF) and EGF causes the release of 20:4ω6 via the activation of phospholipase A<sub>2</sub> involving the mitogen-activated protein (MAP) kinase signalling pathway.<sup>31,32</sup> In the present study, the modulating role of fatty acids and their metabolites, the prostaglandins, on the EGF response in primary hepatocytes was investigated to elucidate the mechanisms concerning the FB<sub>1</sub>-induced mitoinhibitory effect.<sup>10</sup> Investigations from previous studies showed that FB<sub>1</sub> alters the fatty acid metabolic pathways under various experimental conditions *in vitro*<sup>18</sup> and *in vivo*<sup>19</sup> suggesting that it could interfere with growth related responses within the cell.

Fatty acid analyses indicated that the EGF-induced mitogenic response in primary hepatocytes was associated with a decrease in C20:4ω6 (*P* < 0.01) and C16:0 (*P* < 0.05) with a concomitant increase in 18:2ω6 (*P* < 0.01) and C18:0 (*P* < 0.05) (Table 1 and Fig. 1). The decrease in

20:4ω6 could be related to the increased hydrolyses of 20:4ω6 to mediate the subcellular events of EGF. The reason(s) for the accumulation of 18:2ω6 and the changes to C16:0 and C18:0 are not known at present. Addition of 20:4ω6 to the incubation medium stimulated the EGF mitogenic response (Fig. 3A), presumably because the EGF-induced reduction of C20:4ω6 (Fig. 1A vs 1C) was counteracted. The decrease of 18:2ω6 following the addition of 20:4ω6 (Fig. 1A) could be due to a negative feedback mechanism of the latter on the delta-5 and delta-6 desaturases.<sup>33</sup>

In contrast, the addition of 20:5ω3 inhibited the EGF-induced mitogenic response (Fig. 3A). Fatty acid analyses indicated that addition of 20:5ω3 increased the relative levels of 20:5ω3 (*P* < 0.01), 22:5ω3 (*P* < 0.05) and 22:6ω3 (*P* < 0.01) (Fig. 1B). A similar effect was obtained in the presence of EGF, except that 22:6ω3 was not increased significantly (Fig. 1D). The resultant reduction of the mitogenic response could possibly be related to an increased incorporation of ω3 fatty acids in the hepatocyte membranes at the expense of the ω6 fatty acids. It is known that the fatty acid metabolic enzymes have a higher affinity for the ω3 fatty acids and therefore are more readily converted to longer chain metabolites and incorporated at the expense of the ω6 fatty acids in the cellular membranes.<sup>34</sup> The enhanced levels of 20:5ω3 could also facilitate the formation of the 3-series prostaglandins that are known to be metabolically less active than prostaglandins of the 2-series, which are derived from 20:4ω6.<sup>35</sup>

When considering the total fatty acid parameters (Table 2), the enhancement of the EGF response by 20:4ω6 was not associated with a significant increase of the total ω6 fatty acids, ω6/ω3 ratio and PUFA. However, these parameters were increased (*P* < 0.05 to *P* < 0.01) in the absence of EGF. The inhibition of the EGF response by 20:5ω3 was associated with a decrease in the total ω6 fatty acids and PUFA when compared to the parameters in the absence of EGF (Table 2, statistical changes indicated with asterics). The total ω3 fatty acids were not significantly altered as a result of the EGF treatment although there was a significant change in the individual ω3 fatty acid profiles. This is reflected, as discussed above, by the fact that the level of 22:6ω3 was not significantly increased upon the addition of 20:5ω3 as was noted in the absence of EGF (Fig. 1B vs 1D). This seems to imply that 20:5ω3 could be utilised elsewhere as a result of the EGF treatment and not metabolised to the long-chain 22:6ω3 fatty acid product. In addition, there was also an increase in the degree of saturation, i.e. a decrease in the total ω6 fatty acids and PUFA with an increase in total saturated, mono-unsaturated fatty acids which resulted in a decrease in the P/S ratio (Table 2). Therefore, a decrease in the EGF mitogenic response was associated with specific

ic changes to the membrane environment of hepatocytes that are likely to result in a more rigid membrane structure.

The modulating effects of the different fatty acids on the EGF-induced mitogenic response appear to occur via the regulation of prostaglandin production. This became apparent as the cyclooxygenase inhibitor,<sup>26</sup> ibuprofen, inhibits mitogenesis (Fig. 2). Addition of PGE<sub>2</sub> further potentiated the EGF-induced mitogenic response while PGE<sub>1</sub>, derived from 20:3 $\omega$ 6 (dihomo-gamma-linolenic acid), inhibited the EGF response in a dose-dependent manner. Recent studies indicated that 20:3 $\omega$ 6 inhibits phospholipase A<sub>2</sub>, the enzyme that mobilizes 20:4 $\omega$ 6, via its interaction with cAMP<sup>36</sup> suggesting that the metabolism of these two fatty acids could affect growth stimulatory and/or inhibitory effects within a cell. However, in cell culture systems, differences exist concerning the stimulation and/or inhibition of cell proliferation that depends not only on the nature and concentration of a specific prostaglandin, but also on the cell type and density.<sup>28</sup>

In general it would appear that, at low concentrations, prostaglandins stimulate, while at higher concentrations (1  $\mu$ M or greater) they inhibit cell proliferation *in vitro*. Therefore, by manipulating the membrane fatty acid profiles and presumably the prostaglandin levels, the mitogenic signal of growth stimulatory factors, such as EGF, can be modulated.

Recent investigations indicated that FB<sub>1</sub> altered the levels and fatty acid profiles of the major phospholipids and hence the structure of cellular membranes.<sup>18-20</sup> These changes were suggested to be involved in the mitoinhibitory effect of FB<sub>1</sub> on the EGF mitogenic response in primary hepatocytes.<sup>10</sup> The present study provided evidence that changes to the  $\omega$ 6 fatty acid metabolic pathway and the subsequent prostaglandin synthesis could clarify the growth inhibitory effect of FB<sub>1</sub> on the EGF mitogenic response in primary rat hepatocytes. This can be deduced as the addition of PGE<sub>2</sub> counteracted the mitoinhibitory effect of FB<sub>1</sub> (Fig. 4A). The interruption of fatty acid metabolism and more specifically the inhibition of PGE<sub>2</sub> synthesis and the inhibition of growth (mitogenesis) in primary hepatocytes by FB<sub>1</sub> appear to be two closely related physiological events. At present it is not known whether FB<sub>1</sub> affects the activity of the cyclooxygenase, a key enzyme in the synthesis of the 2-series prostaglandins. However, studies in primary hepatocytes, indicated that 18:2 $\omega$ 6 and 20:4 $\omega$ 6 accumulate when exposed to similar concentration levels that inhibit the EGF-induced mitogenic response.<sup>10,18</sup> At cytotoxic concentrations (>300  $\mu$ M) the accumulation of 20:4 $\omega$ 6 became more prominent, implying that FB<sub>1</sub> also altered its metabolism. As suggested previously, the resultant increase in the level of 20:4 $\omega$ 6 could be due to an

inhibitory effect on the prostanoid synthetic pathway.<sup>18</sup> The accumulation of 18:2 $\omega$ 6 could also result from feedback regulation of 20:4 $\omega$ 6 on the delta 6 desaturase enzyme.<sup>33</sup> However, at lower concentrations of FB<sub>1</sub> where the EGF response was disrupted (Fig. 2A), 20:4 $\omega$ 6 was not increased, despite the fact that 18:2 $\omega$ 6 accumulated (Fig. 4B). The accumulation of 18:2 $\omega$ 6 was also effected at low dietary levels *in vivo* in rat liver, while the total  $\omega$ 6 and  $\omega$ 3 fatty acids were decreased. This would imply that the delta 6 desaturase enzyme, a rate-limiting enzyme in the fatty acid metabolic pathway could have been affected by FB<sub>1</sub>.<sup>19</sup> It is not known whether FB<sub>1</sub> inhibits the delta 6 desaturase enzyme and whether alterations of the membranal structure and/or the tight binding of FB<sub>1</sub> to cellular membranes<sup>37</sup> alters the activity of the enzyme. FB<sub>1</sub> also increased the rigidity of hepatocyte membranes by decreasing the membranal cholesterol content resulting in an increase in the PC/cholesterol ratio, a marker for membrane fluidity.<sup>18</sup> *In vivo* data also suggested a more rigid hepatocyte membrane structure following exposure to FB<sub>1</sub>, although it is manifested differently.<sup>19</sup> The inhibitory effect of 20:5 $\omega$ 3 on the EGF mitogenic response in primary hepatocytes was also associated with an increase in the saturation of membranal fatty acids resulting in a decrease in the P/S ratio (Table 1) implying a more rigid membrane structure. Further studies are required to investigate the FB<sub>1</sub>-induced changes to the fatty acid profiles of membrane phospholipids in different cellular compartments and its effect on the disruption of growth factor responses in the liver.

The regulation of fatty acid metabolism and its effect on prostaglandin synthesis is complex. Other effects induced by FB<sub>1</sub> that are not related to prostaglandin synthesis could also alter the growth responses within the cell. For instances, the inhibition of ceramide synthase and the subsequent accumulation of sphinganine have been suggested to be involved in FB<sub>1</sub>-induced mitogenesis in Swiss 3T3 fibroblasts.<sup>15</sup> However, subsequent studies on the same cells indicated that FB<sub>1</sub> activated MAPkinase does not require the accumulation of sphingosine or sphinganine.<sup>23</sup> In primary hepatocytes, these sphingoid bases are not mitogenic, while their accumulation appears not to be directly involved in the mitoinhibitory effect of FB<sub>1</sub> on the EGF-induced mitogenic response.<sup>10</sup> It was shown that sphinganine accumulates maximally between 12 and 24 h in hepatocyte cultures exposed to 1  $\mu$ M FB<sub>1</sub>,<sup>38</sup> that is far below the concentrations which inhibit the mitogenic effect of EGF.<sup>10</sup> Long- and short-term feeding studies in rats indicated that cancer promotion is effected at low levels of FB<sub>1</sub> which have no effect on the sphingolipid levels.<sup>9,20</sup> As the phospholipid and fatty acid profiles of hepatocyte membranes are altered significantly<sup>18,19</sup> it would appear that, as suggested in the present study, these changes are likely to be

involved in the mitoinhibitory effect on the EGF mitogenic response and the cancer promoting activity of FB<sub>1</sub>.

Apart from the role of fatty acids as membrane structural components and a source for prostaglandin production, they are known to play an important role as lipid second messengers that regulate cellular growth and metabolism.<sup>39-41</sup> The mediating effect of 20:4 $\omega$ 6 in the cytolytic effects of tumor necrosis factor (TNF)-induced apoptosis is a classical example, which also involves ceramide as the key second messenger.<sup>39</sup> The suppression of 20:4 $\omega$ 6 cascade-mediated apoptosis by glucocorticoids has been suggested to act as a tumor promoter in aflatoxin B<sub>1</sub> hepatocarcinogenesis involving TGF- $\beta$ 1 and probably TNF whose functions are mediated in part by 20:4 $\omega$ 6.<sup>41</sup> Gamma-linolenic acid (18:3 $\omega$ 6) has been implied in the induction of apoptosis in cell cultures.<sup>42</sup> Several mechanisms have been proposed to trigger the apoptotic response including: (1) the deregulation of c-myc, (2) the activation of 20:4 $\omega$ 6-activated sphingomyelinase with the subsequent release of ceramide, and (3) the elevation of cAMP due to the production of the E-series prostaglandins. However, the subsequent role of 20:4 $\omega$ 6 and its metabolites that are derived further down the line in the  $\omega$ 6 metabolic pathway from 18:3 $\omega$ 6 (Fig. 5), cannot be excluded in these effects. The present study implies that the regulation of the fatty acid metabolic pathways and subsequent prostaglandin synthesis could play an important role in the regulation of cell proliferation by FB<sub>1</sub>. In this regard, a recent study showed that FB<sub>1</sub> increased the expression of TGF- $\beta$ 1 and c-myc in hepatocytes in vivo, which suggested that these events could be involved in the apoptotic and cancer-promoting effects of the compound.<sup>43</sup> As mentioned above, the ceramide-associated signal transduction pathway is also of interest, as FB<sub>1</sub> is known to alter the sphingolipid metabolic pathway by increasing the level of sphinganine and decreasing the level of complex sphingolipids such as sphingomyelin and ceramide.<sup>12,14,19</sup> A recent study in murine lymphocyte and human monoblast leukemia cells indicated that FB<sub>1</sub> blocks daunorubicin-induced ceramide elevation and apoptosis.<sup>44</sup> As daunorubicin does not stimulate sphingomyelin hydrolyses via sphingomyelinase, but instead stimulates ceramide synthase, inhibition of ceramide synthesis by FB<sub>1</sub> provides further evidence for a requirement of ceramide to induce apoptosis. However, FB<sub>1</sub> is known to induce apoptosis in many other cell types and in vivo,<sup>13</sup> suggesting that other pathways could exist that affect the apoptotic signals apart from the disruption of sphingolipid biosynthesis. A novel pathway involving a cascade of lipid messengers that couple glycerol phospholipids and sphingolipids has been suggested<sup>39</sup> resulting in an interplay and/or cross-signalling between the fatty acid metabolic pathway (mainly 20:4 $\omega$ 6) and the sphingomyelin cycle (mainly

ceramide). Further studies are required to investigate how these pathways, both affected by FB<sub>1</sub>, are involved in the growth regulatory effects of FB<sub>1</sub> in rat liver that eventually could lead to adverse biological effects such as the modulation of cell proliferation, apoptosis and cancer promotion.

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# Fumonisin-Induced Hepatocarcinogenesis: Mechanisms Related to Cancer Initiation and Promotion

Wentzel C.A. Gelderblom,<sup>1</sup> Stefan Abel,<sup>1</sup> Cornelius M. Smuts,<sup>2</sup> Jeanine Marnewick,<sup>1</sup> Walter F.O. Marasas,<sup>1</sup> Eric R. Lemmer,<sup>3</sup> and Danica Ramljak<sup>4</sup>

<sup>1</sup>Programme on Mycotoxins and Experimental Carcinogenesis and <sup>2</sup>National Research Programme for Nutritional Intervention, Medical Research Council, Tygerberg, South Africa; <sup>3</sup>MRC/UCT Liver Research Centre, University of Cape Town, Cape Town, South Africa; <sup>4</sup>Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland, USA

We review the hepatocarcinogenic effects of fungal cultures of *Fusarium verticillioides* (= *Fusarium moniliforme*) strain MRC 826 in male BD IX rats. Subsequent chemical analyses of the fumonisin B (FB) mycotoxin content in the culture material used and long-term carcinogenesis studies with purified FB<sub>1</sub> provide information about dose-response effects, relevance of hepatotoxicity during FB<sub>1</sub>-induced carcinogenesis, and the existence of a no-effect threshold. Fumonisin intake levels of between 0.08 and 0.16 mg FB/100 g body weight (bw)/day over approximately 2 years produce liver cancer in male BD IX rats. Exposure levels < 0.08 mg FB/100 g bw/day fail to induce cancer, although mild toxic and preneoplastic lesions are induced. The nutritional status of the diets used in the long-term experiments was marginally deficient in lipotropes and vitamins and could have played an important modulating role in fumonisin-induced hepatocarcinogenesis. Short-term studies in a cancer initiation/promotion model in rat liver provided important information about the possible mechanisms involved during the initial stages of cancer development by this apparently nongenotoxic mycotoxin. These studies supported the findings of long-term investigations indicating that a cytotoxic/proliferative response is required for cancer induction and that a no-effect threshold exists for cancer induction. The mechanisms proposed for cancer induction are highlighted and include the possible role of oxidative damage during initiation and the disruption of lipid metabolism, integrity of cellular membranes, and altered growth-regulatory responses as important events during promotion. **Key words:** fatty acids, fumonisins, *Fusarium verticillioides*, hepatocarcinogenesis, hypothesis, mechanisms, phospholipids. — *Environ Health Perspect* 109(suppl 2):291–300 (2001).

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Investigations into the toxigenic properties of *Fusarium verticillioides* (= *Fusarium moniliforme*) have been the focus of many scientific endeavors following the classic finding that the fungus is responsible for natural outbreaks of equine leukoencephalomalacia (ELEM) (1,2). Many isolates of *F. verticillioides* from different origins in southern Africa were screened on a regular basis at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, Tygerberg, South Africa, in toxicity trials in ducklings and rats (3). Comparative toxicity studies of the fungus in different animal species led to the finding that the major target organ differs in each species, whereas certain organs, including the liver and kidneys, appear to be affected consistently to a greater or lesser degree (4). It was proposed that the rat served as the best experimental model to screen toxigenic isolates of *F. verticillioides* for their potential to induce different lesions in various animal species. Several events following these initial studies have made a major impact on the subsequent research concerning the toxicologic effects of this fungus in animals. One of these was the finding that contamination of corn with *F. verticillioides* was positively associated with the incidence of human esophageal cancer in

the Transkei region of the Eastern Cape Province, South Africa (5,6). Second, toxicity screening of different *F. verticillioides* isolates obtained from corn cultivated in a high-incidence area of esophageal cancer induced hepato- and cardiotoxic lesions in rats (4). The induction of cirrhosis together with bile duct and nodular hyperplasia was of particular interest with respect to the potential carcinogenic activity of different isolates of the fungus. The possible link between occurrence of this fungus on corn and the development of esophageal cancer has initiated intensive investigations to characterize the toxic and carcinogenic principle(s) that occur in corn, the major dietary staple of humans in the Transkei.

## Toxicity and Carcinogenicity Studies in Rats

### Studies with Cultures of *F. verticillioides* Strain MRC 826

An isolate of *F. verticillioides* designated strain MRC 826, obtained from corn grown in a high-incidence area of esophageal cancer in Transkei, induced ELEM in horses and produced the potent mutagenic compound fusarin C (3,4,7). Chronic feeding studies in male BD IX rats with a freeze-dried corn culture (batch MRC 826B) of *F. verticillioides* at

dietary levels ranging from 2 to 4% in a commercial rat feed caused liver cancer in 80% and ductular carcinoma in 63% of the surviving rats after 450 days (8). An important finding was that the hepatocellular carcinomas (HCCs) developed in cirrhotic livers showing nodular hyperplasia. Another prominent lesion was the concurrent development of cholangio- or adenofibrosis, a lesion that appears to develop from the proliferation of hyperplastic epithelial cells, goblet cells, and Paneth cells. The experiment was conducted with both oven-dried (MRC 826, batch 9-20) and freeze-dried (MRC 826, batch B) culture material and identical lesions were induced; however, the degree of the effects was higher with the freeze-dried material. It was suggested that the causative principle(s) was partially destroyed during the oven-drying treatment at 45–50°C. This was of particular interest because the mutagen fusarin C, produced by the strain MRC 826, was highly heat and light labile and not very toxic acutely (7). Three pertinent issues received attention in a subsequent chronic feeding study (9) in rats using the same culture batch of the fungus (MRC 826, batch B): whether the hepatocarcinogenicity was related to the toxic effects of the fungal culture material; whether the nontoxic mutagen fusarin C could be related to the carcinogenic outcome; and whether the diet that was marginally deficient in certain vitamins and lipotropes known to have protective effects against esophageal cancer (10) could sensitize rats to develop esophageal cancer when fed low levels of the fungal culture.

We investigated the relative contributions of fusarin C and toxicity to the carcinogenic effects of the fungus in the liver by including a

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Address correspondence to W.C.A. Gelderblom, PROMEC/MRC, PO Box 19070, Tygerberg 7505, South Africa. Telephone: 27-21-9380286. Fax: 27-21-9380260. E-mail: wentzel.gelderblom@mrc.ac.za

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nontoxic strain of *F. verticillioides*, designated MRC 1069, that produces three times more fusarin C than strain MRC 826 (9). We used a semisynthetic diet marginally deficient in some vitamins and micronutrients to evaluate a possible synergistic effect between nutritional deficiencies and the fungal culture for the induction of esophageal cancer (Table 1). Most lesions found in the rats fed a dietary level of 0.5% of the culture material of MRC 826, batch B, included a high frequency of neoplastic nodules (21/21), ductular hyperplasia (21/21), adenofibrosis (19/21), cholangiocarcinomas (8/21), and HCC (2/21) that metastasized to the lungs. Liver sections of 85% of the animals showed marked increases in the presence of gamma glutamyl transpeptidase positive (GGT<sup>+</sup>) foci and/or nodules. Unlike results in the previous study (8), very little fibrosis, except in association with adenofibrosis, was noticed in the liver, presumably because of the low dietary levels of the culture material used. Basal cell hyperplasia occurred in 12/21 rats fed culture material of strain MRC 826 (batch B), whereas one rat developed an esophageal papilloma. Very few lesions occurred in the liver of the rats fed culture material of MRC 1069 despite its being fed at a dietary level of 5%. Mild ductular hyperplasia was present (18/22); one rat had a focus of cholangiofibrosis and another presented with a neoplastic nodule. Fatty acid changes occurred in the treated rats of both groups. Hepatocytes in the treated and control rats were prominently loaded with glycogen, presumably caused by the high carbohydrate content of the diet used.

When comparing with the first experiment (8), we must consider several aspects. A clear dose–response effect with respect to hepatotoxicity and carcinogenicity became apparent, suggesting that the hepatotoxicity is related to cancer development by the fungus in the liver of rats. Fusarin C was clearly not involved in the carcinogenicity of the fungus because culture material *F. verticillioides* strain MRC 1069, containing at least 3 times as much fusarin C and fed at a dietary level 10 times higher than that of MRC 826, produced only a few neoplastic lesions in the liver. The basal cell hyperplastic lesions failed to progress to neoplasia despite the fact that the diet contained marginal levels of lipotropes and some vitamins.

Following these long-term studies with fungal culture material in South Africa, a feeding study in male Fischer 344 rats showed that corn naturally contaminated with *F. verticillioides* and associated with a field outbreak of ELEM in the United States induced nodules, adenofibrosis, and cholangiocarcinomas in the liver after 4 to 6 months (11). Although the corn-only diet was deficient in many vitamins and lipotropes,

the lesions were very similar to those described by Marasas et al. (8), which were induced by *F. verticillioides* strain MRC 826 in male BD IX rats fed a nutritionally balanced diet. The early appearance of the lesions in the liver of the rats obtained by Wilson et al. (11) seems related to dietary deficiencies, as discussed above. This study further strengthened the hypothesis that the causative toxic principle(s) responsible for ELEM in horses and the hepatotoxicity/carcinogenicity in rats could be identical. Studies of the carcinogenic effects of fusarin C in a short-term cancer initiation/promotion model indicated that culture material of *F. verticillioides* strain MRC 826 exhibited cancer-promoting activity when using diethylnitrosamine (DEN) as a cancer initiator and the induction of GGT-positive foci and/or nodules as end points (12). Subsequently, several other strains of the fungus, isolated from a high-incidence area of esophageal cancer in Transkei, were screened for cancer-promoting activity in a modified version of the resistant hepatocyte rat liver model (13). As described above, DEN was used as a cancer initiator while the culture material of the different strains was fed at a dietary level of 5% for 21 days during promotion with the induction of GGT-positive foci used as end point. Three other strains of *F. verticillioides* in addition to strain MRC 826 exhibited cancer-promoting activity, and a significant correlation was found between toxicity and the cancer-promoting activity. As discussed above, this study also suggested that the compound(s) responsible for the toxic and carcinogenic activity could be identical.

#### Studies with Fumonisin B<sub>1</sub> Isolated from *F. verticillioides* Strain MRC 826

**Long-term studies.** The fumonisin B (FB) mycotoxins were originally isolated (14) using the short-term cancer initiating/promoting model described above and their chemical structures were determined (15). Information about the carcinogenic effects of FB<sub>1</sub>, the main fumonisin produced by *F. verticillioides*, obtained from a short-term study (14) suggested that this mycotoxin could effect both cancer initiation and promotion, and hence could act as a complete carcinogen. Cancer initiation and promotion were associated with a toxic effect characterized with the proliferation of bile ductules, fibrosis, and nodular regeneration similar to those described for *F. verticillioides* MRC 826 in male BD IX rats (16,17). Dosing of horses proved that FB<sub>1</sub> caused the neurotic syndrome ELEM (18). These investigations confirmed the previous hypothesis that the compound responsible for ELEM in horses was also responsible for hepatotoxicity and hepatocarcinogenicity in rats (4). These findings led to carcinogenicity testing of FB<sub>1</sub>

in male BD IX rats performed with the culture material of strain MRC 826 using a marginally deficient diet, as described by Jaskiewicz et al. (9) and Van Rensburg et al. (10).

When male BD IX rats were fed FB<sub>1</sub> at 50 mg/kg diet, regenerative nodules and cholangiofibrosis occurred from 6 months onward (19). The rats that were sacrificed or that died from 18 months until 26 months, when the experiment was terminated, suffered from micro- and macronodular cirrhosis with large expansive nodules of cholangiofibrosis. Histologic changes inside the regenerative nodules varied and included fatty changes, hyaline droplet degeneration, necrosis, and areas with a ground-glass appearance that stained positive for GGT. Of the rats that were killed between 18 and 26 months, 66% developed HCC; in 4 rats this metastasized to the kidney, heart, and lungs. Cholangiofibrosis—manifested as irregular ductlike structures lined with an epithelium consisting of large columnar cells and numerous goblet cells—occurred in 100% of the rats killed between 18 and 26 months. Lesions in the kidneys consisted of diffuse interstitial lymphocytic nephritis and mild membranoproliferative glomerulonephritis and were more pronounced in the rats killed at 26 months. Most of the lesions observed in the liver and kidneys of the rats fed FB<sub>1</sub> (19) were also induced by culture material of strain MRC 826, except that the esophageal basal cell hyperplasia and cardiac lesions induced by the culture material (8) were not present. Other compounds present in the fungal culture material may cause these lesions, either separately or synergistically with the fumonisins. However, it was shown that FB<sub>1</sub> caused the hepatotoxic and hepatocarcinogenic effects of the fungal culture material in male BD IX rats. In a subsequent experiment, low dietary levels of FB<sub>1</sub> were fed to male BD IX rats to establish dose–response effects with respect to cancer development in the liver (20). In short, male BD IX rats were fed a semipurified diet containing 1, 10, and 25 mg FB<sub>1</sub>/kg diet over a period of 24 months. Detailed feed intake profiles were monitored to calculate FB<sub>1</sub> intake profiles during the course of the experiment. No HCC or cholangiofibrotic lesions were noticed in any of the rats terminated between 18 months and 24 months. The major lesions in the liver of the rats fed the high-dose FB<sub>1</sub> diet [25 mg FB<sub>1</sub>/kg (Table 1)], consisted of anisokaryosis (13/17), neoplastic nodules (9/17), oval cell proliferation (2/17), bile duct hyperplasia (3/17), lobular distortion and portal fibrosis (5/17), and ground-glass foci (5/17), whereas the livers of all the rats terminated at 26 months contained positive foci (11/11) of the placental form of glutathione S-transferase (GSTP). In the rats fed the 10 mg FB<sub>1</sub>/kg diet, fewer lesions

appeared and only mild toxic lesions occurred in the livers of the 1 mg FB<sub>1</sub>/kg dietary group. The data indicate that a threshold exists and that a chronic toxic effect is required for FB<sub>1</sub>-induced hepatocarcinogenesis. The results of these toxicity and carcinogenicity studies in rats, together with estimated exposure levels, were used to determine risk-assessment parameters for fumonisins in humans (21).

Since the discovery of the fumonisins in 1988, sensitive analytic detection techniques have been developed (22), which have enabled the retrospective estimation of the FB intake in the initial long-term experiment performed by Marasas et al. (8) and subsequently by Jaskiewicz et al. (9). These calculated data and comparisons with the long-term studies in rats, using purified FB<sub>1</sub> (19,20), are summarized in Table 2. Cancer induction by the fumonisins occurs in the presence of adverse hepatotoxicity, including cirrhosis, cholangiofibrosis, and oval cell proliferation (Table 2). An average dietary intake of 0.08 mg FB/100 g body weight (bw)/day induced mild toxic effects with 50% of the rats having neoplastic nodules in the liver; an average intake of 0.16 mg FB/100 g bw/day causes liver cancer in 55% of BD IX male rats over a period of approximately 2 years.

**Dietary considerations.** Comparisons of the semipurified diet used in the chronic feeding studies with culture material of MRC 826 (8,9) and FB<sub>1</sub> (19) and the synthetic AIN 76 diet (23) used in subsequent experiments (17) are shown in Table 1. The semipurified diet was developed by Van Rensburg

et al. (10) to investigate the role of a diet low in micronutrients on the development of esophageal cancer in rats. The rationale behind the study was to evaluate the role of simulated human diets involving corn and wheat, which are invariably used as a main source of food in high-incidence areas for esophageal cancer, on the development of esophageal tumors in rats. The supplementation of marginally

deficient corn and wheat diets with various combinations of nicotinic acid, riboflavin, zinc, magnesium, molybdenum, and selenium reduced the numbers of esophageal papillomas in rats (10). Indigenous African grains (sorghum and millet) also significantly reduced the incidence of esophageal papillomas compared to the corn-based diet (10). The nutritional composition of the semipurified diet

**Table 1.** Comparison of dietary composition<sup>a</sup> of AIN 76 diet with the semipurified diet used in fumonisin B<sub>1</sub> feeding studies.

	AIN-76	Semipurified		AIN-76	Semipurified
Protein (g/kg)	214.3	109	Vitamins <sup>b</sup>		
Soy protein		30	Thiamin (mg)	6.4	3.9
Casein	200	10	Riboflavin (mg)	6.1	3.55
Egg albumin		10	Nicotinic acid (mg)	31.1	32
Corn meal		59	Vitamin B6 (mg)	7.1	0.6
Methionine	3	1.91	Folate (mg)	2	0.5
Total CHO (g/kg)	592	659.1	Vitamin B12 (µg)	10	8
Corn starch	216.7	750	Pantothenic acid (mg)	16.8	3
Sucrose	216.7		Biotin (mg)	0.2	0.1
Glucose	216.7	111.25	Vitamin A (IU)	4,005	16,672
Dextrin		54.3	Vitamin D (IU)	1,000	250
Total Fat (g/kg)	46.4	47.6	Vitamin E (mg)	79.7	41.33
Saturated	5.9	6.33	Vitamin K (mg)	5	2.95
MUFA	9.0	11.7	Choline	2	0.7
PUFA	29.3	27.0	Minerals <sup>c</sup>		
Sunflower seed oil (g)	50	30	Calcium	5,100	515
Energy			Iron	48	17.3
Kcal	3,779	3,448	Magnesium	604	488
KJ	15,820	14,426	Phosphorus	4,264	1,133
Fibre (g/kg)	55.4	37.2	Potassium	3,925	2,307
			Sodium	1,245	1,028
			Zinc	46	20.8
			Copper	5.7	

Abbreviations: CHO, carbohydrate; KJ, kilojoules. <sup>a</sup>Composition analyses performed using the MRC Food Composition Tables (80). <sup>b</sup>mg/kg or units/kg. <sup>c</sup>mg/kg AIN-76 (23); semipurified diet (18).

**Table 2.** Comparison of body weight gains, total FB intake, and histologic findings between different long-term experiments with culture material of *F. verticillioides* and purified FB<sub>1</sub> in male BD IX rats.

Reference	Body weight		FCM Gain (g)	FCM (% in diet)	FCM intake (mg/100 g bw/day)	FB intake (mg/100 g bw/day)	Duration (days)	Total intake (mg/100 g bw)	Major histologic lesions in the liver
	Initial (g)	Final (g)							
Marasas et al. (8)	63.5 ± 4.7	290.1 ± 46.8	226.6 ± 47.3	4	0.13	0.69	288	198.33	Cirrhosis (20/20)
Control	64.7 ± 4.8	370.8 ± 71.8	306.1 ± 70.2			0.0005	894	0.45	Adenofibrosis (19/20)
Jaskiewicz et al. (9)	113.9 ± 4.7	359.8 ± 78.0	246.0 ± 77.6	0.25	0.01	0.04	211	9.08	Ductular carcinoma (10/20)
Control	113.6 ± 5.1	483.6 ± 88.4	370.0 ± 87.2			0.0005	869	0.39	HCC (12/20)
Gelderblom et al. (19)	68.6 ± 1.9	416.0 ± 38.2	347.5 ± 38.01		50 mg FB <sub>1</sub> /kg diet	0.160	780	124.8	Basal cell hyperplasia (11/15)
Control	68.1 ± 2.7	517 ± 108.8	448.9 ± 107.4				690	55.20	Neoplastic nodules (18/21)
Gelderblom et al. (20)	98.5 ± 7.1	445.1 ± 88.9	346.6 ± 89.9		25 mg FB <sub>1</sub> /kg diet <sup>a</sup>	0.080	690	22.08	GGT-positive foci (18/21)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2		10 mg FB <sub>1</sub> /kg diet	0.032	690	2.21	Fatty change (21/21)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2		1 mg FB <sub>1</sub> /kg diet	0.003	690	0.35	HCC (2/21)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2		0.22 g FB <sub>1</sub> /kg	0.001	690	0.35	Ductular hyperplasia (21/21)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	Adenofibrosis (19/21)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	Cholangiocarcinoma (8/21)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	Cirrhosis (15/15)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	Regenerative nodules (15/15)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	Cholangiofibrosis (15/15)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	HCC (10/15)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	Anisokaryosis (13/17)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	Hyperplastic nodules (9/17)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	Oval cell proliferation (2/17)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	Bile duct hyperplasia (3/17)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	Portal fibrosis (5/17)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	Ground-glass foci (7/17)
Control	95.06 ± 9.2	423.8 ± 107.7	328.7 ± 105.2				690	0.35	GSTP foci (11/11)

FCM, *Fusarium* culture material. <sup>a</sup>Histologic changes recorded in the high-dose group (20).

used in the long-term experiments differs from that of the AIN 76 (23) developed for rats in several respects. These differences included low protein content and marginal to marked deficiencies in lipotropes (2- to 3-fold lower), vitamins (2- to 10-fold lower), and minerals (2- to 10-fold lower). The caloric contents of the two diets were similar. It is well recognized that diet plays a major role in the induction and spontaneous development of cancer in experimental animals (24). The semipurified diet used in the long-term studies, therefore, could have had an important influence on the outcome of liver and esophageal cancer development in the male BD IX rats—for example, the methionine content of the semipurified diet was marginally lower whereas the folate levels were four times lower. Low levels of the lipotropes, methionine, and choline are involved in cancer development of many organs including the liver (25). In addition to folic acid, these lipotropes play important metabolic roles in the utilization of methyl groups (24).

**Short-term studies.** Several short-term cancer models, using rat liver, exist to study the underlying mechanisms of cancer development by genotoxic carcinogens (26). Because FB<sub>1</sub> is a complete carcinogen in the liver, subsequent studies were directed to investigate the cancer-initiating and -promoting potential of this apparently nongenotoxic mycotoxin. The cancer-promoting activity of *F. verticillioides* was first demonstrated in male BD IX rats (13) and, as discussed above, used to purify the different structurally related fumonisin analogues. The basic concepts underlying the processes of initiation and promotion by fumonisins are discussed in detail elsewhere (21,27) and are based on the "resistant hepatocyte" model developed in the liver by Farber (28). In short, there are two basic sequences of which the first is the production or appearance of hepatocytes with a so-called "resistant" phenotype that makes them resist the growth-inhibitory or toxic effects of many carcinogens. Genotoxic carcinogens rapidly (within several minutes or hours) induce this new phenotype (29), whereas nongenotoxic carcinogens such as clofibrate (30), FB<sub>1</sub>, and a choline-deficient diet (25) induce a similar phenotype but over a period of several weeks. The induction of this phenotype is complex, and although a mutation-like event is generally considered an important step, this supposition is critically questioned (31). For the ultimate cancer to develop, the altered or initiated cell first must be stimulated to grow during promotion or selection. During this process, called differential inhibition, the initiated cell proliferates in an environment created by the promoter that inhibits the growth of the surrounding normal hepatocytes (32).

**Cancer initiation.** Initial studies on the cancer-initiating potential of the FB mycotoxins were performed in male Fischer rats fed a purified basal diet (16). Two different protocols were used. In the first, FB<sub>1</sub> was fed in the diet (1 g FB<sub>1</sub>/kg diet) for 26 days followed by partial hepatectomy. Selection occurred 2 weeks later by 2-acetylaminofluorene/carbon tetrachloride (AAF/CCL<sub>4</sub>), and the rats were sacrificed after an additional two weeks. The second protocol consisted of partial hepatectomy (PH) followed by single gavage dosages of FB<sub>1</sub> at various time points before or after PH. The latter regimen is the classic protocol for evaluating the cancer-initiating potential of genotoxic carcinogens (28). Histologic changes induced by feeding FB<sub>1</sub> in the diet for 26 days were similar to those described for BD IX rats and included the generation of early hepatocyte nodules and mild to moderate bile duct proliferation. After the promoting treatment, three to five hepatocyte nodules were visible macroscopically in the liver; the number of GGT positive foci was also significantly increased compared to the controls. In contrast to this finding, neither FB<sub>1</sub> nor FB<sub>2</sub> exhibited any cancer-initiating activity during the gavage treatment before or after PH (second protocol).

Subsequent studies in male Fischer rats fed the AIN 76 diet focused on dosage studies in relation to the initiating and promoting potential of the mycotoxin (17,33). Initiation depended on both the dosage and the duration of the treatment. A dose of 29.7 mg FB<sub>1</sub>/100 g bw over 7 days did not effect initiation, whereas the same dosage over 21 days did. Initiation by FB<sub>1</sub> also depended on the induction of a hepatotoxic effect together with compensatory or regenerative cell proliferation, a prerequisite for initiation (34). FB<sub>1</sub> also appears to be a mitoinhibitor of normal hepatocytes; a dietary treatment of 250 mg FB<sub>1</sub>/kg bw for 3 weeks (16) or a single gavage dose of 50 mg/kg bw (33) inhibits liver regeneration induced by PH. Thus, a balance seems to exist between the induction and inhibition of hepatocyte regeneration, and the effect on cancer initiation may depend on which of these two processes prevails at a specific time. For example, a total dosage of 29.7 mg FB<sub>1</sub>/100 g bw over 7 days is likely to create a strong inhibitory effect on cell proliferation and therefore will not support the process of cancer initiation. However, the same dosage administered over 21 days is likely to support regenerative cell proliferation as a result of FB<sub>1</sub>-induced hepatotoxicity, which then will support cancer initiation (17). The latter concept is not new; initiation by many genotoxic hepatocarcinogens is potentiated either by use of a toxic dosage that stimulates hepatocyte regeneration or by the introduction of PH during the initiating regimen. In combination

with PH, which synchronizes the entry of liver cells into the S-phase by approximately 18 hr, cancer initiation by a genotoxic carcinogen could be effected at very low doses when introduced at this stage (35). The same holds true for initiation by the fumonisins, except that the whole process occurs at a far slower rate, probably because the FB<sub>1</sub>-induced cell proliferation is counteracted by its mitoinhibitory effect, producing a much smaller yield of initiated hepatocytes. In addition, a recent study indicated that FB<sub>1</sub> induced apoptosis (36), which has been suggested to reduce the number of initiated cells in the liver (37).

Another aspect that could determine the kinetics of the initiating step is the nature of the event(s) leading up to the induction of the initiated hepatocytes as a result of FB<sub>1</sub> treatment. The nature of initiation is of particular interest with respect to FB<sub>1</sub> because the compound appears not to exhibit any mutagenic or genotoxic effects in different *in vivo* and *in vitro* tests (16,38). However, Knasmüller et al. (39) reported that FB<sub>1</sub> as well as the mycotoxins moniliformin and deoxynivalenol exhibited clastogenic effects (chromosomal aberrations) at concentrations from 1.4 to 144 μM in primary hepatocyte cultures. At these concentrations FB<sub>1</sub> reduced the mitotic index and the induction of micronuclei markedly to significantly. These data suggested that FB<sub>1</sub> might exhibit some genotoxic effects. It was postulated that lipid peroxidation could play a role in the chromosomal breakage caused by the accumulation of polyunsaturated fatty acids in primary rat hepatocytes after exposure to FB<sub>1</sub> (40). Because cytotoxic effects and lipid peroxidation occur only at high concentrations of FB<sub>1</sub> (> 75 μM), the induction of chromosomal aberrations at levels of 1.4 and 14 μM need to be investigated further to clarify whether FB<sub>1</sub> is directly or indirectly responsible for chromosomal strand breaks. The disruption of sphingolipid metabolism is effected maximally at these concentrations (41), with the accumulation of sphinganine known to affect cell growth and differentiation (42). However, for the purpose of the present review, the fumonisins will be regarded as not causing direct DNA damage (mutations).

As mentioned above, the role of lipid peroxidation during cancer initiation in rat liver must be considered (17) because relatively high cytotoxic dosages over long time periods are required. Recent investigations indicated that FB<sub>1</sub> induces lipid peroxidation in cell membrane preparations (43) and isolated rat liver nuclei (44) and in primary rat hepatocytes and rat liver *in vivo* (45). When egg yolk phosphatidylcholine (PC) bilayers were used (43), FB<sub>1</sub> increased the rate of oxidation, free radical production, and lipid peroxidation, thereby disrupting membrane

structure and permeability. These effects were noticed at high concentration levels—between 1 and 10 mM FB<sub>1</sub>—raising some doubts about inducing similar effects *in vivo*. Cawood et al. (46) showed that when radiolabeled FB<sub>1</sub> is used, the compound is tightly associated with plasma and microsomal membranes. However, a specific binding site for the fumonisins has not yet been characterized. Nuclear membrane lipid peroxidation with concomitant DNA strand breaks occurred in isolated rat liver nuclei treated with FB<sub>1</sub> *in vitro* at concentrations ranging from 40 to 300 μM (44). The formation of hydroxy and the subsequent formation of peroxyl radicals in the vicinity of nuclear material was proposed to cause DNA strand breaks. It was further postulated that metal ions, specifically iron, endogenously associated with cellular DNA could be important sites for metal-catalyzed oxidative DNA damage. *In vitro* studies in primary hepatocytes showed that lipid peroxidation is effected in a dose-dependent manner closely associated with cytotoxicity induced by FB<sub>1</sub> (45). Although α-tocopherol prevented lipid peroxidation by FB<sub>1</sub>, cytotoxicity was not completely abolished, indicating that lipid peroxidation is not solely responsible for the cytotoxic effects and could be secondary to cell cytotoxicity. Except for halogenated hydrocarbons such as carbon tetrachloride, oxidative stress and the resultant lipid peroxidation seem to occur as a result of cell injury induced by toxins (47). At low levels of exposure (75 μM), FB<sub>1</sub> also enhanced the susceptibility of hepatocytes to undergo lipid peroxidation induced by cumene hydroperoxide, probably via the accumulation of polyunsaturated fatty acids in primary hepatocytes (40). In a recent study in rats fed a dietary level of 250 mg FB<sub>1</sub>/kg diet, with or without dietary iron loading, FB<sub>1</sub> augmented iron-induced lipid peroxidation in the liver (48).

Observations from *in vivo* feeding studies support the results from *in vitro* studies that lipid peroxidation occurs in a dose-dependent manner associated with a hepatotoxic effect. Lipid peroxidation appeared to be a secondary effect rather than a causative mechanism of FB<sub>1</sub>-induced hepatic injury (45). Purification of membranal fractions indicated that FB<sub>1</sub> significantly ( $p < 0.05$ ) increased lipid peroxidation in plasma and microsomal membranes at a dietary level of 250 mg/kg, and it was enhanced (not significantly) in the mitochondrial and nuclear membranal fractions. Oxidative damage could well be an important initial event and in addition to the inhibitory effect on cell proliferation, could explain the slow kinetics of the initiating step. Apoptosis also must be considered because it becomes an important biologic phenomenon at high exposure levels of fumonisins (36,49) and

because it is known to remove the genetically altered initiated cells in the liver (50).

With respect to the cancer-initiating potency of FB<sub>1</sub> (45), cancer initiation of the choline-deficient diet occurred only after 9 weeks, preceded by lipid peroxidation and a hepatotoxic effect (25). The peroxisome proliferator clofibrate (30) also causes cancer initiation after prolonged feeding of several weeks. Because FB<sub>1</sub> is considered a weak or slow cancer initiator, the compound may have a strong effect on postinitiation events such as cancer promotion (17). Because the fumonisins occur naturally at relatively low dietary levels compared to the levels that initiate cancer in rats, future research should focus on the events related to later phases of cancer development, including promotion and progression.

**Cancer promotion.** As discussed above, the process of promotion represents an important phase during which the initiated cells are clonally expanded into hepatocyte nodules by a process known as differential inhibition (28,32). Although other mechanisms of the clonal expansion of the initiated hepatocyte have been proposed, considerable evidence exists to support a selection process whereby a few resistant hepatocytes proliferate in an environment where the proliferation of normal cells is inhibited (32). A compound is called a cancer promoter if it can create such an environment, and studies of fumonisins indicate that a similar hypothesis could be developed for the cancer-promoting property of these compounds. This growth selection of resistant hepatocytes has been recognized as a property of different cultures of *F. verticillioides* (12,13) and was successfully used to develop a bioassay for the purification of the fumonisins. Initial studies suggested that hepatotoxicity was associated with the cancer-promoting potential of this mycotoxin (13). A recent study showed that cancer promotion, unlike initiation, was effected at relatively low dietary levels (50 mg FB<sub>1</sub>/kg diet) in the absence of excessive hepatotoxicity (33). The cancer-promoting activity of FB<sub>1</sub> was also associated with an inhibitory effect on hepatocyte proliferation. This suggested an induction of a growth differential whereby the growth of the resistant initiated cells is promoted and that of normal cells inhibited. Inhibition of cell growth occurs in many cell culture systems (51) and in rat liver *in vivo* under different experimental conditions involving PH. A dietary level of 250 mg FB<sub>1</sub>/kg fed over 21 days significantly inhibited regenerative hepatocyte cell proliferation in hepatectomized male Fischer rats after 24 hr (17). Three days after PH the level of DNA synthesis was significantly higher in the FB<sub>1</sub>-treated group, whereas at 7 days there was no difference. FB<sub>1</sub> seems to delay hepatocyte regeneration in a

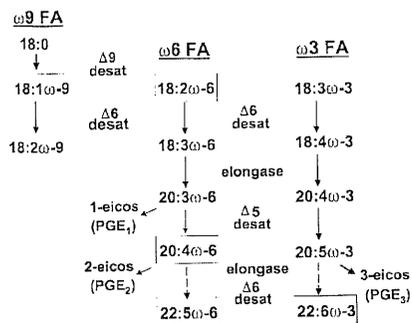
reversible manner. Even a single gavage dosage (50 mg/kg bw and higher) 6 hr after PH significantly inhibited DNA synthesis. Thus, the inhibitory effect on cell proliferation is likely to be an important determinant of cancer promotion in rat liver. Many cancer promoters such as 2-acetylaminofluorene, phenobarbital, orotic acid, and ciprofibrate inhibit the epidermal growth factor (EGF)-induced mitogenic response in primary hepatocytes (33). This inhibitory effect—also known as mitoinhibition of the EGF response by FB<sub>1</sub>—has been used to investigate possible mechanisms involved during cancer promotion (41). Binding of EGF to its receptor was not affected in hepatocytes exposed for 12 hr to FB<sub>1</sub>. The inhibitory effect was also reversible; maximum inhibition seemed to occur late during the G<sub>1</sub>-phase of the cell cycle. Pretreatment of hepatocytes with FB<sub>1</sub> only marginally inhibited the EGF response, indicating that there is very little memory after the mycotoxin is removed. *In vivo* and *in vitro* experiments suggest that FB<sub>1</sub> behaves in a manner similar to most cancer promoters in inducing a growth differential that selectively stimulates the outgrowth of initiated cells.

Although a mechanism for cancer initiation has been proposed for FB<sub>1</sub> (see above), the process whereby FB<sub>1</sub> creates a growth differential in the liver that selectively favors the growth of initiated cells still needs to be elucidated. The final part of this review focuses on a mechanism that is likely to create such a “promoting” environment to sustain the process of differential inhibition.

## Altered Lipid Biosynthesis as a Possible Mechanism for Cancer Promotion by the Fumonisin: A Hypothesis

### General Introduction

The major constituents of cellular membranes are the phospholipids, which contain fatty acids as important constituents of the typical bilayer structure (52). Essential fatty acids (EFA) are normally linked to the 2 position of the glycerol backbone of the phospholipids and sometimes also to the 1 position. Free cholesterol is closely associated with the fatty acids and hence is an important mechanism in determining membrane fluidity. EFA consists of the ω6 and ω3 derived from linoleic acid (C18:2ω6) and α-linolenic acid (C18:3ω3) respectively (Figure 1). A series of alternating desaturations (which add a double bond) and elongations (which add two carbon atoms) are involved in the synthesis of the different long-chained fatty acid metabolites. The desaturation and elongation are not confined to the metabolism of EFA; the saturated fatty acids palmitic and stearic can also be converted to long-chained fatty acids. Apart from the role



**Figure 1.** Essential fatty pathways indicating the key role of  $\Delta 6$  desaturase enzyme and the fatty acids involved in the synthesis of different prostaglandins. The marked fatty acids (boxes) are likely to play a key role in the development of hepatocyte nodules.

of EFA as structural components of all membranes, they are precursors of the eicosanoids, prostaglandins, leukotrienes, and other oxygenated derivatives.

#### *In Vitro* Studies in Primary Hepatocytes

Studies in primary hepatocytes indicate that the incorporation of  $^{14}\text{C}$  palmitic acid decreased in the total lipids and the neutral lipids, triacylglycerol (TG), and the cholesterol esters at a noncytotoxic concentration of 150  $\mu\text{M}$   $\text{FB}_1$  and at cytotoxic concentrations of 250  $\mu\text{M}$  and higher (40). In contrast, the incorporation of radiolabel into phospholipids increased with a concomitant increase in the concentration level of PC and phosphatidylethanolamine (PE), whereas the total cholesterol decreased. The concentration and labeling of sphingomyelin (SM) decreased, presumably as a result of the inhibitory effect on the ceramide synthase, a key enzyme in the sphingolipid biosynthetic pathway (53). Fatty acid changes were restricted mainly to PC and TG with a decrease in the relative levels of C16:0 and the C16:1 $\omega$ 7 fatty acids and C18:1 $\omega$ 9. Changes in polyunsaturated fatty acids (PUFA) were restricted mainly to an increase in C18:2 $\omega$ 6 in TG and PC; C20:4 $\omega$ 6 also increased in both PC and TG. These changes were observed in cytotoxic and noncytotoxic dosage levels. As a result, the PUFA increased in both PC and TG. The increases in PC, PE, and PUFA are likely to counteract the shift to a more rigid membrane structure caused by the decrease in total cholesterol. The increase of C18:2 $\omega$ 6 could be related to the impairment of the  $\Delta 6$  desaturase enzyme, the rate-limiting enzyme in fatty acid metabolism (Figure 1), and the increase in C20:4 $\omega$ 6 could be caused by the disruption of prostaglandin biosynthesis by inhibiting the cyclooxygenase enzyme. This was supported by the finding that the inhibition of monooxygenase by ibuprofen also inhibits the EGF mitogenic response (54). At

lower concentrations of  $\text{FB}_1$ , the relative levels of C20:4 $\omega$ 6 were unaffected, whereas C18:2 $\omega$ 6 still increased, indicating that, depending on the dosage, different effects could be induced in primary hepatocytes. This implies that the disruption of the  $\Delta 6$  desaturase could be an important early event that occurs in hepatocytes exposed to low concentrations of  $\text{FB}_1$ . The accumulation of C20:4 $\omega$ 6 was effected at dosages that significantly inhibit the EGF response (41). The inhibition of C20:4 $\omega$ 6 metabolism by  $\text{FB}_1$  was further strengthened by the following: First, the addition of C20:4 $\omega$ 6 and C20:5 $\omega$ 3 to primary hepatocytes in the presence of EGF respectively stimulates and inhibits the mitogenic response; second, the mitoinhibitory effect of  $\text{FB}_1$  was counteracted by the addition of prostaglandin  $\text{E}_2$  (54). Changes to the fatty acid profiles of hepatocytes membranes and specifically C20:4 $\omega$ 6 also effected the mitogenic response. This was shown by the addition of C20:5 $\omega$ 3, which inhibits the EGF response presumably by replacing C20:4 $\omega$ 6 from the membrane phospholipids. The resulting formation of the prostaglandin  $\text{E}_3$  series has fewer, even opposite, properties from those of the prostaglandin  $\text{E}_2$  series that are produced from C20:4 $\omega$ 6 (51). These results suggest that C20:4 $\omega$ 6 is central to the regulation of the EGF response in primary hepatocytes. This was recognized earlier, as the disruption of C20:4 $\omega$ 6 metabolism affects the mitogenic response of EGF and hepatocyte growth factor (HGF) in primary hepatocytes (55).

#### *In Vivo* Studies in Rats

*In vivo* studies indicate that  $\text{FB}_1$  disrupted lipid biosynthesis differently from that in the *in vitro* studies (56). In contrast to the *in vitro* studies, the major changes were associated with both the PE and the PC phospholipid fractions, and cholesterol increased in both the serum and liver. In the short-term studies in male Fischer rats, a dietary level of 250 mg  $\text{FB}_1/\text{kg}$  increased the PE level, whereas SM decreased. No effect was noticed in the rats that received the 50 and 100 mg  $\text{FB}_1/\text{kg}$  diets. However, in long-term studies using BD IX rats, dietary levels of 1, 10, and 25 mg/kg increased the PE levels in the liver. Fatty acid analyses of the PE and PC fractions of the liver indicated that, as was shown in the *in vitro* study, the relative level of C18:2 $\omega$ 6 increased in PE and markedly in the PC fraction of the rats from the short-term study. The level of C20:4 $\omega$ 6 was not altered, although there was a marked decrease in the PC fraction using the 250 mg/kg dietary level. C22:5 $\omega$ 6, the end product in the n-6 metabolic pathway, was reduced in PE in the liver of the rats that received the 100 and 250 mg/kg diets. A similar pattern was noticed in

the liver of rats of the long-term study in both the PC and PE fractions, but because of the small numbers of rats (3 per group) used for analyses, these changes were not significant. However, analysis of the total fatty acid profiles of the liver, using a larger number of rats per group (4 to 6 animals/group), indicated that the relative levels of C18:2 increased. Fatty acid analyses of serum PC of both the short-term and long-term  $\text{FB}_1$  feeding studies confirm the observations obtained in the liver: an increase in C18:2 $\omega$ 6 but a decrease in C20:4 $\omega$ 6 and C22:5 $\omega$ 6 in the short-term (50 mg  $\text{FB}_1/\text{kg}$  diet and higher) and long-term (10 mg  $\text{FB}_1/\text{kg}$  diet and above) experiments. With regard to the total fatty acid parameters, the monounsaturated fatty acids (MUFA) increased in PE, whereas the total n-6 and PUFA decreased in PC. In the long-term experiment, the MUFA increased in PC while the n-3 fatty acids increased in PC and PE, altering the n6/n3 ratio. The effect of  $\text{FB}_1$  on the n-6 fatty metabolic pathway seems to rely to a greater or lesser extent on the dietary level of  $\text{FB}_1$ , the length of exposure, the specific cellular phospholipid fraction, and differences between *in vitro* and *in vivo* experiments.

Subsequently, the effects of different dietary dosages (10, 50, 100, and 250 mg  $\text{FB}_1/\text{kg}$  diet) of  $\text{FB}_1$  fed for 21 days were evaluated on lipid metabolism in rat liver microsomal membranes (57). These dietary levels of  $\text{FB}_1$  were used to investigate the cancer-promoting potential of the fumonisins in DEN-initiated rats (29). The major changes associated with the microsomes were increased levels of PC, phosphatidyl inositol (PI), PE, and cholesterol. The levels of the saturated fatty acids and MUFA, especially C18:1 $\omega$ 9, increased significantly in the treated groups (100 mg  $\text{FB}_1/\text{kg}$  diet and higher) in all the phospholipids except phosphatidyl serine (PS). The relative (%) and absolute ( $\mu\text{g}$ ) values of C18:2 $\omega$ 6 increased in the PC, PI, PS, and PE phospholipid fractions. C20:4 $\omega$ 6 showed a decrease in the relative values, whereas the absolute values remained constant in PC, PI, and PS despite the fact that the concentration of the phospholipids increased in the high-dosage groups. In PE, however, the relative value was not altered. However, the absolute value of C20:4 $\omega$ 6 in PE increased, presumably because of the prominent increase (> 2 $\times$ ) in the level of PE compared to that of the other phospholipids. A similar effect was noted with C22:4 $\omega$ 6 and C22:5 $\omega$ 6 in PC and PE. The relative values of the n-3 fatty acid, C22:6 $\omega$ 3, also decreased in a dose-responsive manner in all the phospholipids; the absolute values remained the same except PE, where they significantly increased. The relative levels of total PUFA were not altered; the absolute levels increased (PC, PE, and PI) because of

increased phospholipid concentrations. The polyunsaturated/saturated (P/S) fatty acid ratio also decreased in the PC phospholipid fraction because of alterations in the PUFA and saturated fatty acid levels. Changes to membrane environment of FB<sub>1</sub> also expanded and included the plasma, mitochondrial, and nuclear membrane fractions of rat livers exposed to 250 mg FB<sub>1</sub>/kg diet for 21 days. Some differences exist in the lipid profiles of the different membrane fractions with respect to the effect of FB<sub>1</sub> on the levels of cholesterol and PC and PE. In the plasma membrane and nucleus, only PE significantly increased; PC, SM, and cholesterol were unchanged. The mitochondrial membrane structure was also altered differently from the plasma membrane. The level of PE increased, PC and SM decreased, and cholesterol remained unchanged. The fatty acid patterns were similar, with minor differences between PC and PE—e.g., PUFA decreased in PC (both the relative and absolute values), whereas in PE the relative value decreased and the absolute value increased. The absolute values of the saturated fatty acids and MUFA increased, causing a decrease in the P/S ratio of PC and PE and suggesting a less fluid mitochondrial membrane.

It can be argued that some of these changes can be related to the hepatotoxic effects induced by FB<sub>1</sub> in the liver. Apart from cancer promotion, the toxic effects are closely related to cancer initiation by the fumonisins, making it difficult to associate specific changes in lipid metabolism with cancer induction at this stage. However, a characteristic fatty acid pattern seems to emerge in the livers of rats exposed to dietary levels of FB<sub>1</sub> that effect both cancer initiation and promotion. These include the following: First, an increase in saturated fatty acids and MUFA (C18:1ω9) fractions was observed in both PC and PE. Second, the relative level of C18:2ω6 increased in PC, whereas the absolute value was enhanced in PC and PE. Third, the relative and absolute values of C20:4ω6 tend to decrease in PC and increase in PE. Fourth, the relative and absolute values of C22:4ω6 and C22:5ω6 decreased in PC, whereas only the relative value of C22:5ω6 decreased in PE. The n-3 fatty acid, C22:6ω3, also decreased in PC but tended to increase in PE. Fifth, both the relative and absolute total PUFA values decreased in PC but only the relative levels decreased in PE. And, sixth, the P/S ratio decreased in both PC and PE, suggesting a less fluid plasma membrane structure.

#### Mechanistic Implications with Respect to Cancer Promotion

Apart from the role of the PUFA in regulating many processes in the cells via their production

of different classes of prostaglandins, their role in determining the structure and function of cellular membranes also must be considered. A change in saturation could determine the responsiveness of cells to transformation or the expression of specific phenotypes supporting differential growth that produces clonal expansion of certain cell types associated with neoplastic development. With respect to cancer promotion, the disruption of growth-stimulatory responses in primary hepatocytes and regenerating liver could be important in establishing the growth differential.

*Altered lipid parameters associated with the growth of hepatocyte nodules.* Abel et al. (58) recently investigated the role of different lipid parameters in the development and/or progression of hepatocyte nodules at different time intervals (1, 3, 6, and 9 months). The concentration of the phospholipid PE increased, whereas the total cholesterol increased in the 1- and 9-month nodules. Despite the fact that PC increased in the 1-month nodules, the increased level of PE caused a decrease in the PC/PE ratio in hepatocyte nodules. Fatty acid analyses indicated that C18:1ω9 and C18:2ω6 increased in PE and PC, while C20:4ω6 decreased in PC but increased quantitatively in PE. The end products of the n-6 and n-3 pathways, C22:5ω6 and C22:6ω3, decreased both qualitatively and quantitatively in PC, causing a decrease in PUFA. The lipid profiles of the surrounding tissue reflect those of the control tissue. In regenerative liver (over 7 days after partial hepatectomy), used as a control for cell proliferation, the fatty acid profiles of PE and PC are very similar to those of hepatocyte nodules except that C18:1ω9 decreased in PC. Other differences were the increased membrane fluidity and the tendency of PC to decrease in regenerating liver. Apart from a few differences, the lipid parameters associated with increased cell proliferation in the hepatocyte nodules closely mimic those of normal regeneration in the liver. However, one major difference is that the lipid changes in the nodules are persistent whereas they are reversed in regenerating liver. In the hepatocyte nodules the altered lipid metabolic pattern, specifically the fatty acid profiles, could be important in regulating growth in these lesions. In this regard, the increased levels of PE and C20:4ω6 are of interest because the fatty acid regulates many processes related to cell growth, such as proliferation and apoptosis (58). With respect to cell proliferation in hepatocyte nodules, the role of C20:4ω6 and its cyclooxygenase prostaglandin E<sub>2</sub> series products in the activation of protein kinase C and mitogen activation protein kinases should be considered (59). Tang et al. (60) suggested that the metabolism of C20:4ω6 is involved in the evolution of preneoplastic foci

into nodules and hepatocellular carcinomas in rat liver. C20:4ω6 has also been linked to the action of transforming growth factor (TGF)-α and tumor necrosis factor (TNF)-α (61), which together with the deregulation of *c-myc* expression could be important determinants during FB<sub>1</sub>-induced apoptosis in the liver of rats.

The decrease in PUFA and the increase in C18:1ω9 in hepatocyte nodules have been suggested to play important roles in the lower levels of lipid peroxidation normally seen in cancerous lesions (62). Cancer cells have low levels of PUFA and the degree of depletion *in vitro* can be an accurate predictor of its malignancy *in vivo*.

*Disruption of growth control by FB<sub>1</sub>.* The effects of FB<sub>1</sub> on phospholipid and fatty acid metabolism closely mimic those seen in hepatocyte nodules, although there are some differences, as described for nodules and regenerating liver (see above). The decrease in fatty acid saturation, induced by FB<sub>1</sub>, implies a more rigid membrane structure such as found in hepatocyte nodules. The n-6 fatty acid pathway is markedly affected with an accumulation of C18:2ω6 and a decrease in C20:4ω6 as well as in the subsequent products C22:4ω6 and C22:5ω6. This specific altered fatty acid pattern (Figure 1) likely caused an impaired Δ6 desaturase enzyme. This hypothesis was further strengthened by the fact that another substrate for the enzyme, C18:1ω9, increased and an n-3 fatty acid product of the enzyme, C22:6ω3, decreased. However, the modulating effect of FB<sub>1</sub> on this rate-limiting enzyme in fatty acid metabolism still needs to be elucidated. The decrease in PUFA, in addition to the disruption of fatty acid metabolism, could also result from lipid peroxidation induced by FB<sub>1</sub> at high-dosage levels (45). In this regard the accumulation of C18:1ω9 is of interest because C18:1ω9 exhibits potent antioxidant activity (62) that, in the case of FB<sub>1</sub>-induced hepatotoxicity, could provide a specific survival mechanism to hepatocytes under conditions of stress.

The concentration of PE is markedly increased in the membrane fractions of the hepatocyte, increasing the absolute values of C20:4ω6 within the cell. The latter state— together with the increased level of C18:1ω9, which implies a lower oxidative status—is likely to favor cell proliferation, especially in the initiated hepatocyte cell population (60,62). This becomes evident with a similar fatty acid pattern found in hepatocyte nodules (58), presumably sustaining cell proliferation, whereas in the surrounding and normal liver it appears to inhibit growth, thereby creating an environment for the differential inhibition of growth. This is also true for FB<sub>1</sub>, which effects this altered lipid profile in the liver and can inhibit cell proliferation in

regenerating liver (16,17,33). However, in such an environment the initiated hepatocytes would proliferate into hepatocyte nodules, and some of these might develop into tumors after continued exposure to FB<sub>1</sub>. Very little is known about the nodules induced by FB<sub>1</sub> in the liver, but because hepatocyte nodules appear to be very similar with respect to the resistant phenotype regardless of the initiator or promoter used (26), the differential created by FB<sub>1</sub> is likely to promote their growth. Changes to membrane structure and fluidity appear to have important implications with respect to membranal processes related to normal growth and differentiation. In early persistent and late preneoplastic nodules, the binding of EGF, lipoproteins, and desialylated glycoproteins is markedly reduced (63). A decreased ligand binding might play a role in the altered responses to external growth inhibitory and stimulatory factors that regulate cell proliferation and other physiologic factors in the liver.

**Modulation of growth regulatory molecules in the liver.** The molecular mechanisms underlying FB<sub>1</sub>-induced hepatotoxicity and carcinogenesis have not been examined in depth. A recent study employing Northern blot (mRNA) analysis showed increased hepatic expression of HGF, TGF- $\alpha$ , and especially TGF- $\beta$ 1 and *c-myc* during short-term feeding of FB<sub>1</sub> (36). Immunostaining with LC(1-30) antibody for mature TGF- $\beta$ 1 showed that zone 1 and 2 hepatocytes were responsible for the increased expression of TGF- $\beta$ 1. Overexpression of TGF- $\beta$ 1 may be responsible for the prominent proapoptotic effects of FB<sub>1</sub> in the liver. The proto-oncogene *c-myc* is a positive regulator of cell proliferation that is involved in tumor progression (64,65) and has also been implicated in TGF- $\beta$ 1 signaling (66). Increased expression of *c-myc* oncogene and TGF- $\beta$ 1 may cooperate in the promotion of liver tumors during feeding FB<sub>1</sub>, possibly by providing an environment that selects for the growth of TGF- $\beta$ 1-resistant transformed liver cells. Oncogenesis due to overexpression of both *c-myc* and TGF- $\alpha$  appears to involve disruption of the Rb/E2F pathway and deregulation of cell cycle control. Both *c-myc* and TGF- $\alpha$  contribute to induction of cyclin D1 expression and resultant inactivation of the retinoblastoma tumor suppressor protein, and *c-myc* may directly induce E2F (67). With respect to apoptosis, overexpression of *c-myc* together with the depletion of growth factors and/or disruption of growth signaling pathways could cause imbalances of cell cycle progression and hence induction of apoptosis (68). In this regard, FB<sub>1</sub> overexpressed *c-myc* in rat liver (36), whereas it disrupted growth-related responses in different cell types such as primary hepatocytes (41) and in the liver *in vivo* (33).

Recent evidence shows that FB<sub>1</sub> stabilizes cyclin D1, causing accumulation of the protein in the nucleus of altered hepatocytes in foci, nodules, adenomas, and carcinomas (69) in the livers of rats (male BD IX) fed FB<sub>1</sub> over a period of two years (19). In male Fischer rats fed FB<sub>1</sub> over a period of 21 days (17), cyclin D1 protein levels in liver also increased up to 5-fold in a dose-responsive manner, with no simultaneous increase in mRNA. The increase in FB<sub>1</sub>-treated samples of cyclin-dependent kinase (Cdk)4 complexes with cyclin D1 and consequently elevated Cdk4 activity were confirmed by an increased phosphorylation of the retinoblastoma protein. Levels of cyclin E and Cdk2 did not differ between controls and FB<sub>1</sub>-treated livers (short term) except for one sample in which a decrease in both proteins was detected. Alterations in cyclin D1 were specific to the livers, and all other tissues were negative for cyclin D1 overexpression except the kidney. Kidney showed some positive nuclear staining in the proximal tubules in both untreated and treated rats. This finding must be interpreted with caution in view of the tendency of proximal tubules often to stain nonspecifically in immunohistochemistry. Because chronic interstitial nephritis was present in the kidneys and FB<sub>1</sub> can have toxic effects in rat kidneys (70), this may also reflect a role of cyclin D1 in this pathology. To test whether the overexpression of cyclin D1 was a common property of rat HHC, liver sections from paraffin-embedded rat HCC caused by nitroglycerin or diethylnitrosamine/phenobarbital (DEN/PB) (71,72) were compared to those induced by FB<sub>1</sub>. The cyclin D1 overexpression, characteristic of FB<sub>1</sub>-induced preneoplastic lesions and HCC, was not changed in HCC caused by DEN/PB or nitroglycerin. However, HCCs induced by nitroglycerin or DEN/PB showed proliferating cell nuclear antigen staining rates similar to those in the FB<sub>1</sub>-induced tumors. These findings suggest that altered cyclin D1 and Cdk4, as major cell cycle oncogenes, may be role players in the carcinogenic effects of FB<sub>1</sub>. Presently, we are in the process of determining which signaling molecules, known to participate in the regulation of cyclin D1 stability/degradation, could be affected by FB<sub>1</sub>. The modulating effects of FB<sub>1</sub> on both sphingolipids and phospholipids could play a major role in the molecular events involving cyclin D1 protein stability (69).

## Conclusions

The toxicity induced by FB<sub>1</sub> in the liver appears to play an important role during the cancer initiation, and the induction of oxidative damage and lipid peroxidation could be important initial events. Selective inhibition seems to be the likely mechanism during

cancer promotion. Changes in the balance of the different cell regulatory molecules discussed above are likely to be involved in the induction of a growth differential that selectively stimulates the growth of initiated cells. This was shown with the peroxisome proliferator Wy-14,643, which decreased the level of HGF in the liver (73). *In vitro* studies (74) indicate that HGF stimulates the growth of normal hepatocytes while inhibiting the growth of preneoplastic or neoplastic cells. The reduction of HGF therefore could play an important role in the promotion of preneoplastic cell growth. Apoptosis is also very important during the growth of hepatocyte nodules because cancer promoters such as phenobarbital are known to decrease the rate of apoptosis in these lesions (75). FB<sub>1</sub> can induce apoptosis in the liver (36,49) and the disruption of sphingolipid metabolism has been implicated because it disrupts sphingolipid metabolism and therefore ceramide synthesis (53), an important signaling molecule for apoptosis. However, fatty acids, especially C20:4 $\omega$ 6, have been found to be important second messengers during TNF- $\alpha$ -induced apoptosis by the release of ceramide via the stimulation of sphingomyelinase (61). In rat hepatoma cells, C20:4 metabolites were also shown to be involved in apoptotic cell death elicited by TGF- $\beta$ 1 (76). In a recent study in an esophageal cancer cell line, C20:4 $\omega$ 6 and its cyclooxygenase products prostaglandin E<sub>2</sub> and prostaglandin A<sub>2</sub> induced apoptosis, a process that was inhibited by FB<sub>1</sub> (77). The effect of FB<sub>1</sub> can be explained either by the reduction of ceramide or the regulation of C20:4 $\omega$ 6 levels, as discussed above. In contrast, a recent study indicated that FB<sub>1</sub> induced apoptosis in esophageal epithelial cells and neonatal human keratinocytes (51). It would appear that, depending on the cell type, the extent to which different pathways are interrupted could determine whether the cell would undergo apoptosis (78). A unique pathway has been proposed whereby the glycerophospholipids and the sphingolipid cycle interact to control a variety of cellular processes including apoptosis, with C20:4 $\omega$ 6 and ceramide as the key role players (61). A similar interactive pathway is likely to exist for the fumonisins in the liver to regulate processes related to cell proliferation and apoptosis (Figure 2). FB<sub>1</sub> effects a similar phospholipid and hence fatty acid pattern in the liver, as was noted in hepatocyte nodules. However, subsequent effects on sphingolipid and/or prostaglandin production seems to inhibit the growth of normal hepatocytes, which together with the overexpression of TGF- $\beta$ 1 and *c-myc* could effect apoptosis. Oxidative damage and the resultant lipid peroxidative products could also further enhance apoptosis



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## Cancer initiation by fumonisin B<sub>1</sub> in rat liver – role of cell proliferation

W.C.A. Gelderblom<sup>a,\*</sup>, D. Galendo<sup>b</sup>, S. Abel<sup>a</sup>, S. Swanevelder<sup>c</sup>,  
W.F.O. Marasas<sup>a</sup>, C.P. Wild<sup>d</sup>

<sup>a</sup>Programme on Mycotoxins and Experimental Carcinogenesis, P.O. Box 19070, Tygerberg 7505, South Africa

<sup>b</sup>Unit of Gene-Environment Interactions, International Agency for Research on Cancer, 150 Cours Albert-Thomas, 69372 Lyon, France

<sup>c</sup>Biostatistic Unit, Medical Research Council, PO Box 19070, Tygerberg 7505 South Africa

<sup>d</sup>Molecular Epidemiology Unit, Epidemiology and Health Services Research, Algernon Firth Building, School of Medicine, University of Leeds, Leeds, LS2 9JT, UK

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### Abstract

Fumonisin B<sub>1</sub> (FB<sub>1</sub>), a carcinogenic mycotoxin produced by the fungus *Fusarium verticillioides* in corn, causes cancer initiation in rat liver in a similar manner to genotoxic carcinogens although apparently with different kinetics. The present experiment was designed to evaluate the role of regenerative cell proliferation, effected by partial hepatectomy (PH) and carbontetrachloride (CCl<sub>4</sub>) and direct mitogen-induced hyperplasia, induced by lead nitrate (PbNO<sub>3</sub>), on FB<sub>1</sub>-induced cancer initiation. Initiation was effected over a period of 14 days by gavage administration of FB<sub>1</sub> at different daily doses ranging from 0.14 to 3.5 mg FB<sub>1</sub>/100 g body weight while the stimuli for cell proliferation were introduced 7 days after the start of the FB<sub>1</sub> treatment. Based on the proliferative stimulus used, cancer promotion was effected 3 weeks after completion of the initiating treatment by 2-acetylaminofluorene (2-AAF) treatment followed by PH or carbon tetrachloride CCl<sub>4</sub> on day 4. Cancer initiation by FB<sub>1</sub> was associated with a hepatotoxic effect and an increase in lipid peroxidation. In contrast to compensatory liver cell proliferation induced by PH and CCl<sub>4</sub>, mitogen-induced hyperplasia (PbNO<sub>3</sub>) failed to enhance the cancer initiating potential of FB<sub>1</sub> suggesting that cancer induction by a non-genotoxic carcinogen is supported by regenerative cell proliferation. Cognizance of the enhancing role of cell proliferation during cancer initiation by FB<sub>1</sub> is required in assessing the risks posed by this mycotoxin to humans. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Fumonisin B<sub>1</sub>; Cancer initiation; Cell proliferation; Risk assessment

### 1. Introduction

Hepatocyte proliferation is known to be an important parameter in the development of liver cancer induced by genotoxic carcinogens in the rat [1,2].

During cancer initiation, regenerative hepatocyte proliferation is presumably required to ‘fix’ the mutational event. Proliferation is also a major driving force in the clonal expansion of initiated cells to form hepatocyte nodules during cancer promotion. With genotoxins, the efficacy of initiation is enhanced either by regenerative cell proliferation as induced by partial hepatectomy (PH) or a necrogenic dosage of carbon tetrachloride (CCl<sub>4</sub>). This is in sharp contrast with the

\* Corresponding author. Tel.: +27-21-938-0286; fax: +27-21-938-0260.

E-mail address: wentzel.gelderblom@mrc.ac.za (W.C.A. Gelderblom).

inefficacy of cell proliferation induced by mitogens such as lead nitrate ( $\text{PbNO}_3$ ) [3,4]. At present it is not known whether a similar distinct effect on cancer initiation will be obtained with non-genotoxic chemical carcinogens.

Fumonisin B<sub>1</sub> (FB<sub>1</sub>), a mycotoxin produced by *Fusarium verticillioides* (= *F. moniliforme*), was characterized as an active liver cancer-promoter isolated from corn cultures of the fungus [5]. Subsequent studies on the dosimetry of FB<sub>1</sub>-induced cancer initiation indicated that prolonged exposure at relatively high and toxic dietary dosages is required [6]. It would appear that the absolute level of events (DNA mutations, etc.), resulting in initiation, occur at a far slower rate when compared to genotoxic carcinogens. One possible contribution to the slow kinetics could be the inhibitory effect of FB<sub>1</sub> on cell proliferation [7] resulting in an inhibition and/or delay in the cancer initiation process [1]. In this regard, pretreatment of rats with FB<sub>1</sub> decreased the efficacy of cancer initiation by diethylnitrosamine (DEN) in Sprague–Dawley rats [8]. Recently, FB<sub>1</sub> was shown to induce apoptosis in the liver [9], a protective mechanism whereby genetically damaged cells are removed, decreasing the population of initiated cells in the liver [10].

FB<sub>1</sub> is generally regarded as a non-genotoxin, in that it lacks activity in mutagenicity [11] and genotoxicity assays [6,12] and appears not to bind directly to DNA. Studies concerning the cancer initiating activity of FB<sub>1</sub> indicated that a cytotoxic/proliferative threshold exists for cancer initiation in rat liver and levels that fail to induce a toxic effect, lack cancer initiating activity [6]. This was further supported in a long-term study indicating that low dietary levels that cause only mild toxic changes fail to induce hepatocellular cancer in rats [13]. A recent study by Mehta et al. [14] in Sprague–Dawley rats also suggested that compensatory cell proliferation in response to cellular toxicity is a prerequisite for initiation. This is in agreement with the hypothesis set forward by Cohen and Ellwein [15] that, for non-genotoxic carcinogens, a cytotoxic/proliferative threshold is likely to exist for cancer induction. A recent study by Abel et al. [16] proposed that induction of oxidative stress and the resultant lipid peroxidation as secondary events of the FB<sub>1</sub>-induced hepatotoxic effects, could possibly explain the delayed cancer initiating activity of FB<sub>1</sub> as compared to genotoxic carcinogens. A parallel was

drawn with the cancer initiating potential of a choline deficient diet where hepatocyte cell death and lipid peroxidation also precede the initiation event [17]. It would appear that the hepatotoxicity, as proposed previously [13], could therefore be regarded as an initial event in FB<sub>1</sub>-induced hepatocarcinogenesis.

In view of the critical role of regenerative cell proliferation during cancer initiation with genotoxic carcinogens in rat liver [18], the present study investigated the role of different modulators of cell proliferation on the cancer initiating potential of FB<sub>1</sub>.

## 2. Materials and methods

### 2.1. Animals

Male Fischer rats, were obtained from IFFA CREDO, Domaine des ONCINS BP 0109, 69592 L'ARBRESLE Cedex, France. They were approximately 130–140 g in body weight, were caged in pairs, maintained on laboratory chow (Biscuit EXTRALABO, Etablissement. B.P. 59 77482 PROVINS Cedex, France) and cycles of alternating 12-h periods of light and darkness.

### 2.2. Chemicals and solutions

2-acetylaminofluorene (2-AAF), bromodeoxyuridine (BrdU), 2-thiobarbituric acid and  $\text{PbNO}_3$  were obtained from Sigma Chemical Co. (Lyon, France). FB<sub>1</sub> was purified according to the method of Cawood et al. [19]. All solvents used were of analytical grade. The 2-AAF was prepared fresh by dissolving 300 mg in DMSO (1 ml) and sunflower oil (29 ml) to obtain a solution of 10 mg/ml. FB<sub>1</sub> was first dissolved in a small volume of 0.1 M NaOH and then made up to the desired volume with distilled water (pH 5.5). Antibodies (rabbit anti rat) against the placental glutathione S-transferase (GSTP) were obtained from DAKO.

### 2.3. Treatments

#### 2.3.1. Initiation by FB<sub>1</sub>

Cancer initiation was effected by a repeated gavage treatment according to the method described by Gelderblom et al. [6]. Rats were treated on a daily basis with different doses of FB<sub>1</sub>, yielding a total

dose of 2, 6, 20, 30 and 50 mg/100 g body weight (bw) over a period of 14 days (Fig. 1). These total dosages represent a daily dosage of 0.14; 0.42; 1.43; 2.1 and 3.5 mg FB<sub>1</sub>/100 g bw, respectively. The body weight was recorded on a daily basis while the relative liver

weight of a subgroup of animals (three per group), killed after the 14 day treatment, were determined and preserved for thiobarbituric acid reactive substances (TBARS) determination (see below). Promotion was effected 3 weeks later and consisted

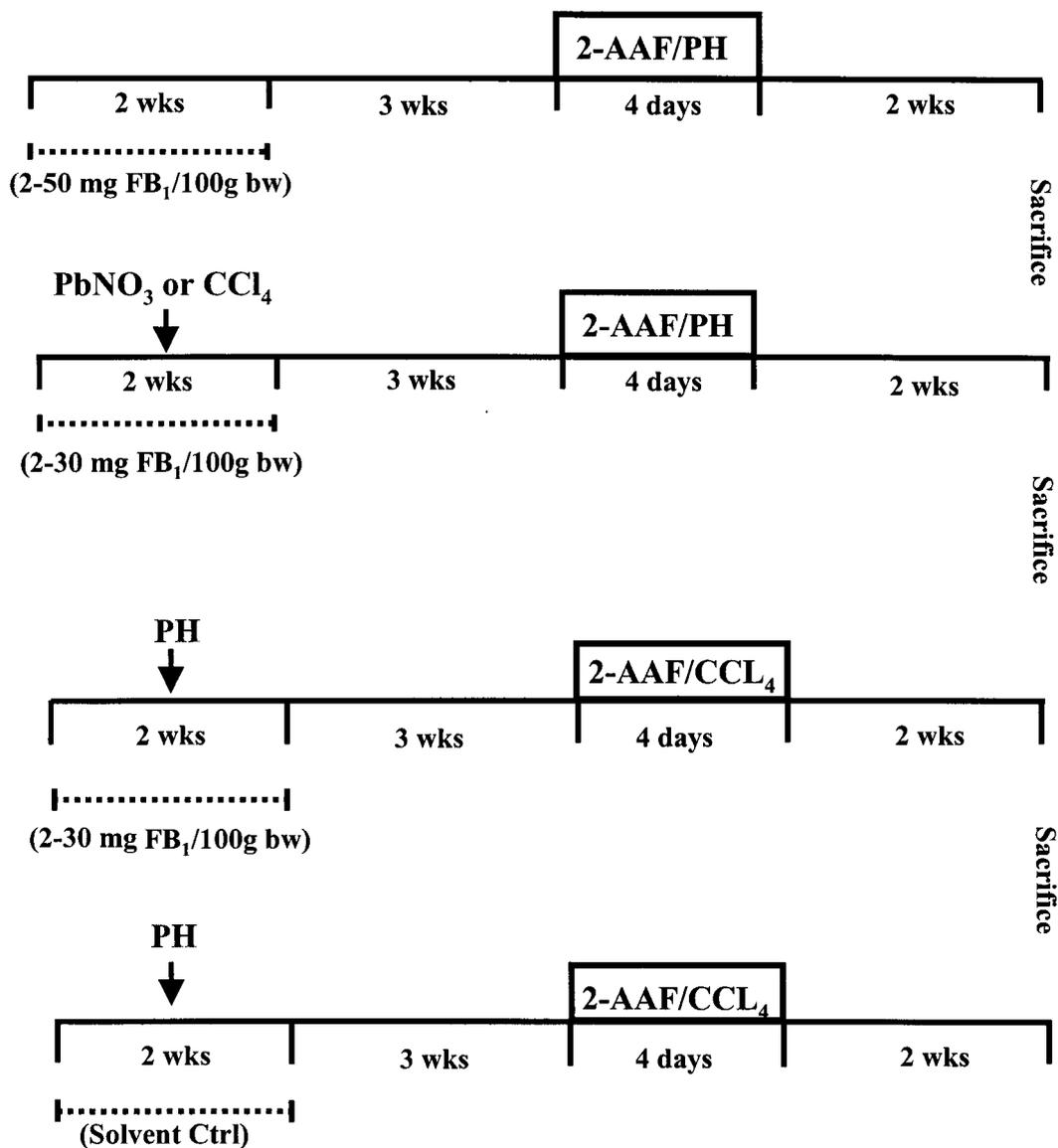


Fig. 1. Experimental protocols for studying the role of different stimuli of cell proliferation on the cancer initiating potential of FB<sub>1</sub>. Rats were treated with different doses of FB<sub>1</sub> by gavage over a period of 14 days followed by the promoting stimuli after 3 weeks consisting of 2-AAF (3 × 20 mg/kg body weight) on 3 consecutive days and either PH or CCl<sub>4</sub> on day 4. Rats were killed after a further 2 weeks. Stimuli for cell proliferation were introduced 7 days after commencement of the FB<sub>1</sub> treatment. Control groups without the FB<sub>1</sub>-initiating treatment were also included.

of three gavage dosages of 2-AAF (20 mg/kg) on 3 consecutive days followed by PH on day 4. The latter was performed under ether anesthesia according to the original method described by Higgins and Anderson [20]. The drinking water of the rats was supplemented with 5% glucose for 24 h postoperatively. Rats were sacrificed 2 weeks after the promoting treatment and liver tissue sections preserved in buffered formalin for GSTP and BrdU staining where applicable. Eight to ten animals were used in each of the treatment groups.

Different cell proliferative stimuli were introduced halfway during the 14-day FB<sub>1</sub> gavage treatment to monitor the role of cell proliferation on the cancer initiating potency.

**2.3.1.1. Effect of PbNO<sub>3</sub> and CCl<sub>4</sub>.** Cancer initiation was effected by gavage treatment of the rats (eight animals per group) with FB<sub>1</sub> over a period of 14 days as described above (Fig. 1). The highest dosage (50 mg FB<sub>1</sub>/100 g bw) was not included due to its marked effect on body weight gain. After 7 days of the FB<sub>1</sub> treatment, rats of the FB<sub>1</sub>-treated groups as well as a control received a single dose of either CCl<sub>4</sub> (0.2 ml/kg bw by gavage) or PbNO<sub>3</sub> (100 µmol/kg: i.v. – femoral vein). Promotion was effected by a 2-AAF/PH treatment, 3 weeks after the initiation treatment and the experiment was terminated as described above.

**2.3.1.2. The effect of PH.** Initiation was effected by gavage treatment of the rats over a period of 14 days to obtain the desired total FB<sub>1</sub> dose indicated in Fig. 1. Once again the highest dose of FB<sub>1</sub> was not included due to its marked affect on the body weight gain (50 mg FB<sub>1</sub>/100 g bw). After 7 days of the FB<sub>1</sub> treatment the rats of each treatment group were subjected to PH. The drinking water of all the rats was supplemented with 5% glucose for 24 h postoperatively. Promotion commenced 3 weeks after the initiation treatment was terminated. It consisted of a gavage treatment of the rats with 2-AAF (20 mg/kg on 3 consecutive days) followed by CCl<sub>4</sub> treatment (2 ml/kg bw). The CCl<sub>4</sub> was dissolved in sunflower oil (1:1) and the animals treated with 0.4 ml/100 g bw. A control group without the PH treatment was also included to monitor the selection potential of the 2-AAF/CCl<sub>4</sub> promotion regimen on FB<sub>1</sub>-induced initiated cells.

## 2.4. Stimuli for hepatocyte cell proliferation

### 2.4.1. Effect of PH, PbNO<sub>3</sub> and CCl<sub>4</sub>

Rats were subjected to PH and killed 20 h later having been treated 1 h prior to sacrifice with BrdU (100 mg/kg; i.p.) dissolved in DMSO:saline (1:3). Male rats of the same age without PH served as controls. The effect of PbNO<sub>3</sub> was examined by injecting 100 µmol/kg (i.v. into the femoral vein) dissolved in sterile distilled water. The rats were sacrificed after 30 h again following treatment 1 h prior to sacrifice with BrdU (100 mg/kg; i.p.). Control rats (three per group) were treated in a similar manner using distilled water. The CCl<sub>4</sub> was dissolved in sunflower oil (1:1) and animals treated with 0.1 and 0.2 ml/100 g bw per rat (five rats per group). The control rats were treated with sunflower oil (0.1 ml/100 g bw). Rats were sacrificed 48 h after the CCl<sub>4</sub> treatment following the BrdU treatment as described above. The different times selected for the BrdU treatment following the different cell proliferative stimuli coincide with the peak of DNA synthesis (3). Liver sections, 2–3 µm in thickness, were preserved in buffered formalin for BrdU antibody staining.

### 2.5. TBARS

To monitor the effect of FB<sub>1</sub> on the level of oxidative damage, the liver of the rats were collected following the 14 day initiating treatment (three animals per group), frozen in liquid nitrogen and stored at –80°C. TBARS were determined in the liver homogenates according to the method described by Esterbauer and Cheeseman [21]. In short, a liver homogenate (10%) was prepared in 1.15% KCl in 3 mM EDTA solution containing 0.01% BHT as an antioxidant. After a further 13× dilution with the above solution, a subsample (1 ml) was mixed with two volumes of ice cold 10% trichloroacetic acid (TCA) to precipitate the proteins. After centrifugation (3000 rev./min for 10 min), the supernatant (2 ml) was mixed with an equal volume of the TBA reagent (0.67% in distilled water) and incubated at 100°C for 10 min. The mixture was allowed to reach room temperature, the absorbance was measured at 532 nm and the lipid peroxidation expressed as nmole malondialdehyde (MDA) equivalents/mg protein using the molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$

for MDA. As the assay is not specific for MDA the term TBARS is used to describe the reaction product

### 2.6. Autopsies and histochemical analyses

Rats were sacrificed under ether anesthesia and the livers were observed macroscopically for any abnormalities. Tissue sections of all the major liver lobes were preserved in buffered formalin for H&E and GSTP analyses, respectively. GSTP staining was performed according to the method of Ogawa et al. [22] using the avidin-biotin-peroxidase complex (ABC) and affinity-purified biotin-labeled goat anti-rabbit IgG serum (Vector Laboratories, Burlingame, CA). Paraffin wax sections (5  $\mu\text{m}$ ) of the formalin preserved material were washed with petroleum benzene and a graded alcohol series before staining with the reagents in the ABC kit. GSTP-antiserum was used at a dilution of 1:800. Sections were counterstained with Carazzi's haematoxylin to provide blue stained nuclei with the GSTP<sup>+</sup> cells showing a reddish-brown pigmentation. Negative controls, omitting the primary antibody, were included to test for the specificity of anti-GSTP antibody binding. The number and size of GSTP<sup>+</sup> foci were monitored by light microscopy (10–20 $\times$  magnification). The GSTP<sup>+</sup> foci were further categorized into lesions <10 cells (mini foci) and >10 cells per focus and expressed as number per cm<sup>2</sup>. BrdU was detected by the ABC method using a monoclonal anti-BrdU antibody (Sigma Chemical Company) and the labeling indices scored by counting at least 1000 cells randomly per liver section ( $\times 40$ ).

### 2.7. Statistical analyses

The Wilcoxon Signed Rank Test, a non-parametric paired test, was used to test for significant changes between time periods, within each group. When two independent groups were tested for significant differences, the *t*-test was used for parametric data, and the Wilcoxon Rank Sum Test for non-parametric data. The ANOVA and Tukey *t*-test were used to identify significant differences between the means of more than two groups.

## 3. Results

### 3.1. Effect on body weight gain

FB<sub>1</sub> significantly reduced the body weight gains at doses of 20 mg/100 g bw and higher over a period of 14 days (Fig. 2A). The relative liver weight was also significantly decreased in the 30 and 50 mg FB<sub>1</sub>/100 g treated groups.

When compared to the body weight gain of control rats over a 14 day period, treatment with the different cell proliferative stimuli, PH and PbNO<sub>3</sub> administered at day 7 resulted in a significant reduction ( $P < 0.05$ ) in body weight gain (control group in Fig. 2A vs control groups in Fig. 2B). No significant effect on the body weight gain was noticed with the CCl<sub>4</sub> gavage treatment. When treated with the different

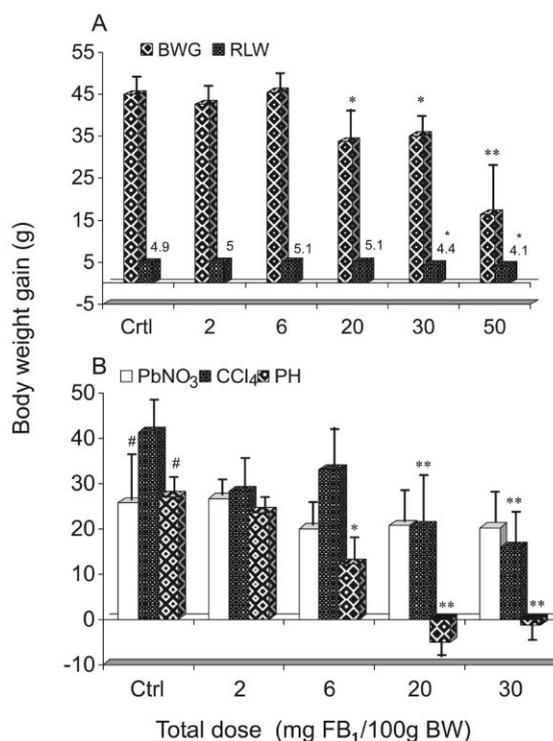


Fig. 2. Effect of FB<sub>1</sub> treatment on the body weight gains (BWG) with different FB<sub>1</sub> doses over a period of 14 days in the absence (A) and presence (B) of the cell proliferative stimuli. Body weight values are means  $\pm$  SD of 8–10 animals per group while the relative liver weights (A) are the mean of three animals per group. \* $P < 0.05$ , \*\* $P < 0.01$ . In B, # symbolizes a significant ( $P < 0.05$ ) reduction in the BWG when compared to the control group (A).

Table 1

The effect of different stimuli for cell proliferation on the cancer initiating effect of FB<sub>1</sub> in rat liver<sup>a</sup>

Treatment	GSTP <sup>+</sup> lesions (no/cm <sup>2</sup> )					
	<10 cells/focus (mini foci)			>10 cells/focus (foci)		
	FB <sub>1</sub> (Ctrl)*	PbNO <sub>3</sub>	CCl <sub>4</sub>	FB <sub>1</sub> (Ctrl)*	PbNO <sub>3</sub>	CCl <sub>4</sub>
Solvent	0.02 ± 0.03a	0	0	0.02 ± 0.03a	0	0.01 ± 0.02a
2	0.02 ± 0.04a	0	0	0.05 ± 0.1a	0.02 ± 0.01a	0.04 ± 0.04a
6	0.01 ± 0.02a	0.03 ± 0.06a	0.04 ± 0.1a	0.03 ± 0.07a	0.03 ± 0.01a	0.05 ± 0.05a
20	0.01 ± 0.03a	0	0.04 ± 0.1a	0.02 ± 0.06a	0.04 ± 0.01a	0.09 ± 0.09(a)
30	0.03 ± 0.04a	0.03 ± 0.04a	0.08 ± 0.1a	0.05 ± 0.06a	0.02 ± 0.01a	0.11 ± 0.06b
50	0.21 ± 0.33a	nd	nd	0.55 ± 0.23A	nd	nd

<sup>a</sup> Values are means of 5–8 animals per treatment group. Means followed by the same letter (column) do not differ statistically. When letter differs then  $P < 0.05$ , and when cases differ then  $P < 0.01$ . \*Normal cancer initiating protocol was used followed by the AAF/PH selection regimen. nd, not determined; Ctrl, control.

doses of FB<sub>1</sub> in combination with the cell proliferative stimuli, there were further significant ( $P < 0.01$ ) reductions in the body weight gains at doses 20 and 30 mg FB<sub>1</sub>/100g with CCl<sub>4</sub> and at 6, 20 and 30 mg FB<sub>1</sub>/100g with PH (Fig. 2B) compared to the proliferative stimuli alone (control, Fig. 2B). However, with PbNO<sub>3</sub>, FB<sub>1</sub> doses up to 30 mg/100 g bw did not lead to any significant reduction in body weight gain compared to the PbNO<sub>3</sub> alone.

### 3.2. Induction of GSTP positive lesions

The results indicated that treatment with FB<sub>1</sub> significantly induced the formation of GSTP + mini foci (<10 cells/focus) and foci (>10 cells/focus) at the

highest dose of 50 mg FB<sub>1</sub>/100 g bw, which is equivalent to a daily dosage of 3.5 mg FB<sub>1</sub>/100 g bw (Table 1). Treatment of the rats with PbNO<sub>3</sub> during the initiating treatment period did not alter the cancer initiating potential of FB<sub>1</sub> up to a dosage of 30 mg per 100 g bw. However, the formation of GSTP<sup>+</sup> foci (>10 cells/focus) was markedly (not significantly) and significantly ( $P < 0.05$ ) increased at the doses of 20 and 30 mg FB<sub>1</sub>/100g bw, respectively, when using CCl<sub>4</sub> (Table 1). With PH as the proliferative stimulus and AAF/CCl<sub>4</sub> as the promoting regimen (Table 2), a significant increase in the number of GSTP<sup>+</sup> foci (>10 cells/focus) was observed only at the 30 mg FB<sub>1</sub>/100g bw dose. Analysis of the total number of GSTP + lesions (minifoci and foci), illustrated in

Table 2

The modulating role of PH on the cancer initiating properties of FB<sub>1</sub> in rat liver<sup>a</sup>

Treatment	GSTP <sup>+</sup> lesions (no/cm <sup>2</sup> )			
	<10 cells/focus (mini foci)		>10 cells/focus (foci)	
	FB <sub>1</sub> (Ctrl)*	PH	FB <sub>1</sub> (Ctrl)*	PH
Solvent	0.03 ± 0.04a	0.03 ± 0.02a	0.01 ± 0.02a	0.02 ± 0.02a
2	0.01 ± 0.02a	0.01 ± 0.02a	0	0.01 ± 0.02a
6	0	0.01 ± 0.02a	0.01 ± 0.02a	0.02 ± 0.02a
20	0	0.03 ± 0.04a	0.01 ± 0.02a	0.02 ± 0.03a
30	0	0.04 ± 0.04a	0.01 ± 0.01a	0.10 ± 0.06A

<sup>a</sup> Values are means of 5–8 animals per treatment group. Means followed by the same letter (column) do not differ statistically. When letter differs then  $P < 0.05$ , and when cases differ then  $P < 0.01$ . \*Normal initiating protocol was used followed by the AAF/CCl<sub>4</sub> promoting regimen; Ctrl, control.

Fig. 3, showed that both the  $\text{CCl}_4$  and PH, stimuli for regenerative cell proliferation, enhanced the induction of  $\text{GSTP}^+$  and therefore the cancer initiating potential of the higher doses of  $\text{FB}_1$ . No effect was obtained with  $\text{PbNO}_3$  at any of the  $\text{FB}_1$  doses used.

### 3.3. Hepatocyte proliferation

The liver of the control rats hardly showed any labeling with BrdU, whilst levels of labeling, of approximately 9.5, 20, and 50%, were measured in the liver of the rats treated with  $\text{PbNO}_3$ ,  $\text{CCl}_4$ , and PH, respectively (Fig. 4). Under the present experimental conditions  $\text{PbNO}_3$  exhibited approximately 2- and 5-fold lower proliferative indices than  $\text{CCl}_4$  and PH, respectively.

### 3.4. Formation of TBARS

A significant ( $P < 0.001$ ) increase of approximately 2-fold in the level of TBARS was detected in the liver of rats that received the 50 mg  $\text{FB}_1/100$  g bw dose (Fig. 5).

### 3.5. Histopathological changes

Histological changes in the liver have been described elsewhere [6,7] and were mainly evident in the 30 and 50 mg  $\text{FB}_1/100$  g bw dose groups. Briefly, these included degenerative changes such as single cell necrosis (apoptosis), hydrophic cell swelling and hyaline droplet accumulation. Mild proliferation in oval cells and increased mitotic figures were also noticed in the 50 mg  $\text{FB}_1/100$ g body weight dose

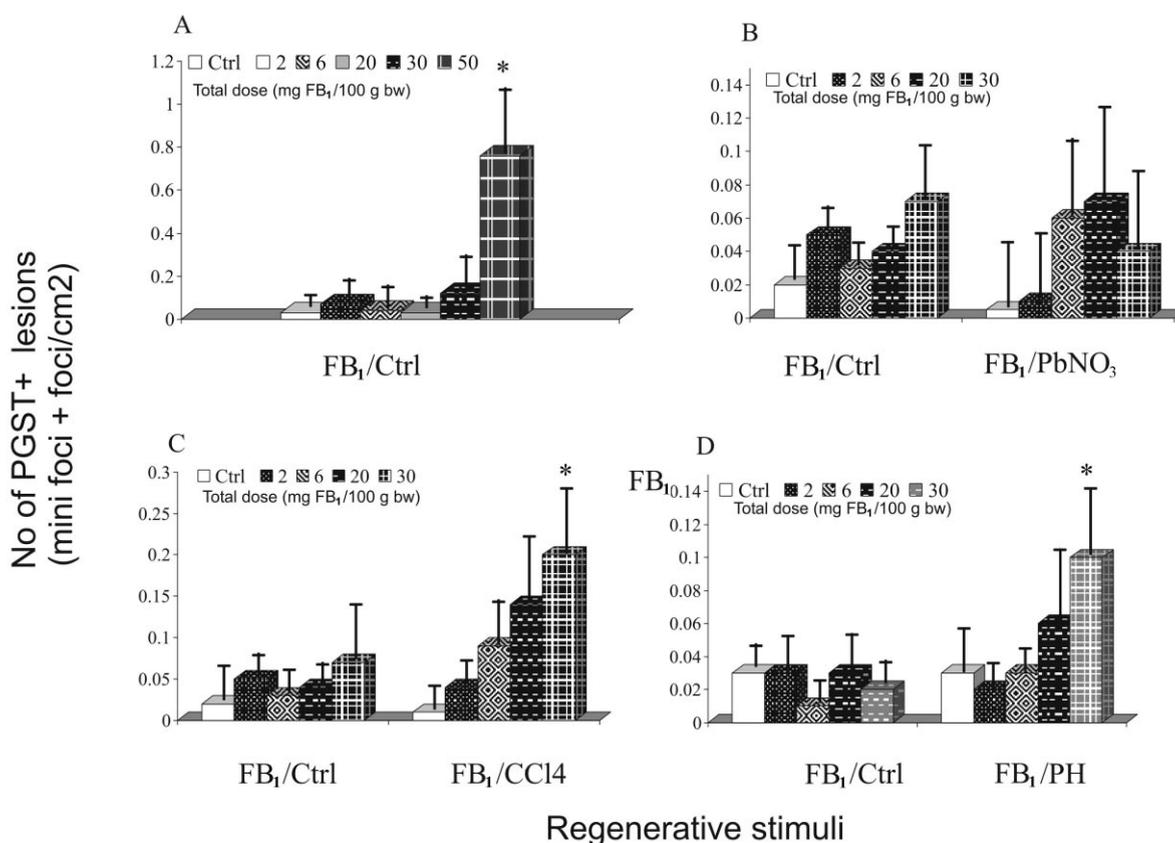


Fig. 3. The dose dependent cancer initiating effect of  $\text{FB}_1$  in rat liver (A) and the modulating role of  $\text{PbNO}_3$  (B),  $\text{CCl}_4$  (C) and PH (D) on  $\text{FB}_1$ -induced cancer initiation. The proliferative stimuli were introduced 7 days after the initiating treatment commenced. AAF/ $\text{CCl}_4$  was used as the promoting sequence when PH was used as the proliferative treatment during initiation (D). Values are the mean of 8–10 rats per group. \* $P < 0.05$ , for comparisons between  $\text{FB}_1$ /proliferative stimuli and  $\text{FB}_1/\text{Ctrl}$  in the same panel. Ctrl, control.

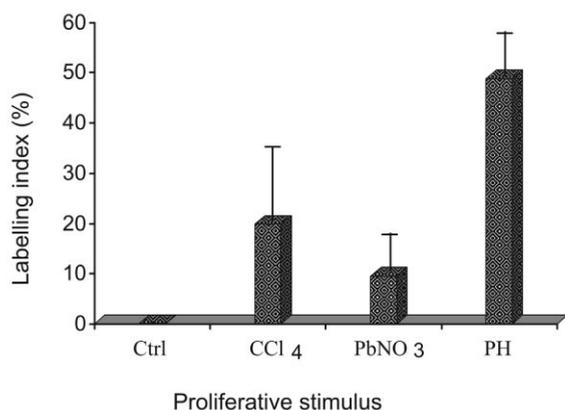


Fig. 4. Effect of PbNO<sub>3</sub>, CCl<sub>4</sub> and PH on the labeling index in the liver monitored by measuring the incorporation of BrdU label at 30, 48, and 20 h following the proliferative treatment, respectively. Values are the mean of 5–6 animals per group and at least a total of 1000 cells were counted in different microscopic fields (40×). Ctrl, control.

group. After 2-AAF/PH selection, 1–3 nodules (2–5 mm) were visible macroscopically in the livers of these rats.

#### 4. Discussion

FB<sub>1</sub>-induced cancer initiation is likely to proceed via a similar pathway to that described for the geno-

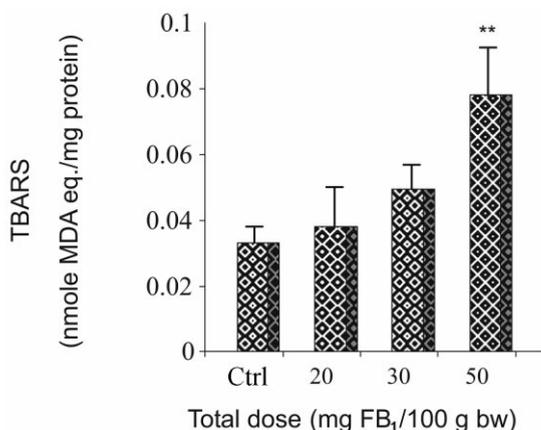


Fig. 5. Dose-response generation of TBARS in the liver of rats treated with different doses of FB<sub>1</sub> (gavage) over a period of 14 days. Values are the mean of three rats per group. \*\**P* < 0.01. Ctrl, control.

toxic class of carcinogens with respect to the induction of initiated hepatocytes and their subsequent resistance to the mitoinhibitory effect of 2-AAF resulting in their development into hepatocyte nodules [6,23]. The only apparent difference appears to be in the induction kinetics of the initiated hepatocyte which, in contrast to genotoxic carcinogens, is only induced after a prolonged exposure of at least 2 weeks [6]. The biochemical phenotype of the FB<sub>1</sub>-induced nodules is not known, although it is likely to be similar to that induced by genotoxic chemicals [24] as the nodules also stain positively with two histochemical markers for preneoplasia, i.e. gamma glutamyl transpeptidase and GSTP [6]. A unique feature of early preneoplastic and cancerous lesions induced by fumonisins in rat liver is the over stabilization of cyclin D1, suggesting that an epigenetic mechanism could be involved [25]. The mechanism involved in the genesis of these FB<sub>1</sub>-induced initiated cells is not known at present but a recent study suggests that oxidative damage as a result of chronic FB<sub>1</sub>-induced hepatotoxic effects could be involved [16]. Three other studies also indicated that FB<sub>1</sub> causes lipid peroxidation in membranal environments [26], rat liver nuclei [27] and cells in culture [28]. In the present study a significant increase in lipid peroxidation, as determined by the TBARS assay, occurred in the liver of rats exposed to the high FB<sub>1</sub> dose (50 mg FB<sub>1</sub>/100 g bw). Marked hepatotoxicity as well as cancer initiation was also observed at this dose level, suggesting a close relationship between hepatotoxicity and cancer initiation as hypothesized in earlier studies [23]. Whether oxidative damage is also responsible for the genotoxic effects of FB<sub>1</sub> reported by Knasmüller et al. [29] in primary hepatocytes is not known at present. Nevertheless, the majority of studies to date indicate that FB<sub>1</sub> is better classified as a non-genotoxic hepatocarcinogen.

Cancer initiation, resulting from treatment with genotoxic carcinogens, is enhanced by PH and CCl<sub>4</sub> treatments which induce regenerative cell proliferation, but not by chemicals such as PbNO<sub>3</sub>, ethylbromide, etc which induce direct hyperplasia [3,30]. However, it is not known whether increased cell proliferation also plays a determining role in the initiation effected by non-genotoxic chemicals such as the fumonisins. With respect to FB<sub>1</sub>, it was hypothesized that the level of cell proliferation, as a

result of hepatotoxicity, plays a critical role during initiation, but that it is counteracted by the inhibitory effect of FB<sub>1</sub> on cell proliferation in normal liver [6,7]. An important balance therefore seems to exist that, as a function of time, will determine whether cell proliferation will exceed a critical level to sustain cancer initiation by the fumonisins [6]. It was therefore of interest to determine whether an increase in cell proliferation during the cancer initiating treatment would support the induction of initiated cells.

Only the two agents inducing regenerative cell proliferation, namely PH and CCl<sub>4</sub>, significantly enhanced the cancer initiating effect of FB<sub>1</sub> and even then only at the highest FB<sub>1</sub> dose used (30 mg FB<sub>1</sub>/100 g bw). The CCl<sub>4</sub>-induced proliferation also showed a marginal effect on cancer initiation in the rats that received 20 mg FB<sub>1</sub>/100 g bw. No direct comparison between the effect of these two regenerative stimuli, namely PH and CCl<sub>4</sub>, can be made as different promoting regimens (AAF/CCl<sub>4</sub> vs AAF/PH) were used. In contrast to PH and CCl<sub>4</sub>, the mitogen-induced hyperplastic effect of PbNO<sub>3</sub> failed to enhance the cancer initiating potential of FB<sub>1</sub>. Of the three proliferative stimuli, PH was the most effective followed by CCl<sub>4</sub> and PbNO<sub>3</sub> in enhancing the BrdU labeling index in the liver. Whether these differences in the rate of cell proliferation could have an effect on initiation is not known at present. However, PH and CCl<sub>4</sub> introduced 7 days after commencing the FB<sub>1</sub> initiating treatment, did result in a further reduction in body weight gain (Fig. 2B), presumably due to an enhanced susceptibility to FB<sub>1</sub>-induced hepatotoxic effects. A recent study indicated that FB<sub>1</sub> was more toxic in regenerating liver following PH [31]. It can be argued that these agents exert their effects on the cancer initiating potency of FB<sub>1</sub> both by enhancing the hepatotoxicity of FB<sub>1</sub> as well as increased regenerative cell proliferation. In contrast, PbNO<sub>3</sub> treatment combined with FB<sub>1</sub> revealed no significant interaction in terms of reduced body weight gain (Fig. 2B). As a relation exists between the reduction in body weight gain and FB<sub>1</sub>-induced hepatotoxic effects [6], it would appear that mitogen-induced cell proliferation induced by PbNO<sub>3</sub> not only failed to enhance FB<sub>1</sub>-induced initiation but also did not enhance the hepatotoxic effects of FB<sub>1</sub>. Lemmer et al. [32] indicated that dietary iron protects against a reduction in the

relative liver weight induced by FB<sub>1</sub>. Of relevance was the finding that dietary iron, a known mitogen in the liver [33], reduced the cancer initiating potency of FB<sub>1</sub> under the specified experimental conditions.

Differences therefore seem to exist between regenerative and mitogen-induced cell proliferation with respect to the biological effects of FB<sub>1</sub> in the liver. The interaction between CCl<sub>4</sub> and FB<sub>1</sub>, both of which induce oxidative damage in the liver, is of interest with respect to initiation. Cell death induced by CCl<sub>4</sub> in zone 4 (central vein) of the lobule resulted from excessive lipid peroxidation in hepatocyte membranes [34]. However, in the case of FB<sub>1</sub>, lipid peroxidation appears to be a secondary event following cell death and has been implicated as a causative factor in cancer initiation [16]. As the combined effect of the cell proliferative stimuli and the FB<sub>1</sub> treatment on oxidative damage was not measured during the initiating treatment, it is not known whether lipid peroxidation contributed to the enhanced cancer initiating potency of FB<sub>1</sub>.

The differences in the type of cell proliferation induced by PH and/or CCl<sub>4</sub> versus that caused by mitogens such as PbNO<sub>3</sub> and their respective role in the initiating events induced by genotoxic carcinogens have been well established [4,35]. In the case of regenerative cell proliferation, the liver is in the process of regaining its critical mass following injury and/or removal of a part by PH. When carcinogen exposure is timed to coincide with the majority of cells passing through the S- or DNA synthesis phase of the cell cycle, a maximum number of initiated cells is produced. However, a single and/or multiple dose of FB<sub>1</sub> in conjunction with PH, failed to cause initiation as compared to the effect of genotoxins [23]. This finding indicated that the kinetics of the initiating step induced by FB<sub>1</sub> is different from the latter compounds. With respect to mitogen-induced hyperplasia, the liver is stimulated to produce increased tissue mass but the original size is restored after removal of the stimulus due to the elimination of the excess cells through the process of apoptosis [4]. As initiated cells seem to be prone to undergo apoptosis [10] it could well be that, in conjunction with the delayed cancer initiating potential of FB<sub>1</sub>, the initiated cells are more readily removed from the liver. It is known that FB<sub>1</sub> also directly induces apoptosis in the liver and hence this could be a further

restriction on the survival of initiated cells [9]. A recent study indicated that pre-treatment of rats with dietary FB<sub>1</sub> significantly reduced the persistence of DEN-induced GSTP + hepatocytes in the liver [8].

The present study suggests that regenerative cell proliferation either supports the process of initiation and/or renders the liver more susceptible to FB<sub>1</sub>-induced hepatotoxicity, which facilitates the process of cancer initiation. As in the case of initiation with genotoxic carcinogens, mitogen-induced hyperplasia did not enhance initiation by FB<sub>1</sub>. The fact that regenerative cell proliferation enhances the cancer initiating potency of FB<sub>1</sub> has to be taken into account, as in the case of genotoxins, when determining tolerance levels and establishing risk assessment parameters for the fumonisins in humans. This is of particular interest as the fumonisins co-occur naturally in corn with aflatoxin B<sub>1</sub> in areas where people are commonly infected with hepatitis B virus [36].

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## Lipids and $\Delta 6$ -Desaturase Activity Alterations in Rat Liver Microsomal Membranes Induced by Fumonisin B<sub>1</sub>

W.C.A. Gelderblom<sup>a,\*</sup>, W. Moritz<sup>a</sup>, S. Swanevelder<sup>b</sup>, C.M. Smuts<sup>c</sup>, and S. Abel<sup>a</sup>

<sup>a</sup>PROME C Unit and <sup>b</sup>Biostatistic Unit, Medical Research Council, Tygerberg 7505 South Africa, and <sup>c</sup>NIRU, Medical Research Council, Tygerberg, South Africa

**ABSTRACT:** Alterations in the membrane structure and function of hepatocyte membranes by fumonisin B<sub>1</sub> (FB<sub>1</sub>) have been proposed to play an important role in the disruption of growth regulatory effects and hence to have cancer-promoting ability. Detailed analyses of lipids in liver microsomal fractions of rats exposed to different dietary levels of FB<sub>1</sub> over a period of 21 d, indicated an increase in PC, PE, PI, and cholesterol (Chol). These changes decreased the PC/PE and increased the total phospholipid/Chol ratios. When considering FA content, the quantities of total FA increased ( $P < 0.05$ ) in the major phospholipid fractions as a result of the increased phospholipid levels. However, when considering the relative levels (mg/100 mg of the total FA) of specific FA, the monounsaturated FA (16:1n-7 and 18:1n-9) and 18:2n-6 increased ( $P < 0.05$ ), whereas the long-chain PUFA decreased ( $P < 0.05$ ) in the main phospholipid fractions. Enzyme analyses indicated that the activity of the  $\Delta 6$ -desaturase was significantly reduced in liver microsomal preparations in a dose-dependent manner. An increase in the 20:3n-6/20:4n-6 ratio also suggested a decrease in the activity of the  $\Delta 5$ -desaturase. Disruption of microsomal lipid metabolism at different levels by FB<sub>1</sub> could play an important role in the alteration of growth regulatory effects in the liver.

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Fumonisin B<sub>1</sub> (FB<sub>1</sub>), a naturally occurring mycotoxin produced by *Fusarium verticillioides* in corn, alters growth regulatory responses in the liver. The inhibitory effect of FB<sub>1</sub> on growth-stimulating responses *in vitro* in different cell culture systems (1,2) and *in vivo* in rat liver has been related to the cancer-promoting activity of the compound (3,4). Studies in primary rat hepatocytes indicated that FB<sub>1</sub> increased the level of PC and PE, whereas sphingomyelin (SM) decreased (5). Total cholesterol (Chol) levels were decreased, and the relative levels of 18:2n-6 and 20:4n- increased. Sphingolipid metabolism was disrupted *via* the inhibition of ceramide synthase (6) resulting in the accumulation of sphinganine and to some extent sphingosine. The disruptions of lipid metabolism at different levels involving phospholipid, sphingolipid, and FA biosynthesis were suggested to be key events in the cytotoxicity of the fumonisins to hepatocytes (5). Subsequent in-

vestigations in primary hepatocytes indicated that FB<sub>1</sub> inhibited the epidermal growth factor (EGF) mitogenic response, a common property of many cancer promoters in rat liver (1). This mitoinhibitory effect was not related to the disruption of sphingolipid metabolism; and events such as the disruption of Chol, phospholipid and FA and metabolism or combined effects seem to play a determining role. Recent findings indicated that the mitoinhibitory effect of FB<sub>1</sub> was counteracted by prostaglandin (PG) E<sub>2</sub>, suggesting that arachidonic acid (20:4n-6) metabolism is a key determinant (7). Alterations of FB<sub>1</sub> on 20:4n-6 and PG-induced effects on cell cycle progression and apoptosis have been reported (8,9).

When considering the FA profiles in hepatocytes exposed to FB<sub>1</sub>, it has been suggested that the rate-limiting enzyme in FA metabolism,  $\Delta 6$ -desaturase, is impaired (5). *In vivo* changes in the lipid profiles include increased Chol and PE levels and decreased SM in the liver of rats fed a dietary level of 250 mg FB<sub>1</sub>/kg for 21 d (10). A long-term feeding study in rats indicated an increased PE level at a dietary level as low as 1 ppm FB<sub>1</sub> (10). The relative level of 18:2n-6 was again significantly increased in PE, and 18:1n-9 was also markedly increased. The final FA of the n-6 pathway, 22:5n-6, was significantly decreased, indicating a disruption of the n-6 metabolic pathway, presumably owing to an impaired  $\Delta 6$ -desaturase enzyme. A similar effect was noticed in plasma PC, where the relative levels of 20:4n-6 and 22:5n-6 were reduced while 18:2n-6 accumulated.

It was proposed that changes in lipid metabolism in the liver, including phospho- and sphingolipids, Chol, and FA metabolism, play a determining role in the toxicological effects of the fumonisins (11,12). The interaction of these different lipid parameters is likely to be important in determining cell survival by altering cell proliferative and/or apoptotic pathways (13,14). The present study investigated lipid profiles of liver microsomal preparations of rats exposed to different dietary levels of FB<sub>1</sub> and the possible modulating effect on FA desaturation.

### MATERIALS AND METHODS

**Reagents.** [1-<sup>14</sup>C]Linoleic acid (LA; 18:2n-6) and [1-<sup>14</sup>C]- $\gamma$ -linolenic acid (GLA; 18:3n-6) were obtained from DuPont, Belgium. LA, GLA, NADH, ATP, nicotinamide, coenzyme A, and BHT [2,6-di-*tert*-butyl-*p*-cresol] were ordered from Sigma Chemical Corporation. FB<sub>1</sub> was purified according to

\*To whom correspondence should be addressed at PROMEC Unit/MRC, P.O. Box 19070, Tygerberg, 7505, South Africa.  
E-mail: wentzel.gelderblom@mrc.ac.za

Abbreviations: Chol, cholesterol; EGF, epidermal growth factor; FB<sub>1</sub>, fumonisin B<sub>1</sub>; GLA,  $\gamma$ -linolenic acid; LA, linoleic acid; PG, prostaglandin; SM, sphingomyelin; TFA, total fatty acids; TPL, total phospholipid.

the method described by Cawood *et al.* (15) to a purity of 90–95%.

**Treatment of animals.** Fischer 344 male rats were fed diets containing 10, 25, 50, 100, 250, and 500 mg FB<sub>1</sub>/kg diet over a period of 21 d, as described previously (3). Rats were terminated by decapitation and the livers collected in ice-cold saline and frozen at –70°C.

**Preparation of microsomal preparations.** Liver homogenates were prepared by a modification of the methods described by Koba *et al.* (16) and De Antueno *et al.* (17). Liver was homogenized at 4°C with three parts of a homogenizing buffer consisting of a 0.1 M potassium phosphate buffer containing 0.25 M sucrose, 0.15 M KCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, and 1.5 mM GSH using a Potter-Elvehjem homogenizer. The homogenate was first centrifuged at 10,000 × *g* for 20 min, then the supernatant was collected and centrifuged at 105,000 × *g* for 1 h. The microsomal pellet was resuspended in homogenizing buffer using a glass homogenizer to a level of 10 mg protein/mL and stored at –70°C.

**Lipid analyses.** The microsomal preparations (2 mg protein/mL) were extracted with chloroform/methanol/BHT (2:1:0.01; vol/vol/%) according to the method of Smuts *et al.* (18). No lipid analyses were performed on the liver microsomal fraction of the rats fed the 500 mg FB<sub>1</sub>/kg diet. The phospholipid classes in the extracts were separated by TLC according to the method of Gilfillan *et al.* (19) using chloroform/methanol/petroleum ether/acetic acid/boric acid (40:20:30:10:1.8 g, by vol) as developing solvent. The spots corresponding with the major phospholipids were visualized under UV light and collected for phospholipid and FA analyses. Transmethylation of the FA was achieved by MeOH/H<sub>2</sub>SO<sub>4</sub> (95:5) treatment for 2 h at 70°C in glass stoppered tubes. FAME were analyzed by GC on a Varian model 3700 gas chromatograph using fused-silica megabore DB-225 columns (J&W Scientific, Folsom, CA). The FA peaks were identified by comparison of retention times to those of a standard mixture of free FA 14:0 to 22:6 and quantified using an internal standard (17:0) and expressed as µg FA/mg protein.

Phospholipid analyses were carried out by the method of Itaya and Ui (20) utilizing malachite green. The collected phospholipid fractions were digested in perchloric acid at 170°C for 2 h and diluted in an appropriate volume of H<sub>2</sub>O. Quantification was effected colorimetrically using phosphate as an external standard. The total Chol of the chloroform/methanol extracts was determined by the enzymatic iodine method using Chol oxidase and esterase enzyme preparations (18).

**Δ6-desaturase assay.** Enzyme activity was monitored by the method of Koba *et al.* (16). Test tubes containing 0.5 µC LA (ca. 100 nmol/mL = 100 µM; 20 µL ethanol), 0.25 M sucrose, 0.15 M KCl, containing NaF (45 mM), NADH (1.0 mM), ATP (1.5 mM), CoA (0.25 mM), nicotinamide (0.5 mM), MgCl<sub>2</sub> (5 mM), GSH (1.5 mM), and 100 mM phosphate buffer (pH = 7.4) in a total volume of 1 mL were preincubated at 37°C for 5 min. The microsomal solution was incubated separately for 5 min at 37°C, and 1 mL (2 mg protein) added to the incubation mixture to obtain a final protein

concentration of 1 mg/mL. This mixture was incubated for a further 30 min at 37°C. The assay was terminated by the addition of freshly prepared 10% KOH (1.8 N) in ethanol (2 mL), and the mixture was saponified at 100°C for 30 min. After acidifying with 0.4 mL of 11.6 N HCl (36%), the mixture was extracted with hexane (2 × 5 mL), the hexane was evaporated (N<sub>2</sub>), and the samples were transmethylated as described above. FAME were extracted with hexane/H<sub>2</sub>O (2 × 2 mL hexane + 1 mL H<sub>2</sub>O), and the hexane layer was evaporated to dryness under nitrogen. After addition of 80 µL of hexane/chloroform (20:5) the methylated products were fractionated on AgNO<sub>3</sub>-TLC plates using chloroform/methanol (50:2) as developing solvent. For the preparation of the AgNO<sub>3</sub>-TLC plates, silica gel plates were impregnated with 10% AgNO<sub>3</sub> in acetonitrile for 15 min, dried at 100°C for 20 min, and kept desiccated in the dark prior to use. The spots were visualized under UV light after spraying with 0.1% dichlorofluorescein in chloroform/methanol (1:1 vol/vol). The spots corresponding with the substrate [1-<sup>14</sup>C]18:2n-6 (LA) and the product [1-<sup>14</sup>C]18:3n-6 (GLA) were scraped into scintillation vials; scintillation liquid was added, and the radioactivity determined. The control treatments consisted of incubations with heat-inactivated microsomes and microsomal preparations without the incubation mixture. Enzyme activity, which served to monitor the formation of GLA, was expressed as pmol GLA/min/mg protein.

**Statistical analyses.** Data were analyzed by two-way ANOVA using the GLM procedure, and Tukey's Studentized Range Test was used to determine whether the means differed statistically. Values were considered significant if *P* < 0.05.

## RESULTS

Details concerning the effects of the different dietary levels of FB<sub>1</sub> on the liver and body weight gains have been reported previously (3). In short, body weight gain was significantly reduced in rats fed the 250 mg FB<sub>1</sub>/kg diet (*P* < 0.01) and markedly affected in the animals that received the 100 mg FB<sub>1</sub>/kg diet.

**Chol and phospholipids.** The Chol content of the microsomes was significantly (*P* < 0.01) and marginally (*P* < 0.1) increased in the rats fed the 250 and 100 mg FB<sub>1</sub>/kg diets, respectively (Fig. 1A). Of the different phospholipid fractions analyzed, no significant effect on the concentration of PS was noticed as a result of the FB<sub>1</sub> treatment. In contrast, the levels of PC, PE, and PI were significantly increased (*P* < 0.05 to *P* < 0.01) in the 250 mg FB<sub>1</sub>/kg dietary group (Tables 1 and 2). In the case of PE, the concentration was also marginally (*P* < 0.1) increased in the 100 mg FB<sub>1</sub>/kg dietary group. The total phospholipid (TPL) concentration was also significantly (*P* < 0.05) increased from 100 mg FB<sub>1</sub>/kg, whereas it was marginally (*P* < 0.1) increased at dietary levels of 10 and 50 mg FB<sub>1</sub>/kg diet (Fig. 1A). When considering the relative levels of each phospholipid, expressed as a percentage of the TPL pool, the level of PC remained constant as PE increased marginally (*P* < 0.1) and significantly (*P* < 0.05) at 100 and 250

**TABLE 1:**  
Concentration and FA Content ( $\mu\text{g}/\text{mg}$  protein) of the Major Liver Microsomal Phospholipids PC and PE of Rats Fed Different Dietary Levels of FB<sub>1</sub> for 21 d<sup>a</sup>

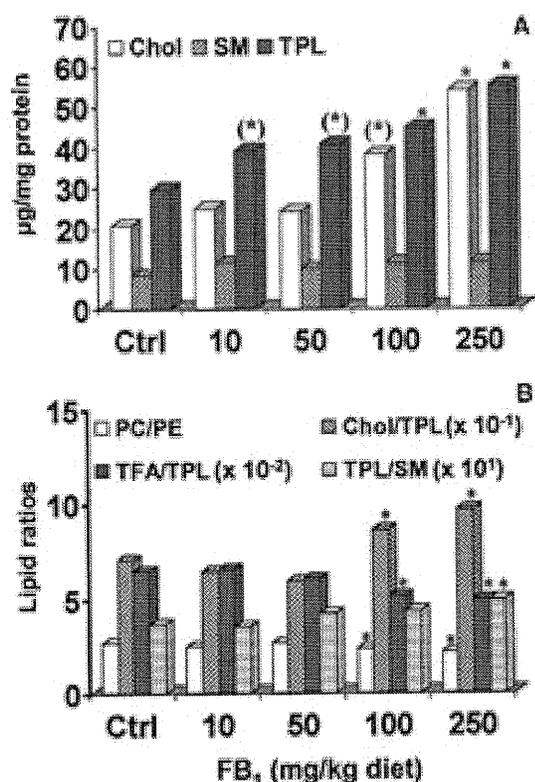
Treatment (mg FB <sub>1</sub> /kg diet)	Phosphatidylcholine (PC)					Phosphatidylethanolamine (PE)				
	Control	10	50	100	250	Control	10	50	100	250
Phospholipids (% of TPL)	177.7 ± 27.1a 61.7a	226.0 ± 51.4a 63.3a	230.0 ± 44.2a 62.4a	254.3 ± 56.3a 60.5a	323.0 ± 40.5b 59.0a	69.8 ± 2.83a 21.6a	93.3 ± 17.2a 21.0a	87.9 ± 8.3a 21.9a	108.5 ± 9.1(b) 26.2(b)	153 ± 24.5A 28.3b
Fatty acids										
16:0	22.13 ± 0.54a	27.40 ± 1.62(b)	26.70 ± 2.68a	26.49 ± 2.63a	31.83 ± 2.27a	7.96 ± 1.42a	9.88 ± 1.00a	9.87 ± 0.36a	10.73 ± 1.82a	14.03 ± 1.08A
18:0	24.70 ± 1.97a	31.64 ± 2.55(b)	29.60 ± 3.46a	25.60 ± 3.28a	30.09 ± 2.18a	10.08 ± 2.57a	14.48 ± 2.16a	13.14 ± 0.56a	14.26 ± 1.18a	18.74 ± 1.73A
Total SFA	46.83 ± 2.48a	59.04 ± 0.94b	56.29 ± 5.87a	52.09 ± 5.19a	61.92 ± 4.45b	18.05 ± 3.99a	24.36 ± 3.15a	23.01 ± 0.65a	24.99 ± 2.85a	32.77 ± 2.82A
16:1	0.62 ± 0.15a	1.01 ± 0.31a	0.92 ± 0.25a	1.46 ± 0.28b	1.52 ± 0.28b	0.11 ± 0.01a	0.17 ± 0.03a	0.15 ± 0.03a	0.29 ± 0.06b	0.25 ± 0.05(b)
18:1	9.46 ± 0.42a	12.44 ± 1.42(b)	12.24 ± 0.82(b)	12.56 ± 0.61b	15.58 ± 2.20A	3.20 ± 0.58a	4.52 ± 0.08a	4.39 ± 0.65a	5.17 ± 0.38b	6.34 ± 0.89A
Total MUFA	10.08 ± 0.54a	13.46 ± 1.73(b)	13.16 ± 1.06a	14.03 ± 0.87b	17.10 ± 2.48A	3.31 ± 0.58a	4.69 ± 0.07a	4.54 ± 0.68a	5.46 ± 0.42b	6.60 ± 0.94A
18:2n-6	10.64 ± 1.52a	14.57 ± 1.06a	13.04 ± 0.20a	16.54 ± 2.00b	18.29 ± 4.19b	2.71 ± 0.94a	4.11 ± 0.60a	3.60 ± 0.41a	6.43 ± 1.10A	6.14 ± 0.80b
18:3n-6	0.34 ± 0.02a	0.50 ± 0.04a	0.40 ± 0.05a	0.72 ± 0.33a	0.81 ± 0.16(b)	0.08 ± 0.03a	0.14 ± 0.01a	0.10 ± 0.01a	0.22 ± 0.12a	0.19 ± 0.02a
20:3n-6	0.35 ± 0.01a	0.57 ± 0.03a	0.46 ± 0.12a	0.73 ± 0.29a	0.51 ± 0.34a	0.15 ± 0.02a	0.23 ± 0.04a	0.22 ± 0.05a	0.41 ± 0.13b	0.37 ± 0.01(b)
20:4n-6	36.12 ± 2.40a	48.88 ± 0.52A	47.52 ± 2.46A	37.80 ± 4.61a	40.73 ± 3.26a	13.32 ± 3.58a	19.99 ± 2.28(b)	18.22 ± 1.33a	20.70 ± 2.48b	21.38 ± 1.13b
22:4n-6	0.96 ± 0.20a	1.24 ± 0.14a	1.33 ± 0.23a	1.11 ± 0.30a	1.84 ± 0.11b	1.07 ± 0.21a	1.46 ± 0.03a	1.50 ± 0.05a	1.65 ± 0.048a	2.72 ± 0.05A
22:5n-6	4.59 ± 0.36a	4.74 ± 0.36a	5.73 ± 1.68a	3.33 ± 0.90a	6.64 ± 0.52a	4.06 ± 0.80a	4.29 ± 0.38a	4.85 ± 0.47a	3.33 ± 1.22a	8.08 ± 0.01A
Total n-6	53.01 ± 3.77a	70.50 ± 0.64A	68.48 ± 4.10b	60.23 ± 6.12a	68.81 ± 7.33b	21.39 ± 5.59a	30.22 ± 2.65(b)	28.48 ± 1.27a	32.72 ± 2.91b	38.87 ± 1.86A
22:5n-3	0.32 ± 0.11a	0.56 ± 0.06	0.39 ± 0.15	0.37 ± 0.03	0.42 ± 0.11	0.37 ± 0.12	0.62 ± 0.02	0.54 ± 0.06	0.44 ± 0.13	0.39 ± 0.16
22:6n-3	3.16 ± 0.71a	4.51 ± 0.29b	4.01 ± 0.50a	2.56 ± 0.28a	2.98 ± 0.06a	3.14 ± 1.04a	4.82 ± 0.55(b)	4.17 ± 0.49a	2.60 ± 0.20a	3.84 ± 0.30a
Total n-3	3.60 ± 0.84a	5.24 ± 0.32b	4.57 ± 0.34a	3.12 ± 0.29a	3.60 ± 0.08a	3.54 ± 1.14a	5.53 ± 0.57b	4.78 ± 0.55a	3.14 ± 0.31a	4.37 ± 0.16a
PUFA	56.61 ± 4.60a	75.74 ± 0.86A	73.04 ± 4.28b	63.34 ± 6.40a	72.41 ± 7.41b	24.94 ± 6.73a	35.75 ± 3.16(b)	33.26 ± 1.76a	35.86 ± 3.13b	43.25 ± 2.02A
Total FA	113.5 ± 5.2a	148.2 ± 1.9b	142.5 ± 10.5b	129.5 ± 11.4a	154.8 ± 30.1b	46.4 ± 7.1a	64.8 ± 6.2a	60.8 ± 3.0a	66.3 ± 6.0a	84.0 ± 14.8b
n-6/n-3 ratio	15.12 ± 2.43a	13.49 ± 0.74a	15.02 ± 1.07a	19.31 ± 0.46b	19.10 ± 1.6(b)	6.13 ± 0.35a	5.48 ± 0.30a	6.00 ± 0.46a	10.46 ± 0.79A	8.87 ± 0.07A
P/S ratio	1.21 ± 0.11a	1.28 ± 0.02a	1.30 ± 0.06a	1.22 ± 0.01a	1.17 ± 0.04a	1.38 ± 0.08a	1.47 ± 0.09a	1.44 ± 0.04a	1.44 ± 0.07a	1.32 ± 0.05a

<sup>a</sup>Values are the means ± SD of triplicate determinations. FB<sub>1</sub>, fumonisin B<sub>1</sub>; TPL, total phospholipids; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated/saturated ratio. Means (rows) followed by the same letter do not differ statistically. If the letter differs, then  $P < 0.05$ . If the letters and cases differ (e.g., a vs. A) then  $P < 0.01$ . If the letter is in parentheses then  $P < 0.1$ .

**TABLE 2**  
**Concentration and FA Content ( $\mu\text{g}/\text{mg}$  protein) of the Major Liver Microsomal Phospholipids PI and PS of Rats Fed Different Dietary Levels of FB<sub>1</sub> for 21 d<sup>a</sup>**

Treatment (mg FB <sub>1</sub> /kg diet)	Phosphatidylserine (PS)					Phosphatidylinositol (PI)				
	Control	10	50	100	250	Control	10	50	100	250
Phospholipids % of TPL	9.63 ± 0.91a 3.7a	12.6 ± 1.3a 3.5a	13.1 ± 1.1a 3.3a	10.0 ± 2.7a 2.2b	12.0 ± 3.2a 2.1b	39.8 ± 8.4a 13.0a	44.2 ± 6.0a 12.3a	46.9 ± 5.5a 12.4a	45.9 ± 5.0a 11.1b	59.1 ± 9.1b 10.6b
Fatty acids										
16:0	0.38 ± 0.03a	0.38 ± 0.07a	0.46 ± 0.11a	0.44 ± 0.12a	0.63 ± 0.14a	1.28 ± 0.08a	1.46 ± 0.37a	1.58 ± 0.17a	1.93 ± 0.21a	1.80 ± 0.13a
18:0	2.82 ± 0.83a	4.09 ± 0.29a	3.77 ± 0.15a	2.39 ± 0.96a	2.94 ± 0.59a	9.74 ± 1.00a	12.70 ± 0.53a	12.64 ± 0.63a	9.61 ± 0.38a	10.31 ± 0.76a
Total SFA	3.20 ± 0.86a	4.48 ± 0.36a	4.23 ± 0.26a	2.84 ± 1.04a	3.57 ± 0.73a	11.01 ± 1.04a	14.16 ± 0.70a	14.22 ± 0.75a	11.53 ± 0.58a	12.11 ± 0.90a
16:1	0.30 ± 0.02a	0.33 ± 0.03a	0.31 ± 0.06a	0.31 ± 0.02a	0.39 ± 0.12a	0.30 ± 0.02a	0.30 ± 0.03a	0.34 ± 0.03a	0.37 ± 0.12a	0.40 ± 0.17a
18:1	0.17 ± 0.04a	0.22 ± 0.01a	0.22 ± 0.02a	0.28 ± 0.04a	0.46 ± 0.15a	0.37 ± 0.06a	0.51 ± 0.11a	0.58 ± 0.09a	0.85 ± 0.11a	0.93 ± 0.15a
Total MUFA	0.47 ± 0.05a	0.56 ± 0.03a	0.54 ± 0.07a	0.58 ± 0.04a	0.85 ± 0.27b	0.67 ± 0.08a	0.81 ± 0.09a	0.92 ± 0.11a	1.22 ± 0.23b	1.33 ± 0.32b
18:2n-6	0.13 ± 0.04a	0.17 ± 0.01a	0.16 ± 0.02a	0.21 ± 0.07a	0.28 ± 0.07b	0.67 ± 0.08a	0.94 ± 0.06a	0.88 ± 0.14a	1.83 ± 0.65b	1.62 ± 0.31(b)
18:3n-6	0.02 ± 0.01a	0.04 ± 0.01a	0.03 ± 0.01a	0.03 ± 0.02a	0.02 ± 0.01a	0.02 ± 0.01a	0.03 ± 0.01a	0.03 ± 0.01a	0.07 ± 0.05a	0.06 ± 0.01a
20:4n-6	2.00 ± 0.71a	3.02 ± 0.08a	2.76 ± 0.09a	1.73 ± 0.79a	1.76 ± 0.37ba	8.74 ± 0.42a	11.57 ± 0.76a	11.54 ± 0.28a	9.27 ± 0.19a	9.52 ± 0.11a
22:5n-6	0.46 ± 0.13a	0.52 ± 0.11a	0.53 ± 0.06a	0.13 ± 0.03a	0.25 ± 0.06b	0.29 ± 0.04a	0.39 ± 0.03a	0.44 ± 0.02b	0.29 ± 0.08a	0.42 ± 0.02(b)
Total n-6	2.73 ± 0.93a	3.92 ± 0.12a	3.65 ± 0.11a	2.34 ± 0.89a	2.83 ± 0.62a	10.17 ± 0.31a	13.46 ± 0.67a	13.53 ± 0.37a	12.15 ± 0.70a	12.24 ± 0.37b
22:5	0.02 ± 0.02a	0.03 ± 0.00a	0.02 ± 0.00a	0.09 ± 0.03a	0.03 ± 0.01a	0.10 ± 0.03a	0.16 ± 0.01a	0.15 ± 0.11a	0.08 ± 0.02a	0.11 ± 0.02a
22:6	0.26 ± 0.10a	0.42 ± 0.05a	0.33 ± 0.04a	0.16 ± 0.06a	0.19 ± 0.04a	0.26 ± 0.04a	0.43 ± 0.02a	0.41 ± 0.04a	0.27 ± 0.03a	0.30 ± 0.01a
Total n-3	0.31 ± 0.12a	0.49 ± 0.05a	0.40 ± 0.04a	0.20 ± 0.07a	0.26 ± 0.06a	0.41 ± 0.04a	0.65 ± 0.02a	0.62 ± 0.06a	0.41 ± 0.01	0.47 ± 0.03a
Total PUFA	3.04 ± 1.05a	4.41 ± 0.18a	4.04 ± 0.08a	2.54 ± 0.95a	3.09 ± 0.67a	10.58 ± 0.27a	14.11 ± 0.67a	14.15 ± 0.41a	12.56 ± 0.71a	12.71 ± 0.40b
Total FA	7.6 ± 2.2a	9.4 ± 0.5a	8.8 ± 0.2a	6.0 ± 2.0a	7.9 ± 2.6a	22.3 ± 1.3a	29.1 ± 0.8b	29.3 ± 1.0b	25.6 ± 1.3a	26.5 ± 4.8a
n-6/n-3 ratio	9.06 ± 0.33a	8.04 ± 0.61a	9.28 ± 1.22a	11.37 ± 0.82(b)	11.19 ± 0.01(b)	26.48 ± 4.16	20.74 ± 0.98a	21.84 ± 1.51a	29.90 ± 1.41a	26.01 ± 0.66a
P/S ratio	0.93 ± 0.07a	0.99 ± 0.06a	0.95 ± 0.08a	0.90 ± 0.06a	0.87 ± 0.02a	0.97 ± 0.07a	1.00 ± 0.07a	1.00 ± 0.05a	1.09 ± 0.01a	1.06 ± 0.04a

<sup>a</sup>For footnote see Table 1.



**FIG 1.** (A) Effect of fumonisin B<sub>1</sub> (FB<sub>1</sub>) on the cholesterol (Chol), sphingomyelin (SM), and total phospholipid (TPL) levels in liver microsomes from rats exposed to different dietary levels over a period of 21 d. (B) Different lipid ratios, that are possible factors modifying membrane fluidity. TFA, total FA; TPL, total phospholipids. \* $P < 0.1$ , \*\* $P < 0.05$ .

mg/kg dietary levels, respectively (Table 1). The relative levels of PI and PS significantly ( $P < 0.05$ ) decreased at the 100 and 250 mg FB<sub>1</sub>/kg dietary levels. As a result of the increase in the TPL pool, the total FA (TFA) were also increased marginally ( $P < 0.1$ ) to significantly ( $P < 0.05$ ) in the two high-dose groups, respectively. Owing to the changes in the lipid parameters of the microsomal membranes, the Chol/TPL ratio increased and the TFA/TPL and PC/PE ratios decreased in the 100 and 250 mg FB<sub>1</sub>/kg dietary groups (Fig. 1B).

**FA profiles.** The FA content, expressed as µg/mg protein, of the different phospholipids is summarized in Tables 1 and 2.

(i) **Saturated FA (16:0 and 18:0).** The total saturated fats increased significantly in the PC ( $P < 0.05$ ) and PE ( $P < 0.01$ ) phospholipid fractions in the 250 mg FB<sub>1</sub>/kg dietary group. In PC it was also significantly ( $P < 0.05$ ) increased in the 10 mg FB<sub>1</sub>/kg dietary group. Both 16:0 and 18:0 increased ( $P < 0.01$ ) in PE in the high-dose group whereas in PC only 16:0 increased significantly ( $P < 0.01$ ) in the high-dose group, and 18:0 was markedly increased in all FB<sub>1</sub>-treated groups. No changes were observed in PS and PI.

(ii) **Monounsaturated FA (16:1 and 18:1).** A marked to significant ( $P < 0.01$  to 0.05) increase in the monounsaturated FA was noticed in all the phospholipid fractions in the livers of the two high-dose groups of rats (100 and 250 mg FB<sub>1</sub>/kg

diet). In PC ( $P < 0.1$ ) and PE marked increases were also noticed at the 10 and 50 mg FB<sub>1</sub>/kg dietary levels. Both 16:1 and 18:1 contributed to the increased levels at the two high-dose levels; 18:1 was marginally ( $P < 0.1$ ) to significantly ( $P < 0.05$ ) increased in PC in all the FB<sub>1</sub>-treated groups.

(iii) **PUFA (n-6 PUFA: 18:2n-6, 20:4n-6, 22:4n-6, 22:5n-6).** LA (18:2n-6) was significantly ( $P < 0.1$  to 0.01) increased in all the phospholipid fractions in the 100 and 250 mg FB<sub>1</sub>/kg dietary groups whereas it was markedly higher in PC and PE in the 10 and 50 mg FB<sub>1</sub>/kg groups. Arachidonic acid (20:4n-6) was significantly ( $P < 0.05$ ) increased in PC at dietary levels of 10 and 50 mg FB<sub>1</sub>/kg, but it was similar to the control levels in the two high-dose groups. No changes were observed in PS while it was markedly to significantly ( $P < 0.1$  to 0.01) increased in PE and PI at all dose levels. The terminal FA in the n-6 pathway, 22:5n-6, was significantly ( $P < 0.01$ ) increased in PE at the high-dose level (250 mg FB<sub>1</sub>/kg). In PI, 22:5n-6 was significantly ( $P < 0.05$  to 0.01) increased at all the FB<sub>1</sub> dose levels. The total n-6 FA marginally to significantly increased in PC, PE, and PI at all the dose levels whereas PS was not affected.

(n-3 PUFA, 22:5n-3, 22:6n-3). The level of 22:6n-3 was marginally ( $P < 0.1$ ) to significantly ( $P < 0.05$  to 0.01) increased in the PC, PE, and PI phospholipid fractions at the 10 mg FB<sub>1</sub>/kg dietary levels. At higher dietary levels it tended to mimic the level in the microsomes of the control rats despite the increase in the level of the respective phospholipid. The total n-3 FA tended to follow the same pattern in PC, PE, and PI, whereas PS was not affected.

**FA parameters.** Changes in the levels of the FA profiles presented above are related to the concentration of the respective phospholipid. As a result, PUFA were marginally ( $P < 0.1$ ) to significantly ( $P < 0.05$  to  $P < 0.01$ ) increased in the microsomal PC, PE, and PI phospholipid fractions of the FB<sub>1</sub>-treated animals. The n-6/n-3 FA ratio was also significantly ( $P < 0.05$ ) increased in PC and PE at the 100 and 250 mg FB<sub>1</sub>/kg dietary levels mainly due to an increase in the n-6 FA. The polyunsaturated/saturate FA ratio was not altered. The TFA increased markedly in the microsomes of the rats fed the 10 mg FB<sub>1</sub>/kg dietary level and significantly at the high-dose levels as a result of the increased levels of the individual phospholipids (Tables 1 and 2).

**FA desaturation.** When considering the activity of the FA  $\Delta$  desaturases the levels of the substrates and products of the enzyme are relevant. Since the concentration of phospholipids may vary, as in the case of the present study, the relative amount of each FA, expressed as a percentage of the TFA, needs to be considered. There was a significant increase in the relative levels of C16:1n-7, C18:1n-9, and C18:2n-6 and a significant decrease in the long-chain FA, C20:4n-6, and C22:6n-3 in PC (Fig. 2A) and PE (Fig. 2B) at a dietary level of 100 mg FB<sub>1</sub>/kg and above. The terminal FA of the n-6 metabolic pathway, 22:5n-6, significantly ( $P < 0.05$ ) decreased in both PE and PC in the 100 mg FB<sub>1</sub>/kg dietary group while no decrease was noticed in the 250 mg FB<sub>1</sub>/kg dietary group. A similar relative FA pattern was also noticed

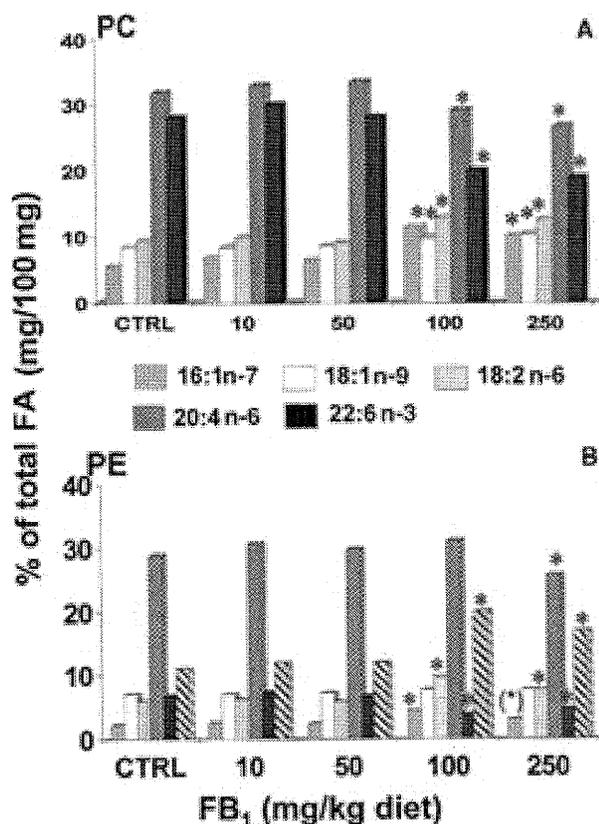


FIG. 2. The relative levels (% of total) of the FA in the major microsomal phospholipids PC (A) and PE (B) of the liver of rats fed different dietary levels of  $\text{FB}_1$  for 21 d. \* $P < 0.05$ . For abbreviation see Figure 1.

in PS and PI phospholipid fractions (data not shown). When considering specific FA ratios, using the relative concentration levels, the 18:2n-6/18:3n-6 ratio decreased significantly ( $P < 0.05$ ) in PC whereas the 18:3n-6/20:4n-6 and 20:3n-6/20:4n-6 ratios increased significantly ( $P < 0.05$ ) in both PC and PE at the two high-dose  $\text{FB}_1$  dietary levels (Fig. 3A). No changes were observed in the 18:3n-6/20:3n-6 ratio in the PC and PE phospholipid fractions.

Enzyme analyses indicated that the conversion of  $^{14}\text{C}$ -LA to  $^{14}\text{C}$ -GLA, catalyzed by the  $\Delta 6$  desaturase, was significantly reduced at dietary levels of 100 mg  $\text{FB}_1$  and higher (Fig. 3). The activity of the  $\Delta 5$  desaturase was not determined.

## DISCUSSION

Lipid analyses of the livers of rats exposed to different dietary levels of  $\text{FB}_1$  indicated that the metabolism of Chol, phospholipid, FA, and sphingolipid is altered (10). The present study indicated that the phospholipids were significantly altered in the rat liver microsomal fraction due to elevated concentrations of PE, PC, and PI. When considering the relative contribution of each phospholipid to the TPL pool, PC remained constant at about 60% whereas PE increased significantly from 20 to 28%. The relative contribution of both PI and PS to the TPL

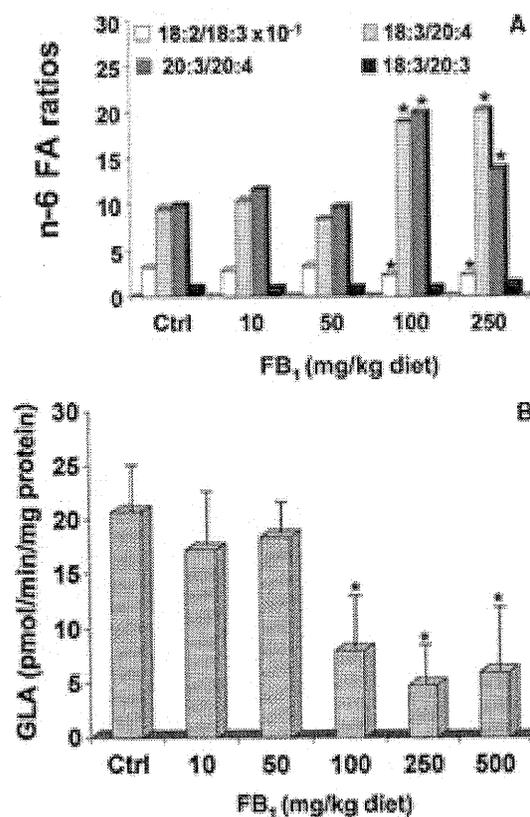


FIG. 3. Alterations of the relative FA ratios associated with changes in n-6 FA metabolism (A), and the effect of  $\text{FB}_1$  on the activity of the microsomal  $\Delta 6$ -desaturase enzyme as a function of different dietary doses fed to rats over a period of 21 d (B). \* $P < 0.05$ . For abbreviation see Figure 1.

pool tended to decrease, from 12 to 10% and from 4 to 2%, respectively. It appears that  $\text{FB}_1$  mainly altered the PE phospholipid compartment in rat liver microsomes, resulting in a significant decrease in the PC/PE ratio. Chol was also significantly increased (2.5 times) at the two higher dietary levels owing to the fact that, despite the increase in TPL, the Chol/TPL ratio increased significantly. The increase in the TPL also resulted in the increase in the TFA content. In this regard the relative level of PUFA decreased significantly in PC, PE, and PI, and the saturated and monounsaturated FA compartment significantly increased.

The impact of these altered lipid parameters on membrane fluidity has been discussed in detail elsewhere (21). These changes suggest that an increased Chol/TPL ratio, decreased PC/PE ratio, and accumulation of the relative levels of the saturated FA together with the decrease of the long-chain PUFA could result in a more rigid microsomal membrane structure. To what extent the increase in the relative levels of the monounsaturated FA, C18:1n-9, and C18:2n-6 counteracted the impact of the other parameters in maintaining membrane fluidity is unknown. However, in persistent hepatocyte nodules, similar changes to the same lipid parameters were associated

with an increase in fluidity (22). No changes in the concentration of the membrane sphingolipid, SM, were noticed as a result of the FB<sub>1</sub> treatment. Apparently, the disruption of sphingolipid metabolism, more specifically, the ceramide synthase by FB<sub>1</sub>, is not reflected in the microsomes. The TPL/SM ratio, however, increased significantly owing to the increase in the PC and PE concentration. As both phospholipids are involved in the synthesis of SM *via* PC:ceramide-phosphocholine and the PE:ceramide-phosphocholine transferases (23), the accumulation of PC and PE could be related to a decreased synthesis of SM due to a reduced level of ceramide caused by the inhibition of ceramide synthase by FB<sub>1</sub>. Increased microsomal Chol is also known to induce the synthesis of SM by increasing the activities of the two phospholipid transferase enzymes (23). However, as the level of SM was not reduced, the above hypothesis does not explain the accumulation of PC and PE, and/or other mechanisms that regulate the level of SM in the microsomes may exist. Studies should be conducted on the modulating effect of FB<sub>1</sub> on Chol and phospholipid-metabolizing enzymes.

The increased TPL pool also resulted in an increase in the TFA concentrations in individual phospholipid fractions. This occurred at dietary levels as low as 10 mg FB<sub>1</sub>/kg. However, at the two higher levels the TFA/TPL ratio decreased mainly owing to an increase in TPL, whereas TFA levels did not increase to the same extent. The latter have been associated with the decrease in the relative values of PUFA, owing to the impaired  $\Delta 6$ -desaturase and/or increased lipid peroxidation induced by the hepatotoxic effects of FB<sub>1</sub> in the liver (4). Abel *et al.* (24) indicated that lipid peroxidation was significantly enhanced in rat liver microsomes after chronic feeding at a dietary level of 250 mg FB<sub>1</sub>/kg over a period of 21 d. Several other studies confirmed that FB<sub>1</sub> induces oxidation damage in membranal environments (25,26). Therefore, despite the increase in the concentration of the phospholipids, the PUFA levels, especially the relative levels of 20:4n-6 and 22:6n-3, which are prone to undergo lipid peroxidation, significantly decrease in both the PC and PE phospholipid fractions of the rats fed the high-dose FB<sub>1</sub> levels. It will not be possible to distinguish between the relative contribution of lipid peroxidation and an impaired FA desaturation toward the reduction of PUFA in the microsomes.

The activity of the  $\Delta 5$ - and  $\Delta 6$ -desaturase enzymes is affected by many factors including changes in the fluidity of the microsomal membrane (16), age (27–29), dietary status (30), different animal species, and different organs in the same species (16). Studies indicated that, with respect to dietary Chol and protein, altering the Chol/phospholipid and PC/PE ratios affects the fluidity of the microsomal membrane as well as the activity of the enzyme and hence the FA content of the microsomes (21,31). The FB<sub>1</sub>-induced alteration of FA patterns in the major phospholipid fractions in primary hepatocytes and in rat liver suggests an impaired  $\Delta 6$ -desaturase enzyme system (5,10). The present study indicates that FB<sub>1</sub> significantly decreased the activity of the  $\Delta 6$ -desaturase, resulting in the accumulation of 18:2n-6 in the major phospholipids.

When considering the 18:3n-6/20:4n-6 and 20:3n-6/20:4n-6 FA ratios, it appears that the activity of the  $\Delta 5$ -desaturase also is impaired. The increase in 18:3n-6 could explain the significant decrease in the 18:2n-6/18:3n-6 ratio, i.e., 18:3n-6 was proportionately higher than 18:2n-6. As no change was observed in the 18:3n-6/20:3n-6 ratio it would appear that the activity of the elongase enzyme was not affected.

Whether the disruption of the activity of the FA  $\Delta$  desaturases is related to a direct interaction with FB<sub>1</sub> or indirectly to a disruption of the membranal structure is unknown. The fact that Cawood *et al.* (32) indicated that FB<sub>1</sub> is tightly associated with hepatocyte membranes emphasizes the importance of monitoring the *in vitro* effects of FB<sub>1</sub> on the activity of the enzyme. Whatever the reason, the impairment of the FA  $\Delta$  desaturases and the resultant disruption of FA metabolism are likely to disrupt the membrane integrity of the microsomes further. When considering the structural changes of the microsomal membranal environment, one must recognize that the activities of other important enzymes are also likely to be altered. Changes in the Chol and phospholipid membrane constituents have been shown to alter the activities of drug-metabolizing enzymes, such as cytochrome P450 (33–37). In this regard recent studies indicated that the activity of certain isozymes of the hepatic P450 enzyme system are selectively inhibited and/or stimulated by FB<sub>1</sub> (38,39).

Structural changes with respect to Chol, phospholipids, and FA in the membrane environment of neoplastic cells have been reported to be important in the progression of these lesions into cancer (22). Membranal changes in hepatocyte nodules are associated with increased Chol and PE, resulting in an increased Chol/TPL ratio, a decrease in the PC/PE ratio, and changes in membrane fluidity. The  $\Delta 6$ -desaturase has been reported to be impaired in hepatocyte nodules and other cancerous tissue resulting in a specific FA pattern in the membranal phospholipids (22,40). The increased concentration of PE and subsequent increased level of 20:4n-6 have been implied to be important stimuli for the altered growth pattern in these lesions (22). A specific role for 20:4n-6 in the development of hepatocyte nodules has been proposed (22,41), suggesting a critical role of this FA in cancer development. Impairment of the  $\Delta 6$ -desaturase, presumably owing to structural changes in the membrane, is an early event in the genesis of hepatocyte nodules (22) and appears to be closely associated with the altered growth pattern of cancerous lesions. Recently it was proposed that the disruption of the membrane structure by FB<sub>1</sub>, and more specifically the effect on PE and 20:4n-6 levels, could be important for the cancer-promoting ability of this compound in the liver (4,11). Addition of PG E<sub>2</sub> counteracts the mitoinhibitory effect of FB<sub>1</sub> on the EGF mitogenic response in primary hepatocytes, suggesting the disruption of 20:4n-6 metabolism (7). FB<sub>1</sub> also inhibits the effects of 20:4n-6, PG-E<sub>2</sub> and PG-A<sub>2</sub> on apoptosis in esophageal cancer cells *in vitro* presumably related to decreased ceramide production (9).

Alterations in rat liver microsomal membrane lipid profiles and  $\Delta 6$ - and possibly  $\Delta 5$ -desaturase activity, leading to

altered FA metabolism, could have important implications regarding signaling pathways that determine cell survival and cancer development in the liver.

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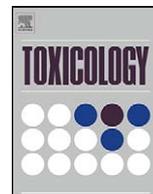
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## Cancer initiating properties of fumonisin B<sub>1</sub> in a short-term rat liver carcinogenesis assay

W.C.A. Gelderblom<sup>a,c,\*</sup>, W.F.O. Marasas<sup>a</sup>, S. Lebepe-Mazur<sup>a</sup>, S. Swanevelder<sup>b</sup>, S. Abel<sup>a</sup>

<sup>a</sup> Program on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, PO Box 19070, Tygerberg 7505, South Africa

<sup>b</sup> Biostatistics Unit, Medical Research Council, PO Box 19070, Tygerberg 7505, South Africa

<sup>c</sup> Department of Biochemistry, University of Stellenbosch, Private Bag X1, Matieland, Stellenbosch, South Africa

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### ABSTRACT

The nature of cancer initiation by fumonisin B<sub>1</sub> (FB<sub>1</sub>) was investigated in rat liver by monitoring the effect of phenobarbital (PB) as cancer promoter and evaluating the involvement of spontaneously initiated cells. A PB promoting regimen (0.05% in the diet) stimulated the outgrowth of FB<sub>1</sub>-induced placental glutathione S-transferase (GSTP) positive initiated hepatocytes. Reversion of the FB<sub>1</sub>-induced GSTP<sup>+</sup> foci was noticed in the absence of a promoting regimen. Younger rats were shown to be more sensitive to the induction of GSTP<sup>+</sup> foci by FB<sub>1</sub>. Cancer initiation by FB<sub>1</sub> was associated with a hepatotoxic effect, which was less pronounced in older rats presumably due to a reduced intake. A specific role of spontaneously initiated cells and their promotion by FB<sub>1</sub> into the development of eosinophilic clear cell foci could not be established under the present experimental conditions. The ability of different stimuli to selectively promote the outgrowth of FB<sub>1</sub> initiated cells further verifies the cancer initiating potency of this apparent non-genotoxic mycotoxin. The underlying mechanism(s) involved in the genesis of the initiated hepatocytes is not known at present.

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### 1. Introduction

Fumonisin B<sub>1</sub> (FB<sub>1</sub>) is a food-borne mycotoxin produced by the fungus *Fusarium verticillioides* that occurs worldwide and causes a variety of naturally occurring toxicoses in animals, including fatal illnesses in horses and pigs, and recently shown to induce neural tube defects in mice (Gelderblom et al., 1988; Marasas et al., 1988; Harrison et al., 1990; Gelineau-van Waes et al., 2005). Human dietary consumption of *Fusarium*-contaminated corn products has been linked epidemiologically to increased rates of esophageal cancer (Rheeder et al., 1992) hepatocellular carcinoma (Ueno et al., 1997) and neural tube defects (Marasas et al., 2004) in regions of the world in which corn is the staple grain, such as South Africa, China and southern USA. Short-term feeding of FB<sub>1</sub> causes hepatotoxic effects, while continued FB<sub>1</sub> administration leads to a chronic toxic hepatitis and fibrosis, which progresses to cirrhosis, and sometimes terminates in hepatocellular carcinoma or cholangiocarcinoma (Gelderblom et al., 1991a; Lemmer et al., 2004).

FB<sub>1</sub> is a cancer promoter stimulating the outgrowth of pre-neoplastic lesions in rat liver (Gelderblom et al., 1996a). However, this mycotoxin also exhibits weak cancer-initiating properties (Gelderblom et al., 1994), which is in agreement with long-term studies indicating that FB<sub>1</sub> acts as a complete carcinogen. This would imply that FB<sub>1</sub> acts similarly to many genotoxic carcinogens, although conflicting data exists as to whether the fumonisins exhibit genotoxic properties. FB<sub>1</sub> lacks genotoxicity when tested in several *in vitro* tests including the *Salmonella* mutagenicity test (Gelderblom and Snyman, 1991b; Knasmüller et al., 1997) and unscheduled DNA test in primary hepatocytes (Norred et al., 1992; Gelderblom et al., 1992). However, studies show that FB<sub>1</sub> induces micronuclei and chromosomal aberrations in primary hepatocytes (Knasmüller et al., 1997) and Hep-G2 cells (Ehrlich et al., 2002). In addition, FB<sub>1</sub> causes DNA damage in astrocytes and human fibroblasts (Galvano et al., 2002), and an increased oxidative DNA damage in rats liver and kidneys (Domijan et al., 2007). The mechanism for potential genotoxicity of FB<sub>1</sub> is not known as no direct interaction with DNA could be demonstrated while it is not metabolized by liver microsomal and/or cytosolic enzyme preparations to any reactive DNA interactive intermediate(s) (Cawood et al., 1994; Pocsfalvi et al., 2001). A potential underlying mechanism linking this non-mutagenic mycotoxin with the initiation of liver cancer *in vivo*, is the FB<sub>1</sub>-induced hepatotoxic effects associated with the induction of oxidative damage and lipid peroxidation (Gelderblom et al.,

\* Corresponding author at: Program on Mycotoxins and Experimental Carcinogenesis (PROMEC), Medical Research Council, PO Box 19070, Tygerberg 7505, South Africa. Tel.: +27 21 938 0244.

E-mail address: [wentzel.gelderblom@mrc.ac.za](mailto:wentzel.gelderblom@mrc.ac.za) (W.C.A. Gelderblom).

1996b; Abel and Gelderblom, 1998). *In vitro* studies showed that FB<sub>1</sub> induced membrane lipid peroxidation and oxidative DNA damage (Sahu et al., 1998; Abado-Becongnee et al., 1998).

Cancer promotion of FB<sub>1</sub>-induced initiated cells is effected by the 2-acetylaminofluorene with partial hepatectomy (2-AAF/PH) or 2-AAF/carbon tetrachloride (CCl<sub>4</sub>) cancer-promoting regimens (Gelderblom et al., 1992, 1994) that selectively stimulates the outgrowth of the genetically altered cell population resistant to the mitoinhibitory effects of 2-AAF (Farber, 1991). FB<sub>1</sub>-induced cancer initiation was stimulated by regenerative cell proliferation while no effect was obtained by mitogenic-induced hyperplasia effected by lead nitrate (Gelderblom et al., 2001a). The occurrence of spontaneously initiated liver tumors in rats also needs to be considered regarding FB<sub>1</sub>-induced hepatocarcinogenesis, particularly when it is regarded as a nongenotoxic carcinogen (Schulte-Hermann et al., 1993; Kraupp-Grasl et al., 1991). These spontaneously occurring liver foci occur more frequently in older rats, and account for the prevalence of pre-neoplastic lesions in the liver of control animals (Ward and Henneman, 1990).

FB<sub>1</sub> induced hepatocytes and their subsequent promotion by phenobarbital (PB), a known cancer promoter in the liver of rats (Kraupp-Grasl et al., 1991), for either 10 or 30 weeks were investigated to further characterize the cancer-initiating properties of FB<sub>1</sub>. Male Fischer (F344) rats, aged 7, 26 and 52 weeks served to elucidate the role of spontaneously initiated cells in FB<sub>1</sub>-induced hepatocarcinogenesis. The induction of the placental form of glutathione S-transferase (GSTP<sup>+</sup>) foci was taken as the endpoint for cancer initiation in the liver

## 2. Materials and methods

### 2.1. Chemicals

All reagents were of analytical grade. PB was obtained from Sigma Chemical Company (St. Louis, MO). FB<sub>1</sub> was purified by high-performance liquid chromatography (HPLC) according to the method described by Cawood et al. (1991) to a purity of between 92 and 95%. Placental glutathione S-transferase (polyclonal rabbit anti-rat) antibody was purchased from Novocastra (Newcastle upon Tyne, UK).

### 2.2. Animals and diets

The study was approved by the Ethics and Research Committee of the South African Medical Research Council, and the experiments were conducted in accordance with the laws and regulations controlling experiments on live animals in South Africa. Male Fischer 344 (F344) rats weighing between 150 and 180 g (7–8 weeks of age) were housed in wire bottom cages with free access to food and water. Rats were caged individually in a controlled environment with a 12-h light and dark cycle, humidity of 45–50% and ambient temperature of 23–25 °C. The AIN-76 control diet was prepared according to standard guidelines (AIN, 1980) with the exception that glucose was replaced with a sucrose:dextrose (1:1) mixture. Rats were weighed three times per week during the treatment protocols.

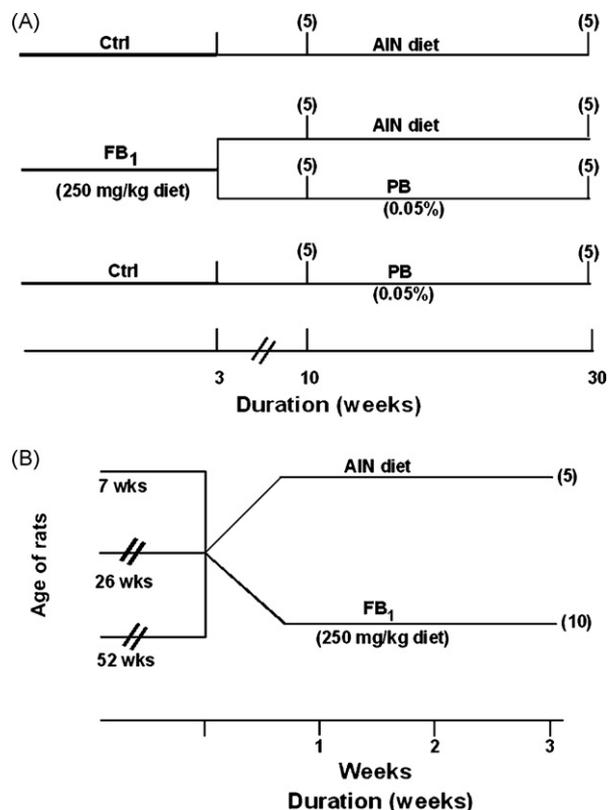
### 2.3. Cancer initiation by FB<sub>1</sub>

#### 2.3.1. Phenobarbital promotion

Male F344 rats (aged 7 weeks) were randomly divided into four groups consisting of 10 animals each: (i) AIN-76 diet only; (ii) PB only; (iii) FB<sub>1</sub> only; (iv) FB<sub>1</sub> followed by phenobarbital (Chart 1A). Cancer initiation was effected by a 3-week dietary treatment of 250 mg FB<sub>1</sub>/kg that represents the threshold level for initiation by FB<sub>1</sub> for the selected period (Gelderblom et al., 1994). Following cancer initiation, the rats were allowed to recover by feeding the control AIN-76 diet for 2 weeks. Cancer promotion was effected using PB, dietary level 0.05% (w/w), administered for periods of either 10 or 30 weeks. Control groups received either the AIN diet or PB dietary treatment, whereas treatment groups received the FB<sub>1</sub> initiating regimen with either the AIN diet or the PB promotion treatment.

#### 2.3.2. Spontaneously initiated cells

Male F344 rats were stagger-bred to obtain rats of ages of 7, 26 and 52 weeks. Rats were randomly divided into the treatment ( $n=10$ ) and control groups ( $n=5$ ) for each age group and fed FB<sub>1</sub> (250 mg/kg diet) or AIN diet for 3 weeks (Chart 1B).



**Chart 1.** Experimental designs to (A) investigate the cancer promoting potential of phenobarbital on FB<sub>1</sub>-induced hepatic foci in rat liver and (B) investigate the effect of age on the generation of FB<sub>1</sub>-induced hepatic foci. Values in parenthesis indicate number of rats sacrificed a specific time point.

### 2.4. Histochemical and histological analyses

Following pentobarbital anesthesia, rats were sacrificed by cervical dislocation and the livers excised and sectioned (5 mm) for processing in buffered formalin. Immunohistochemical staining for GSTP<sup>+</sup> was performed according to the method of Ogawa et al. (1980). GSTP<sup>+</sup> hepatic foci were classified microscopically as "mini" (<5 cells), "small" (5–20 cells), or "large" (>20 cells). The total number of foci containing >5 cells (small or large) per cm<sup>2</sup> of the tissue section, the area of which was determined by image analyses.

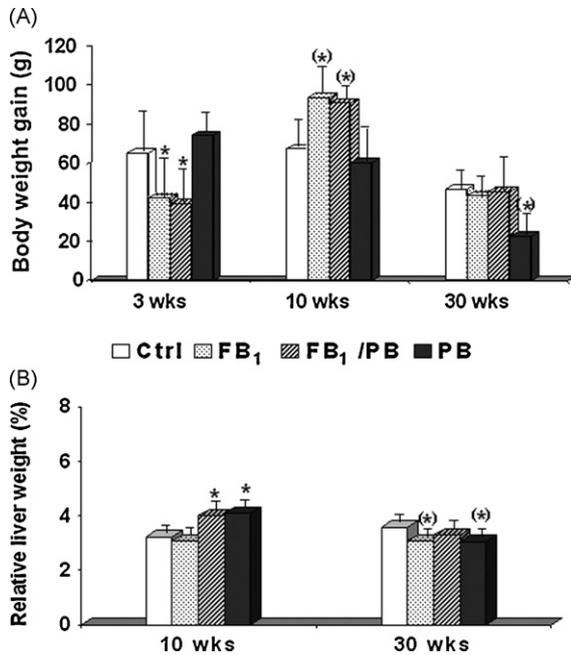
### 2.5. Statistical analysis

The data of this study was analysed using a one-way analysis ANOVA design where only one main effect was present, either with 3 or 4 group levels. When the main effect was significant, the post hoc Tukey's Studentized Range test was used, testing for multiple pair-wise comparisons between the means of the different levels of the factor. Where variances were not equal the Welch test was substituted for the *F*-test. As the data were unbalanced, the Tukey–Cramer adjustment was made automatically. Where data was non-parametric, group differences were tested using the Kruskal–Wallis test, which was followed by the non-parametric Tukey-type test. For parametric data were only two levels were present, Student's *t*-test was used, by the pooled method for equal variances, and the Satterthwaite method for unequal variances. Statistical differences were considered at <0.05.

## 3. Results

### 3.1. Body weight parameters

Seven-week-old rats subjected to the 3-week FB<sub>1</sub>/AIN diet and FB<sub>1</sub>/PB treatments had significantly ( $P<0.05$ ) reduced body weight gains compared to the control rats (Fig. 1A). The body weight gain of the FB<sub>1</sub>/AIN diet and FB<sub>1</sub>/PB-treated groups were marginally ( $P<0.1$ ) increased after 10 weeks compared to the AIN and PB-control groups, respectively. The PB-treated rats showed a marginal ( $P<0.1$ ) reduction in body weight gain after 30 weeks compared to



**Fig. 1.** (A) Body weight gain of rats following 3-week FB<sub>1</sub> induction prior to PB promotion for either 10 or 30 weeks. (B) Liver to body weight ratio (%) of rats following PB promotion for either 10 or 30 weeks. Data (error bars) represent the mean  $\pm$  S.D. of 10 animals per group. \*Differs significantly ( $P < 0.05$ ) and (\*) marginally ( $P < 0.1$ ) from treatment control groups.

the control rats. The relative liver weight (% of the body weight) was significantly ( $P < 0.05$ ) increased in the FB<sub>1</sub>/PB- and PB-treated rats after 10 weeks compared to the control rats (Fig. 1B). After 30 weeks, the relative liver weights of the FB<sub>1</sub>- and PB-treated rats were marginally ( $P < 0.1$ ) reduced compared to the control rats.

### 3.1.1. Phenobarbital promotion

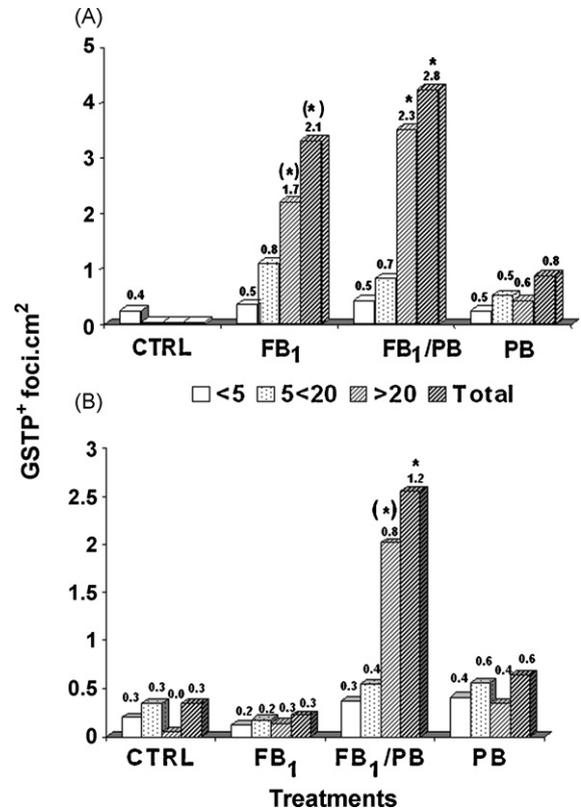
There was a significant ( $P < 0.05$ ) increase in the total number of GSTP<sup>+</sup> foci/cm<sup>2</sup> (>5 cells per focus) after 10 weeks in the FB<sub>1</sub>/PB- and FB<sub>1</sub>/AIN-treated groups as a result of a marginal ( $P < 0.1$ ) increase in the number of large foci (>20 cells per focus) (Fig. 2A). After 30 weeks there was a significant ( $P < 0.05$ ) increase in the total number of GSTP<sup>+</sup> foci/cm<sup>2</sup> (>5 cells per focus) in the FB<sub>1</sub>/PB group as compared to the FB<sub>1</sub>/AIN- and PB-control groups mainly due to an increase in the number of large foci (Fig. 2B). In contrast to the 10-week treatment regimen, no significant effects were noticed in the liver of the FB<sub>1</sub>/AIN-treated rats.

Inter-time comparisons (between 10 and 30 weeks) regarding the development of the GSTP<sup>+</sup> lesions between the different groups showed a marginal ( $P = 0.08$ ) increase in the small (between 5 and 20 cells) foci category and the total number of foci in the control rats after 30 weeks. In contrast the total number of foci in the FB<sub>1</sub>/AIN-treated rats decreased significantly ( $P < 0.05$ ) after 30 weeks. There were no significant differences in the number of GSTP<sup>+</sup> lesions between the 10- and 30-week treatment period for the FB<sub>1</sub>/PB- and PB-treated groups.

### 3.1.2. GSTP<sup>+</sup> foci

The FB<sub>1</sub> feeding treatment significantly ( $P < 0.05$ ) reduced the body weight gain at 7-, 26- and 52-week-old rats as compared to the controls (Fig. 3A). In the 26- and 52-week-old rats there was an actual loss in body gain following FB<sub>1</sub> exposure.

In the 7-week-old rats there was a significant increase in all the PGST<sup>+</sup> focal size categories while it was significantly decreased in the livers of the 26- and 52-week-old rats (Fig. 3B). The induc-



**Fig. 2.** PB cancer promotion of FB<sub>1</sub>-induced GSTP<sup>+</sup> hepatic foci. Male F344 rats fed 250 mg FB<sub>1</sub>/kg diet for 3 weeks were subjected to a 0.1% (w/w) PB dietary regimen of either (A) 10 weeks or (B) 30 weeks. Data are mean  $\pm$  S.D. (indicated on bars) of 5 animals per group. \*Differs significantly ( $P < 0.05$ ) and (\*) differs marginally ( $P < 0.1$ ) from the treatment control groups.

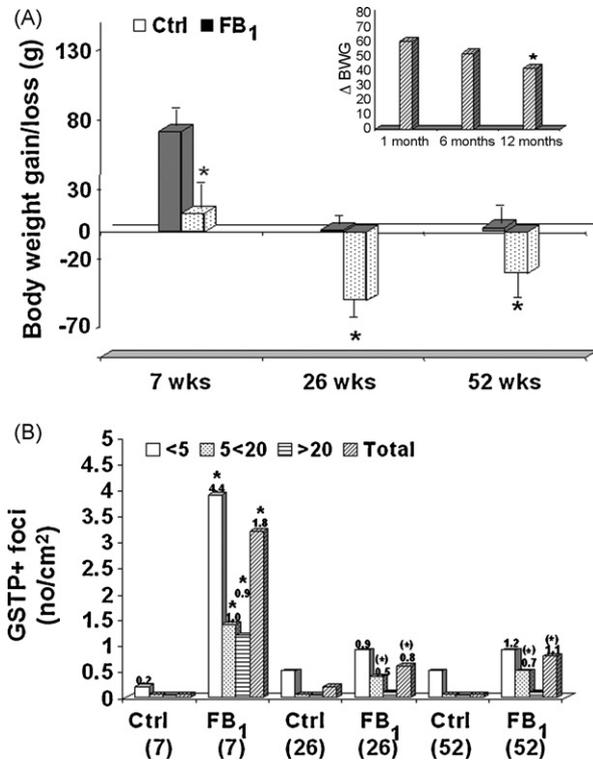
tion of mini ( $P < 0.09$ ) and large foci was only marginally ( $P < 0.08$ ) increased above the control rats in the liver of the 26- and 52-week-old rats. Of the control rats only the 26-week-old rats exhibited a marginal ( $P < 0.1$ ) increase in the level of the minifoci (<5 cells per foci).

### 3.2. Spontaneously initiated cells

Treatment with FB<sub>1</sub> (250 mg/kg) AIN diet resulted in marked hepatotoxic effects histologically, as previously described (Gelderblom et al., 1988; Lemmer et al., 2004). Briefly, the livers showed zone 3 (pericentral) injuries with collapse of the reticulin framework, apoptosis and necrosis of hepatocytes, appearance of hepatic foci, oval cell proliferation, and early fibrosis (Fig. 4). These hepatotoxic lesions were present in rats from all three age groups, but less severe in the 26- and 52-week-old rats (data not shown).

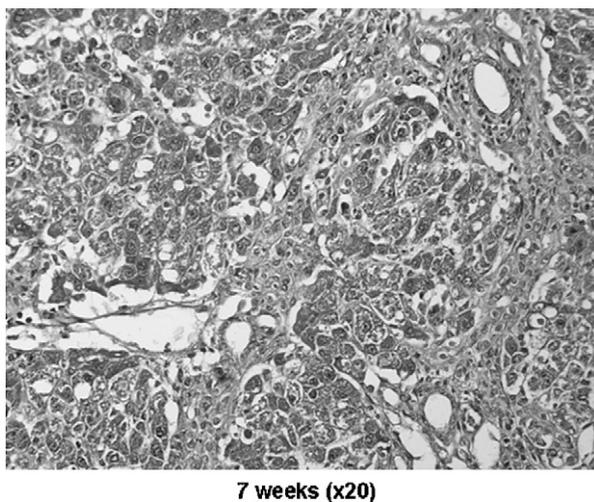
## 4. Discussion

Several mechanisms have been proposed to explain the cancer promoting activity of FB<sub>1</sub> in rat liver including the disruption of sphingolipids, phospholipids and fatty acid metabolism (Riley et al., 2001; Gelderblom et al., 2001b). Long-term studies in rats showed that FB<sub>1</sub> is a complete carcinogen in both the liver and kidney, suggesting that FB<sub>1</sub> induces the different stages involved in the process of carcinogenesis (Gelderblom et al., 1991a; Howard et al., 2001). The fumonisins were characterised as liver cancer promoters while some debate exists whether these compounds can effect cancer initiation. However, with respect to FB<sub>1</sub>-induced cancer initiation in rat liver the following needs to be considered.



**Fig. 3.** (A) Effect of FB<sub>1</sub> feeding regimen (250 mg FB<sub>1</sub>/kg diet) on the body weight gain of rats aged 7, 26 and 52 weeks [inset—differences in the body weight gain between rats fed FB<sub>1</sub> or control AIN diet for 1, 6 and 12 months]. (B) FB<sub>1</sub> induction of GSTP<sup>+</sup> hepatic foci in rats aged 7, 26 and 52 weeks. Data represent the mean ± S.D. (indicated on bars) of 10 animals in the FB<sub>1</sub> group and 5 animals in the control AIN group. \*Differs significantly ( $P < 0.05$ ) and (\*) marginally ( $P < 0.1$ ) from AIN control treatment.

- (i) FB<sub>1</sub> initiates cancer in rat liver similarly to the genotoxic carcinogens, by inducing “resistant” hepatocytes with a dependence on cell proliferation, although the kinetics differs. Phenotypically these initiated cells develop into eosinophilic clear cell foci and nodules that stained positively for GSTP and  $\gamma$ -glutamyl-transferase (GGT). The absolute level of events (DNA mutations, etc.), resulting in initiation, occurs at a



**Fig. 4.** Degenerative changes such as lipid accumulation and single cell necrosis, oval cell proliferation and early fibrosis in liver of a 7-week-old rat following a 3-week 250 mg FB<sub>1</sub>/kg dietary treatment.

far slower rate when compared to genotoxic carcinogens (Gelderblom et al., 1992, 1994).

- (ii) Similarly to genotoxins, cell proliferation associated with hyperplasia failed to enhance the cancer initiating potential of FB<sub>1</sub>, while stimulation of regenerative cell proliferation enhanced the cancer initiating potency of FB<sub>1</sub> (Gelderblom et al., 2001a).
- (iii) A no-effect threshold related to hepatotoxicity and hepatocyte regeneration exists for cancer initiation, which in turn is determined by the dosage and the duration of exposure (Gelderblom et al., 1994, 2001c).
- (iv) Studies using male BD IX, Fischer 344 and Sprague–Dawley rats showed that FB<sub>1</sub> exhibits cancer-initiating properties (Gelderblom et al., 1988, 1992; Mehta et al., 1998).
- (v) Irreversibility of cancer initiation by FB<sub>1</sub> was demonstrated as a 5-week exposure resulted in liver adenomas after 1 year in male Fischer 344 rats (Lemmer et al., 2004).
- (vi) Different cancer promoting stimuli, 2-AAF/PH, 2-AAF/CCL<sub>4</sub> (Gelderblom et al., 1992; and PB (present study)) promote the formation of altered pre-neoplastic hepatic lesions.
- (vii) FB<sub>1</sub> lacks peroxisome proliferative activity (Gelderblom et al., 1996b) while the promotion of spontaneous initiated cells in older rats seems not to play a role when utilising a short-term cancer initiating/promoting liver model (present study).

Several other alleged non-genotoxic carcinogen treatment regimens have been shown to effect cancer initiation in rat liver. These include dietary treatments with the peroxisome proliferators, ciprofibrate and clofibrate (Rao and Reddy, 1991; Nagai and Farber, 1999) and a choline deficient diet (Ghoshal and Farber, 1993). The hepatocyte nodules induced by clofibrate exhibited phenotypical changes regarding the induction of GSTP, GGT and ATPase, indistinguishable from that of genotoxic carcinogens. Feeding studies using ciprofibrate in rats of different ages showed no difference in the induction of tumors (Rao and Reddy, 1991). The peroxisome proliferators exhibited similar genotoxic effects to the fumonisins in rat primary hepatocytes, which included chromosomal aberrations and the induction of micronuclei (Reisenbichler and Eckl, 1993). However, FB<sub>1</sub>-induced changes in the liver are not associated with peroxisome proliferation (Gelderblom et al., 1996b). It would appear that, as in the case of FB<sub>1</sub>, non-genotoxic treatment regimens induce early pre-neoplastic changes in the liver similar to those induced by genotoxic carcinogens that should be taken into account when setting risk assessment parameters. When the provisional maximal tolerable daily intake (PMTDI) value for humans was established by the Joint FAO/WHO Evaluation Committee on Food Additives (JECFA), a no observed effect level (NOEL) for nephrotoxicity and a safety factor of 100 was employed (Bolger et al., 2001). Generally a higher safety factor is required reflecting the carcinogenic characteristics of a compound (Kuiper–Goodman, 1990). Therefore, the cancer initiation potential of the fumonisins should be considered if the risk assessment parameters are revisited in the future.

Cancer initiation in the liver is associated with the induction of individual cells with the ability to clonally expand under the influence of one or more promoting stimuli. When utilizing DEN as a cancer initiator, “resistant” hepatocytes are induced that have the ability to proliferate in the presence of different cancer promoting stimuli including, 2-AAF/PH, phenobarbital and orotic acid (Farber, 1996). The mechanism involved could either be inhibition of the growth of normal hepatocytes whereby the resistant cells are allowed to proliferate or the selective stimulation of initiated hepatocytes (Farber, 1996; Butterworth and Goldsworthy, 1991). Depending on the promoting regimen used and the mechanism involved, different subsets of the initiated cells have the capabil-

ity to develop into altered foci and/or nodules (Dragan and Pitot, 1992). It has been postulated the initiated cells are a heterogeneous population that possesses a variety of genetic lesions (Lee and Cameron, 1993). Cancer promotion by PB consisted in the development of altered eosinophilic clear cell phenotype stained positively for GGT while the peroxisome proliferator, nafenopin, promotes the formation of basophilic foci that stains negatively for GGT (Kraupp-Grasl et al., 1991). FB<sub>1</sub> initiated hepatocytes developed into GGT and GSTP<sup>+</sup> foci in response to the 2-AAF/PH or 2-AAF/CCL<sub>4</sub> cancer-promoting regimens (Gelderblom et al., 1992, 1994, 2001a). The present study indicated that PB, known to select an initiated population into eosinophilic clear cell foci, could also selectively promote FB<sub>1</sub>-induced initiated cells. At 10 weeks no significant difference between the number of GSTP<sup>+</sup> foci between the FB<sub>1</sub>- and FB<sub>1</sub>/PB-treated rats which reflect the cancer promoting potency of FB<sub>1</sub> during the 3-week feeding period, despite the fact that the rats did not receive FB<sub>1</sub> in their diet for 7 weeks (Fig. 2A). However, the number of GSTP<sup>+</sup> cells in the absence of the FB<sub>1</sub> treatment, 27 weeks after the initial feeding treatment, was significantly reduced similar to the control levels, presumably due to the spontaneous reversion of foci into normal tissue (Fig. 2B). These initiated cells populations are known to be susceptible to undergo apoptosis (Schulte-Hermann et al., 1993). Although cancer initiation is irreversible, the sustained presence of FB<sub>1</sub> is required for the selection of initiated cells and their subsequent development into foci and nodules (Gelderblom et al., 1996b). The mechanism by which PB selectively stimulates the outgrowth of FB<sub>1</sub>-induced initiated cells is not known at present. As the PB treatment significantly reduced the rat body and relative liver weight the selective outgrowth of the initiated cells by a mitoinhibitory effect, similar to FB<sub>1</sub> promotion, has to be considered. Previous studies support this finding as PB inhibited the growth stimulatory effect of EGF in primary hepatocyte cultures while prolonged exposure inhibits cell proliferation in rat liver (Manjeshwar et al., 1992; Barbason et al., 1983; Gelderblom et al., 1996b).

The promotion of spontaneously induced initiated hepatocytes resulting in the development of tumors in the liver of older rats has been proposed as a possible explanation for the induction of liver tumors by non-genotoxic carcinogens (Schulte-Hermann, 1983). As both eosinophilic and basophilic foci occur spontaneously in older rats, the continued presence of a non-genotoxic carcinogen acting as a cancer promoter would then eventually result in the development of tumors (Schulte-Hermann et al., 1989). PB and the peroxisome proliferator, nafenopin were shown to enhance the development of tumors in older rats by selectively promote different focal subtypes (Kraupp-Grasl et al., 1991). FB<sub>1</sub> behaves similarly to PB in selecting eosinophilic clear-foci in a two-stage cancer initiating promotion model using DEN as the cancer initiator (Gelderblom et al., 1996a). A chronic feeding study in rats showed that FB<sub>1</sub> induced both basophilic and eosinophilic adenomas in the liver staining positively for GSTP (Gelderblom et al., 2001c; Lemmer et al., 2004). As, FB<sub>1</sub> did not effect the proliferation of peroxisomes, it would appear that, as stated previously, that it induces pre-neoplastic lesions similar to other genotoxic carcinogens such as aflatoxin B<sub>1</sub> (Gelderblom et al., 1992).

The present study showed that FB<sub>1</sub> is more effective in inducing GSTP<sup>+</sup> foci in younger rats presumably reflecting its combined cancer initiation and promoting capacity. The reduced capacity of FB<sub>1</sub>-induced formation of GSTP<sup>+</sup> foci in older rats could be related to a reduction in feed and therefore, FB<sub>1</sub> intake. Studies in male BD IX rats showed that compared to 7- to 8-week-old rats, 8 and 14 months-old rats had a reduced feed intake of approximately 20 and 40%, respectively (Gelderblom et al., 2001c). Assuming a similar scenario in the present study, it would imply that the rats were

exposed to a diet equivalent to 200 and 150 mg FB<sub>1</sub>/kg, respectively, after 26 and 52 weeks. The reduced hepatotoxic effects observed at these time points confirmed a reduced FB<sub>1</sub> intake over the 3-week period of exposure. As the FB<sub>1</sub>-induced hepatotoxic effect is a prerequisite for initiation (Gelderblom et al., 2001a), the mild pathological changes in the liver of the aged rats conform to the low initiating response of FB<sub>1</sub>. In contrast, the apparent dietary levels of 150 and 200 mg FB<sub>1</sub>/kg diet at 52 weeks should still have resulted in cancer promotion as a dietary level of 50 mg FB<sub>1</sub>/kg effectively selected the outgrowth of DEN-initiated cells over 3 weeks (Gelderblom et al., 1996a). It would appear that FB<sub>1</sub> failed to promote the growth of spontaneously induced initiated hepatocytes into eosinophilic clear cell foci in older rats and the induction of these foci in younger rats is related to the combined cancer initiating and promoting potential of FB<sub>1</sub>, as suggested previously (Gelderblom et al., 1994).

In female B6C3F<sub>1</sub> mice, FB<sub>1</sub> significantly enhanced the incidence of spontaneously induced adenomas and carcinomas in a 2-year feed restricted study in (Howard et al., 2001). Although FB<sub>1</sub> is regarded as hepatocarcinogenic in mice, it should be taken into consideration that it only provides evidence for the promotion of spontaneous tumors after prolonged dietary treatment. Exposure of rats to lower dietary levels of FB<sub>1</sub> over a period of 2 years showed a significant increase of pre-neoplastic foci and nodules stained positively for GSTP at a dietary level of 10 mg FB<sub>1</sub>/kg diet (Gelderblom et al., 2001c). In a recent study young male Fischer 344 rats on an initial 5-week feeding (250 mg FB<sub>1</sub>/kg diet) regimen developed liver adenomas 1 year later in the absence of any additional exposure (Lemmer et al., 2004). This observation provides additional evidence about the cancer initiating capacity of FB<sub>1</sub>, and argues against the role of spontaneously initiated hepatocytes. The present study provided further evidence that FB<sub>1</sub> initiates cancer in rat liver as PB promotes the selective outgrowth of these initiated hepatocytes. FB<sub>1</sub> also failed to enhance the formation of eosinophilic clear cell subtype foci stained positively for GSTP in older rats under the present experimental conditions. Although the bulk of evidence thus far point towards cancer initiating capabilities of FB<sub>1</sub>, the induction of liver GSTP<sup>+</sup> hepatocyte nodules and/or foci in rats exposed to low dietary levels for an extended period of time, still cannot rule out the promotion of spontaneous initiated hepatocytes (Gelderblom et al., 2001c).

Studies on the fumonisins as possible human carcinogens are focused on the cancer promoting potential via epigenetic mechanisms involving membrane lipid alterations (Riley et al., 2001; Gelderblom et al., 2001b). However, the genotoxic properties of FB<sub>1</sub> (Ehrlich et al., 2002; Galvano et al., 2002; Domijan et al., 2007), presumably via indirect mechanisms related to oxidative damage (Sahu et al., 1998) resulting in the cancer initiating properties, should be considered. Furthermore synergistic interactions with other carcinogens such as aflatoxin B<sub>1</sub> and dietary iron could, depending on the experimental conditions, enhance the carcinogenic potency of FB<sub>1</sub> (Gelderblom et al., 2002; Lemmer et al., 1999). The modulating effects of dietary constituents could also play an important role in the carcinogenicity of fumonisins (Gelderblom et al., 2004). Depending on specific environmental conditions various parameters may influence the cancer initiating potency of fumonisins that should be taken into account when assessing the risk of fumonisins to human health.

#### Conflict of interest

None.

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## Genotoxic effects of three *Fusarium* mycotoxins, fumonisin B<sub>1</sub>, moniliformin and vomitoxin in bacteria and in primary cultures of rat hepatocytes

Siegfried Knasmüller<sup>a,\*</sup>, Nikolaus Bresgen<sup>b</sup>, Fekadu Kassie<sup>a</sup>,  
Volker Mersch-Sundermann<sup>c</sup>, Wentzel Gelderblom<sup>d</sup>, Edith Zöhrer<sup>a</sup>, Peter M. Eckl<sup>b</sup>

<sup>a</sup> Institute of Tumorbiology-Cancer Research, University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria

<sup>b</sup> Institute of Genetics and Developmental Biology, University of Salzburg, Salzburg, Austria

<sup>c</sup> Institute of Clinical Microbiology, University of Heidelberg, Heidelberg, Germany

<sup>d</sup> PROMEC, Medical Research Council, Tygerberg, South Africa

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### Abstract

The genotoxic effects of three widespread *Fusarium* toxins, vomitoxin (VOM), moniliformin (MON) and fumonisin B<sub>1</sub> (FB<sub>1</sub>) were investigated in bacterial tests and in micronucleus (MN) and chromosomal aberration (CA) assays with primary rat hepatocytes. All three toxins were devoid of activity in gene mutation assays with *Salmonella typhimurium* strains TA98 and TA100 and in SOS chromotests with *E. coli* strain PQ37 in the presence and absence of metabolic activation. FB<sub>1</sub> and VOM gave negative results in differential DNA repair assays with *E. coli* K-12 strains (343/753, *uvrB/recA* and 343/765, *uvr<sup>+</sup>/rec<sup>+</sup>*); with MON, a marginal effect was seen in the absence of metabolic activation mix at relatively high concentrations ( $\geq 55$   $\mu\text{g/ml}$ ). In metabolically competent rat hepatocytes stimulated to proliferate with EGF and subphysiological Ca<sup>2+</sup> concentrations, a decrease of cell division was observed with all three toxins at concentrations  $\geq 10$   $\mu\text{g/ml}$ , VOM was strongly cytotoxic at 100  $\mu\text{g/ml}$ . All three mycotoxins caused moderate increases of the MN frequencies at low concentrations ( $\leq 1$   $\mu\text{g/ml}$ ), but no clear dose–response effects were seen and at higher exposure levels the MN frequencies declined. In the CA experiments with hepatocytes, pronounced dose-dependent effects were observed with all three toxins. MON caused a 9-fold increase over the spontaneous background level after exposure of the cells to 1  $\mu\text{g/ml}$  for 3 h, with FB<sub>1</sub> and VOM, the increases were 6- to 7-fold under identical experimental conditions. This is the first report on clastogenic effects of VOM and FB<sub>1</sub> in mammalian cells, with MON induction of CAs in V-79 cells has been described earlier. Since all three mycotoxins caused CAs at very low concentration levels in liver cells *in vitro*, it is possible that such effects may also occur in humans and mammals upon consumption of *Fusarium*-infected cereals.

**Keywords:** Fumonisin B<sub>1</sub>; Moniliformin; Vomitoxin; Chromosomal aberration; Primary rat liver cell; Mutagenicity; Bacterial mutagenicity test

\* Corresponding author. Tel.: +43 (1) 4015-4348; Fax: +43 (1) 406-0790.

## 1. Introduction

The mycotoxins fumonisin B<sub>1</sub> (FB<sub>1</sub>), vomitoxin (4-deoxynivalenol, VOM) and moniliformin (MON) are produced by toxicogenic *Fusarium* species, and have been identified in substantial amounts in cereals in various continents. Table 1 summarizes the concentration ranges of the different mycotoxins found in European countries, the percentage of contaminated samples and the *Fusarium* species by which they are produced.

The chemical structures of the three compounds

differ substantially (Fig. 1): VOM is a trichothecene derivative, the structure of FB<sub>1</sub> resembles that of sphingosines and the structure of MON is derived from cyclobutene.

Although human foods and animal chows are often contaminated with these compounds, and the acute toxic effects of these toxins are well documented [1,3–7], information on genotoxic and carcinogenic effects of these compounds is limited. A number of epidemiological studies in China and South Africa has indicated that consumption of *Fusarium*-infected food is associated with an in-

Table 1  
Occurrence of vomitoxin, fumonisin B<sub>1</sub> and moniliformin in Europe<sup>a</sup>

Compound	Producer	Cereals contaminated	No. of studies (total number of samples)	Positive samples (%)	Dose range (mg/kg)
Vomitoxin	<i>F. graminearum</i>	wheat, barley	19	42	0.01–500
	<i>F. culmorum</i>	maize, rye	(3817)		
Fumonisin B <sub>1</sub>	<i>F. moniliforme</i> and related species	maize	2 (59)	66	0.01–10
Moniliformin	<i>F. moniliforme</i>	maize	26 (288)	35	0.05–11

<sup>a</sup> Data are from IARC [1] and Smith and Solomons [2]. For Europe, only two studies on fumonisin B<sub>1</sub> are available, but, in addition, data from 9 US studies are available (283 samples, 79% of them positive, dose range 0.05–150 mg/kg).

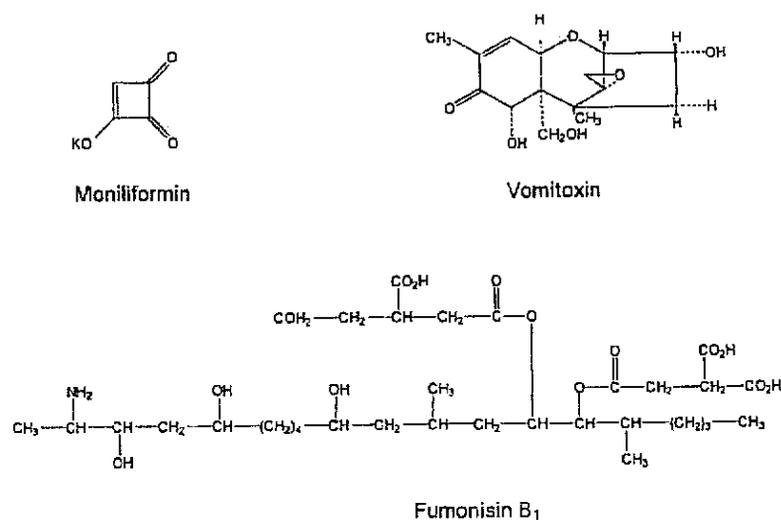


Fig. 1. Chemical structures of the three mycotoxins. Vomitoxin (VOM), trichothec-9-en-8-one,12,13-epoxy-3,7,15-trihydroxy-(3 $\alpha$ ,7 $\alpha$ ); fumonisin B<sub>1</sub> (FB<sub>1</sub>), 1,2,3-propanetricarboxylic acid,1,1'-[(12-amino-4,9,11-trihydroxy-2-methyltridecyl)-2-(1-methylpentyl)-1,2-ethanediy]ester; moniliformin (MON), hydroxy-cyclobutenedion-sodium salt.

creased risk of esophageal cancer [1], but it is still unclear which mycotoxins are responsible for these effects. Regarding the carcinogenicity of the three mycotoxins, FB<sub>1</sub> has been shown to be hepatocarcinogenic in rats, but the underlying molecular mechanisms are not well understood [8]. For VOM and MON, no data of long-term studies in laboratory rodents are available at present.

The aim of the study was to extend the data base on genotoxic effects of the three compounds. Due to the causal relation between DNA damage in somatic cells and cancer, data on mutagenic properties of the toxins will contribute to a better understanding of the potential carcinogenic effects of these toxins. The genotoxic potential of the three mycotoxins was studied in a panel of bacterial tests, namely in gene mutation assays with *Salmonella typhimurium* strains TA98 and TA100, in the SOS chromotest with *E. coli* PQ37 which is based on the induction of  $\beta$ -galactosidase caused by activation of the SOS system and [9,10] and in differential DNA repair assays in which induction or reparable DNA damage is measured by comparing the viability of two *E. coli* strains (*E. coli* 3434/765, *uvr*<sup>+</sup>/*rec*<sup>+</sup> and *E. coli* 343/753, *uvrB*/*recA*) [11] in the presence and absence of metabolic activation. Furthermore, the compounds were tested for induction of chromosomal aberrations (CAs) and micronuclei (MN) in metabolically competent cultures of primary rat hepatocytes [12–14].

The information on mutagenic properties of the three mycotoxins is limited; a few results in gene mutation assays with bacterial indicators are available, in which consistently negative results were obtained; the findings in mammalian mutagenicity assays are quite controversial (for details see Section 4: Discussion).

## 2. Materials and methods

### 2.1. Chemicals

FB<sub>1</sub> and MON were purified according to the method described by Cavood et al. [15] and Steyn et al. [16], respectively, to a purity of 98%. DON was purified from ethyl acetate extracts prepared from rice cultures of *Fusarium graminearum* by subse-

quent chromatographic column separation using silica gel 60 and Sephadex LH 20. Final purification was obtained by crystallisation. Stock solutions of all test compounds were made in sterile dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany). Benzo[*a*]pyrene (BaP), aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), 4-nitroquinoline-*N*-oxide (4-NQO), ethylnitrosourea (ENU), 2-nitrofluorene (2-NF) streptozotocin (SZ), cyclophosphamide (CP), 4-nitrophenyl- $\beta$ -D-galactopyranoside and 4-nitrophenylphosphate were purchased from Sigma (St. Louis, MA). Aroclor 1254 induced rat liver S-9 homogenate (protein concentration, 32 mg/ml) was purchased from Organon Teknika (Durham, NC).

### 2.2. Media

Media for the bacterial assays were purchased from Difco (Detroit, USA), Nutrient Broth No. 2 which was used for the cultivation of the *Salmonella* strains was from Oxoid (Basingstoke, UK). Minimal essential medium (MEM) with Earls salts and non-essential amino acids were from Grand Island Supply Comp. (Paisly, Scotland). The selective agar media for *Salmonella* tests were made as described by Maron and Ames [17]; for DNA repair assays, the media were composed according to Mohn [11].

### 2.3. Bacterial indicator strains

*Salmonella* strains TA98 and TA100 were obtained from B. Ames (Berkeley, CA), *E. coli* PQ37 used for the SOS chromotest was provided by M. Hofnung (Paris, France) and the *E. coli* strains used in the differential DNA repair assays (343/765, *uvr*<sup>+</sup>/*rec*<sup>+</sup> and 343/753, *uvrB*/*recA*) were a gift of G.R. Mohn (RIVM, Bilthoven, The Netherlands).

### 2.4. Animals

Female Fisher 344 rats weighing approximately 100 g were obtained from HARLAN, Zeist (The Netherlands). They were housed in hanging cages at a temperature of 22  $\pm$  2°C and relative humidity of 65  $\pm$  10% in a controlled room with a 12-h light-dark cycle and fed with laboratory chow T783 from Tagger AG (Graz, Austria). Water was provided ad libitum. Sawdust, which served as bedding material,

was obtained from Chemie Linz AG (Linz, Austria). The animals were allowed to acclimatize for at least 2 weeks prior to hepatocyte isolation.

### 2.5. Genotoxicity tests with bacteria

The gene mutation assays with *Salmonella typhimurium* strains were carried out as plate incorporation experiments according to the protocol of Maron and Ames [17]: 0.1 ml of stationary phase cells were plated with 100  $\mu$ l of various concentrations of the test compounds (dissolved in DMSO) with and without 0.5 ml S-9 mix (standard recipe) and 2.0 ml of top agar on selective media plates. The plates were incubated for 48 h, then the number of His<sup>+</sup> revertant colonies was counted manually. Per experimental point, three plates were enumerated.

Differential DNA-repair assays with *E. coli* strains were performed as liquid preincubation assays as described by Knasmüller et al. [18]. The differential survival of the repair deficient relative to the repair proficient strain was calculated as described [18]. One hundred percent survival indicates lack of a genotoxic effect, survival rates < 100% indicate induction of reparable DNA damage.

The SOS chromotest was carried out according to Qillardet and Hofnung [19] with modifications described by Mersch-Sundermann et al. [20]. On the basis of the absorbance values, the ratio of  $\beta$ -galactosidase activities and alkaline phosphatase activities (*R*) were calculated and the induction factor (IF) determined ( $IF = R_D/R_O$ , where  $R_D$  is the ratio at a certain dose and  $R_O$  is the control ratio; for details see Mersch-Sundermann et al. [21]). Per experimental point three measurements were made in parallel.

### 2.6. Experiments with primary rat hepatocytes

Hepatocytes were isolated from female Fischer 344 rats by the in situ two-step collagenase perfusion technique [22]. The isolated hepatocytes were plated at a density of 20 000 viable cells/cm<sup>2</sup> on collagen-coated 60-mm-diameter plastic culture dishes and cultured according to Eckl et al. [23].

The test compounds (dissolved in DMSO) were added to the cultures at different concentrations (5 plates per concentration) and incubated for 3 h. Then

the medium was removed, the plates washed twice with fresh medium and proliferatively stimulated with EGF (40 ng/ml).

For the determination of the mitotic indices and the number of cells with micronuclei, the cultures (two plates per concentration) were fixed 51 h later with cold 3:1 methanol–glacial acetic acid for 5 min on the Petri dishes, rinsed with distilled water for 2 min and air dried. The fixed cells were stained with DAPI according to Eckl et al. [24]. To determine the mitotic index and the number of cells with micronuclei, 1000 cells per Petri dish were analyzed under a fluorescence microscope.

For chromosomal aberration, analyses were carried out according to Reisenbichler and Eckl [25]. At least 20 well-spread metaphases were scored per experimental point to calculate the means  $\pm$  SD for a single experiment. The number of aberrations is given per diploid cell (i.e., 42 chromosomes).

### 2.7. Statistical analysis

Except for SOS chromotests, one or more repetitive experiments were carried out. The data given in Tables 2, 3 and 5 and in Fig. 2 are weighted means and the standard deviations were computed from the pooled variance estimates. Statistical significance was proven in *Salmonella*/microsome assays and in differential DNA repair assays with the Dunnett test. In experiments with primary rat hepatocytes, Student's *t*-test for independent variables was used to calculate the levels of significance.

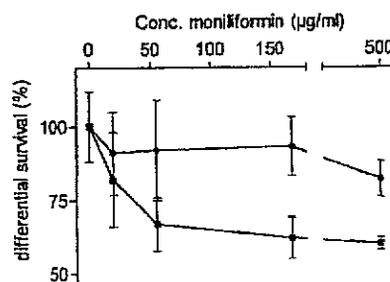


Fig. 2. Induction of reparable DNA damage in *E. coli* by moniliformin in the presence (○) and absence (●) of metabolic activation (experimental points give means  $\pm$  SD of two experiments, 3 plates per experiment).

### 3. Results

The results of the genotoxicity assays with bacterial indicators are summarized in Tables 2–4. It can be seen that all three mycotoxins were devoid of activity in the Salmonella/microsome assays (Table 2) in strains TA98 and TA100 and in the SOS chromotests (Table 3) with *E. coli* PQ37 in the absence and presence of metabolic activation. Also, in preliminary experiments with Salmonella tester strains, in which even higher concentrations of the compounds (up to 2000 µg/plate) were tested, consistently negative results were obtained (data not shown).

In differential DNA-repair assays with *E. coli*, VOM and FB<sub>1</sub> did not induce reparable DNA dam-

age, but with MON, a dose-dependent effect was seen at the two lower concentrations, only with one concentration (55 µg/ml) the results were statistically significant (Table 4, Fig. 2). The addition of S-9 mix resulted in a decrease of this effect.

The results obtained with the three compounds in experiments with primary cultures of rat hepatocytes are listed in Table 5. All three compounds were tested over a broad dose range. The negative control values (MI, MN and CA rates) are in agreement with those found in earlier experiments [25]. CP and AFB<sub>1</sub>, which were used as positive controls, caused significant increases of MN or CA frequencies as expected [12,14]. All three mycotoxins inhibited cell division at the higher dose levels (10–100 µg/ml), with VOM, a pronounced cytotoxic effect was seen

Table 2  
Induction of His<sup>+</sup> revertants in Salmonella strains TA98 and TA100 by vomitoxin, fumonisin B<sub>1</sub> and moniliformin in the presence and absence of metabolic activation<sup>a</sup>

Test compound	Dose (µg/plate)	TA98		TA100	
		Without S-9	With S-9	Without S-9	With S-9
Vomitoxin	0.7	23 ± 1	27 ± 8	99 ± 11	159 ± 10
	2.1	23 ± 5	31 ± 7	108 ± 10	158 ± 12
	6.2	25 ± 5	33 ± 6	87 ± 17	149 ± 12
	19	18 ± 6	29 ± 7	85 ± 17	172 ± 9
	55	24 ± 4	31 ± 15	92 ± 15	159 ± 21
	167	24 ± 3	27 ± 5	104 ± 14	165 ± 7
	500	27 ± 3	27 ± 4	88 ± 13	161 ± 12
Fumonisin B <sub>1</sub>	0.7	28 ± 5	29 ± 9	87 ± 16	152 ± 34
	2.1	20 ± 4	29 ± 10	80 ± 17	157 ± 13
	6.2	25 ± 5	25 ± 5	90 ± 14	171 ± 14
	19	28 ± 10	32 ± 4	87 ± 6	155 ± 9
	55	28 ± 5	26 ± 4	92 ± 18	166 ± 11
	167	21 ± 2	26 ± 8	98 ± 20	160 ± 12
	500	24 ± 4	28 ± 9	80 ± 17	163 ± 14
Moniliformin	0.7	22 ± 9	37 ± 6	112 ± 31	139 ± 29
	2.1	24 ± 7	33 ± 5	92 ± 21	142 ± 12
	6.2	22 ± 4	31 ± 5	103 ± 15	156 ± 20
	19	20 ± 5	34 ± 3	130 ± 21	159 ± 13
	55	26 ± 11	32 ± 4	111 ± 16	161 ± 17
	167	23 ± 3	35 ± 12	96 ± 21	169 ± 31
	500	23 ± 1	39 ± 7	107 ± 20	1621 ± 14
Positive control <sup>c</sup>	400/2	82 ± 10	431 ± 32 <sup>b</sup>	272 ± 34 <sup>b</sup>	157 ± 23 <sup>b</sup>
Negative control	–	24 ± 7	32 ± 8	101 ± 18	552 ± 40

<sup>a</sup> Values are means ± SD His<sup>+</sup> revertants/plate of two representative experiments (3 plates per experimental point per experiment).

<sup>b</sup> Statistically significant (Dunnnett's test,  $p \leq 0.05$ ).

<sup>c</sup> ENU (400 µg/plate) was used as a positive control in tests without metabolic activation; AFB<sub>1</sub> (2 µg/plate) was used in assays with S-9 mix.

with 100 µg/ml; therefore, MN and CAs could not be determined at this dose level. The results of the MN experiments are inconclusive: the MN frequencies were slightly elevated at the lower concentrations (up to 1 µg/ml) and decreased at the higher concentrations, but no clear dose-response effects could be established and statistically significant effects were seen only with VOM and FB<sub>1</sub> at certain doses. Overall, the effects caused by all three mycotoxins were only moderate and far less pronounced than that seen in earlier experiments with other mutagens, such as peroxisome proliferators [25], lipid peroxidation products [24] and CP [14].

The results obtained in the CA experiments clearly indicate that all three *Fusarium* toxins are clastogenic. The CA numbers increased gradually at the lower dose levels and declined at the highest concentrations evaluated (100 µg/ml for MON and FB<sub>1</sub>,

Table 3  
Effects of the mycotoxins vomitoxin, fumonisin B<sub>1</sub> and moniliformin in SOS chromotests with *E. coli* strain PQ 37<sup>a</sup>

Compound	Concentration (µg/assay)	Induction factor	
		Without S-9	With S-9
Vomitoxin	5	0.93 ± 0.044	1.04 ± 0.053
	16	1.01 ± 0.187	0.99 ± 0.014
	50	0.97 ± 0.158	0.99 ± 0.070
	166	0.97 ± 0.122	1.10 ± 0.132
	500	0.91 ± 0.129	0.99 ± 0.134
Fumonisin B <sub>1</sub>	5	1.22 ± 0.123	1.14 ± 0.067
	16	1.06 ± 0.125	0.98 ± 0.055
	50	1.01 ± 0.162	1.15 ± 0.049
	166	1.05 ± 0.068	1.05 ± 0.024
	500	1.14 ± 0.157	1.09 ± 0.072
Moniliformin	5	0.98 ± 0.062	0.96 ± 0.065
	16	0.97 ± 0.090	0.97 ± 0.065
	50	0.92 ± 0.064	0.90 ± 0.053
	166	1.00 ± 0.069	1.02 ± 0.059
	500	1.08 ± 0.196	1.00 ± 0.087
Positive control <sup>c</sup>	0.06/0.6	29.10 ± 4.2 <sup>b</sup>	4.13 ± 0.385 <sup>b</sup>
Negative control	–	1.00 ± 0.000	1.00 ± 0.000

<sup>a</sup> Data indicate means ± SD of induction factors which were calculated on the basis of the ratios of β-galactosidase activities vs. alkaline phosphatase activities. Per experimental point three measurements were made (one experiment).

<sup>b</sup> Statistically significant (Dunnett's test,  $p \leq 0.05$ ).

<sup>c</sup> 4-NQO (60 ng/assay) was used as a positive control in assays without activation, B[a]P (600 ng/assay) was used as a positive control in tests with metabolic activation.

Table 4  
Induction of reparable DNA damage in *E. coli* by vomitoxin, fumonisin B<sub>1</sub> and moniliformin<sup>a</sup>

Test compound	Concentration (µg/ml)	Differential survival (%)	
		Without S-9 mix	With S-9 mix
Vomitoxin	500	104 ± 5	91 ± 11
	167	98 ± 7	89 ± 5
	55	106 ± 5	104 ± 5
	19	99 ± 9	104 ± 12
	6.2	102 ± 14	107 ± 9
	2.1	100 ± 12	99 ± 12
	0.7	95 ± 6	102 ± 5
Fumonisin B <sub>1</sub>	0.7	98 ± 8	100 ± 6
	2.1	102 ± 4	97 ± 12
	6.2	105 ± 12	104 ± 17
	19	104 ± 5	96 ± 25
	55	102 ± 8	103 ± 20
	167	106 ± 6	104 ± 11
	500	99 ± 6	94 ± 7
Moniliformin	0.7	101 ± 8	97 ± 12
	2.1	103 ± 7	96 ± 9
	6.2	109 ± 6	104 ± 12
	19	82 ± 16	91 ± 14
	55	67 ± 9 <sup>b</sup>	92 ± 17
	167	62 ± 7 <sup>b</sup>	93 ± 10
	500	60 ± 2 <sup>b</sup>	82 ± 6 <sup>b</sup>
Positive control <sup>d</sup>	2/2	15 ± 3 <sup>c</sup>	5 ± 3 <sup>c</sup>
Negative control	–	100 ± 12	100 ± 12

<sup>a</sup> Values are means ± SD of differential survival rates of the two strains (*E. coli* 343/753, *uvr*<sup>+</sup>/*rec*<sup>+</sup> vs. *E. coli* 343/765, *uvr*B/*rec*A) measured in two independent experiments (three plates per experimental point per experiment). Different concentrations of the test compounds were incubated with mixtures of the two indicator strains (0.1 ml,  $1-2 \times 10^7$ ) viable cells of each strain and 0.8 ml of PBS or rat liver S-9 mix for 1 h at 37°C. Subsequently, the mixtures were diluted ( $10^{-4}$ ) and 0.1-ml aliquots plated on Neutral red agar plates. After 2 days, the individual strain survival was determined (see Section 2: Materials and methods).

<sup>b,c</sup> Statistically significant (Dunnett's test,  $p \leq 0.05$ ).

<sup>d</sup> SZ (2 µg/ml) was used in tests without metabolic activation, AFB<sub>1</sub> (2 µg/ml) was used in assays with metabolic activation.

10 µg/ml for VOM), probably due to inhibition of cell division. With VOM, the maximal effect was seen at 1 µg/ml at which the CA rate was approximately 6-fold over the background level, with FB<sub>1</sub> the peak was seen with 10 µg/ml (7-fold increase over the background). The strongest effect was seen with MON, which caused a ca. 9-fold increase over

Table 5

Summary of experiments on the induction of micronuclei and chromosomal aberrations by vomitoxin, fumonisin B<sub>1</sub> and moniliformin in primary cultures of rat hepatocytes<sup>a</sup>

Compound	Concentration (µg/ml)	Mitotic index (%)	Micronuclei	Chromosomal aberrations
Vomitoxin	0.001	2.10 ± 0.13 (3)	8.19 ± 2.67 (3)	0.021 ± 0.029 (2)
	0.010	2.05 ± 0.27 (3)	9.81 ± 2.10 (3) <sup>b,d</sup>	0.044 ± 0.076 (3)
	0.100	1.73 ± 0.58 (3)	9.92 ± 5.71 (4)	0.149 ± 0.150 (4)
	1.000	1.38 ± 0.46 (2)	5.80 ± 1.54 (2)	0.200 ± 0.071 (3) <sup>b</sup>
	10.000	1.33 ± 0.03 (2) <sup>b</sup>	6.13 ± 1.09 (2)	0.140 ± 0.028 (2) <sup>cd</sup>
	100.000	cytotoxic	ND	ND
Fumonisin B <sub>1</sub>	0.010	1.98 ± 0.15 (3)	8.24 ± 1.19 (3) <sup>b,d</sup>	0.025 ± 0.043 (3)
	0.100	1.66 ± 0.55 (4)	7.41 ± 1.56 (4)	0.093 ± 0.007 (5)
	1.000	1.03 ± 0.10 (2) <sup>b</sup>	8.12 ± 0.18 (2) <sup>b</sup>	0.212 ± 0.097 (3) <sup>b,d</sup>
	10.000	1.33 ± 0.46 (2)	5.49 ± 0.72 (2)	0.235 ± 0.040 (3) <sup>c</sup>
	100.000	0.95 ± 0.14 (2) <sup>b</sup>	7.89 ± 0.46 (2) <sup>b</sup>	0.145 ± 0.047 (2) <sup>b,d</sup>
Moniliformin	0.010	1.92 ± 0.12 (3)	7.67 ± 1.33 (3)	0.000 ± 0.000 (2) <sup>b</sup>
	0.100	1.69 ± 0.27 (4)	7.94 ± 4.47 (4)	0.149 ± 0.186 (5)
	1.000	1.07 ± 0.46 (2)	8.41 ± 6.14 (2)	0.289 ± 0.153 (3) <sup>b,d</sup>
	10.000	1.30 ± 0.92 (2)	5.87 ± 3.27 (2)	0.285 ± 0.154 (3) <sup>b,d</sup>
	100.000	1.10 ± 0.07 (2) <sup>b</sup>	6.51 ± 2.88 (2)	0.266 ± 0.106 (3) <sup>b</sup>
Positive control <sup>b</sup>				
CP	0.025	0.90 ± 0.14 (4) <sup>c</sup>	9.84 ± 0.80 (4) <sup>c</sup>	ND
AFB <sub>1</sub>	0.031	0.98 ± 0.30 (4) <sup>c</sup>	ND	0.178 ± 0.029 (4) <sup>c</sup>
Negative control	–	1.76 ± 0.46 (5)	6.31 ± 1.27 (4)	0.033 ± 0.025 (4)

<sup>a</sup> Primary cultures of rat hepatocytes were exposed for 3 h to different concentrations of the test compounds and subsequently cultured in EGF-supplemented medium. Values are means ± SD of MN and CA. In each experiment, 1000 cells were evaluated for the determination of MN frequencies and of the MIs. For the determination of CAs, at least 20 well-spread metaphases were evaluated per experimental point. The number of independent experiments is given in brackets. ND, not determined.

<sup>b–d</sup> Statistically significant (Student's *t*-test for independent variables, <sup>b</sup>  $p \leq 0.05$ , <sup>c</sup>  $p \leq 0.005$ , <sup>d</sup> significance under the assumption of equal variances).

the spontaneous rate at 1 and 10 µg/ml. CAs were mainly deletions of both the chromosome and chromatid type; besides, dicentric and ring chromosomes were also found.

#### 4. Discussion

The results of the present experiments indicate that the three mycotoxins are, in general, inactive in bacterial genotoxicity assays, but cause chromosome breakage in primary rat hepatocytes at relatively low concentrations (between 1 and 10 µg/ml). The reasons for the discrepancies of the results seen with all three mycotoxins in bacterial genotoxicity assays and in the CA experiments with metabolically competent cells might either be due to differences in the cellular

organisation of the indicator cells and/or due to differences in the endpoints used.

The negative results obtained with FB<sub>1</sub>, MON and VOM in Ames tests are in agreement with earlier findings [16–29]. In addition, MON was also reported to be devoid of activity in SOS-spot tests with *E. coli* K-12 [30] and in *recA* assays with *Bacillus subtilis* [31].

The data on genotoxic effects of the three mycotoxins in eukaryotic cells are scarce: MON was reported negative in unscheduled DNA synthesis (UDS) experiments with primary rat hepatocytes by Norred et al. [32] and no other data on clastogenic effects or induction of gene mutations in mammalian cells have been published, according to our knowledge. HPLC purified extracts of VOM from contaminated corn have been tested in CA experiments with

V-798 cells by Hsia et al. [33]. In agreement with our observations, they found that VOM induces aberrations (mainly deletions and breaks) at low dose levels and, as in our experiments, a maximal effect was seen with 1.0  $\mu\text{g}/\text{ml}$  which declined at higher dose levels. On the contrary, VOM did not cause gene mutations at the HPRT locus in the same indicator cells [34] and did not induce UDS in primary rat hepatocytes in the 0.1–1000  $\mu\text{g}/\text{ml}$  dose range [35]. Positive results were found in transformation assays with BALB/3T3 mouse embryo cells and the compound also inhibited intercellular gap junctional communication in V-79 cells [36,37], a feature which is characteristic for many tumor promoting agents. These later findings indicate potential carcinogenicity of VOM, but as described above, no data from long-term studies with laboratory rodents are available. It has been shown in a recent study by Rizzo et al. [38] that VOM causes lipid peroxidation in livers of rats following oral administration of a single dose (28 mg/kg). This effect could be inhibited by dietary selenium, vitamin E and ascorbic acid. Based on these observations, the authors concluded, that the acute toxic effects of the mycotoxin are due to the release of free radicals. These observations support the assumption that VOM may cause mutations, as free radicals as well as reaction products of the lipid peroxidation chain (LP) reaction cause DNA damage [39–41]. These findings may also partly explain the discrepancies seen in the present genotoxicity experiments, since it is known that bacterial indicators are relatively insensitive towards radical mediated DNA damage, whereas CA assays with hepatocytes appear to be highly sensitive, as exemplified by results obtained earlier [23].

It has been shown that  $\text{FB}_1$  causes liver cancer in rats and liver foci experiments indicated that the compound acts as a tumor initiator as well as a tumor promoter (for review see [8,42]). The lack of activity in gene mutation assays with *Salmonella* [26] and the negative results obtained in UDS experiments with rat hepatocytes [32,42] have led to the hypothesis that the cancer-initiating effects of the toxin are not associated with DNA damage, but might be due to promotion of spontaneously initiated cells [8]. The present results, however, indicate that  $\text{FB}_1$  causes chromosomal breaks in rat liver cells at

low concentrations and strengthen the assumption that the compound has to be classified as a genotoxic carcinogen. The mechanisms which lead to the induction of chromosomal aberrations are unclear at present. Note in this context that it has been recently [43] found that exposure of primary rat hepatocytes to  $\text{FB}_1$  causes an accumulation of polyunsaturated fatty acids (PUFA). Since it has been shown that the toxic effects of PUFA on mammalian cells are associated with an increase in lipid peroxidation it might be possible that the chromosome-breaking effects of  $\text{FB}_1$  are causally related to this phenomenon.

The present findings show that all three mycotoxins are clastogenic under in vitro conditions at very low concentration levels. As the concentrations of these mycotoxins in *Fusarium*-infected cereals may reach several mg/kg (see Table 1), it is conceivable that internal concentrations in humans might be similar to those found effective under the present experimental conditions. For  $\text{FB}_1$ , data from pharmacokinetic studies with monkeys, pigs and rats are available which indicate that after oral administration of a few mg/kg b.wt., the plasma concentrations reach several hundred ng/ml [44–46]. Therefore it cannot be excluded that chromosome breakage effects may take place under normal exposure conditions in vivo and further experimental work to elucidate this possibility should be conducted.

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## Histopathology and gene expression changes in rat liver during feeding of fumonisin B<sub>1</sub>, a carcinogenic mycotoxin produced by *Fusarium moniliforme*

Eric R.Lemmer<sup>1,5</sup>, Pauline de la Motte Hall<sup>2,3</sup>, Nobuhiko Omori<sup>5</sup>, Masako Omori<sup>5</sup>, Enid G.Shephard<sup>1,6</sup>, Wentzel C.A.Gelderblom<sup>4</sup>, J.Peter Cruse<sup>2</sup>, Rochelle A.Barnard<sup>2</sup>, Walter F.O.Marasas<sup>4</sup>, Ralph E.Kirsch<sup>1</sup> and Snorri S.Thorgeirsson<sup>5</sup>

<sup>1</sup>MRC/UCT Liver Research Centre and <sup>2</sup>Departments of Anatomical Pathology, University of Cape Town, Observatory, Cape 7925, South Africa, <sup>3</sup>Flinders University of South Australia, Bedford Park 5042, South Australia, <sup>4</sup>MRC Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg 7505, South Africa and <sup>5</sup>Laboratory of Experimental Carcinogenesis, National Cancer Institute, Bethesda, MD 20892, USA

<sup>6</sup>To whom correspondence should be addressed  
Email: enid@liver.uct.ac.za

**Fumonisin B<sub>1</sub> (FB<sub>1</sub>) is a carcinogenic mycotoxin produced by the fungus *Fusarium moniliforme* in corn. Feeding of FB<sub>1</sub> to rats causes acute liver injury, chronic liver injury progressing to cirrhosis, and sometimes terminates in hepatocellular carcinoma or cholangiocarcinoma. This study describes the histopathology and changes in gene expression in the rat liver during short-term feeding of FB<sub>1</sub>. Male Fischer rats were fed either FB<sub>1</sub> 250 mg/kg or control diet, and were killed weekly for 5 weeks. FB<sub>1</sub> caused a predominantly zone 3 'toxic' liver injury, with hepatocyte death due to necrosis and apoptosis. Hepatocyte injury and death were mirrored by hepatic stellate cell proliferation and marked fibrosis, with progressive disturbance of architecture and formation of regenerative nodules. Despite ongoing hepatocyte mitotic activity, oval cell proliferation was noted from week 2, glutathione *S*-transferase  $\pi$ -positive hepatic foci and nodules developed and, at later time points, oval cells were noted inside some of the 'atypical' nodules. Northern blot (mRNA) analysis of liver specimens from weeks 3 to 5 showed a progressive increase in gene expression for  $\alpha$ -fetoprotein, hepatocyte growth factor, transforming growth factor alpha (TGF- $\alpha$ ) and especially TGF- $\beta$ 1 and *c-myc*. Immunostaining with LC(1-30) antibody demonstrated a progressive increase in expression of mature TGF- $\beta$ 1 protein by hepatocytes over the 5 week feeding period. The overexpression of TGF- $\beta$ 1 may be causally related to the prominent apoptosis and fibrosis seen with FB<sub>1</sub>-induced liver injury. Increased expression of *c-myc* may be involved in the cancer promoting effects of FB<sub>1</sub>.**

### Introduction

The fumonisin B mycotoxins are natural contaminants of corn infected with the fungus *Fusarium moniliforme* (1).

**Abbreviations:** AAF, acetylaminofluorene; AFP,  $\alpha$ -fetoprotein; AIN, American Institute of Nutrition; FB<sub>1</sub>, fumonisin B<sub>1</sub>; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST $\pi$ ,  $\pi$  class glutathione *S*-transferase; H&E, haematoxylin and eosin; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; PH, partial hepatectomy; TGF- $\alpha$ / $\beta$ , transforming growth factor alpha/beta.

Ingestion of fumonisin B<sub>1</sub> (FB<sub>1</sub>), the major fumonisin produced by the fungus, causes a variety of toxicoses in animals, including equine leukoencephalomalacia (2) and porcine pulmonary edema (3). Human dietary consumption of *Fusarium*-contaminated corn products has been epidemiologically linked to increased rates of esophageal cancer in regions of the world in which corn is the staple grain, such as South Africa (4) and China (5). In rats, feeding with FB<sub>1</sub> causes acute and chronic liver toxicity, bile duct proliferation ('hyperplasia'), fibrosis progressing to cirrhosis, cholangiofibrosis and, often, hepatocellular carcinoma (HCC) and/or cholangiocarcinoma (1,6,7). These carcinogenic mycotoxins are non-genotoxic, and appear to act mainly as promoters (and possibly weak initiators) of tumors (6,8,9).

In addition to initiated hepatocytes, another group of cells, the 'oval cells', proliferate in the liver during experimental hepatocarcinogenesis. This non-specific term was introduced by Farber in 1956 (10), although these cells had been described previously (11). Oval cells have elongated nuclei, scanty cytoplasm and appear very early in the development of several experimental hepatic cancer protocols. Oval cells may either arise from the biliary epithelium (12,13), or else be derived from pluripotential hepatic stem cells (14-16). Oval cells were shown to have the capacity to differentiate to both biliary duct cells and hepatocytes (17-19). Some authors claim that oval cells play no role in hepatocarcinogenesis (20,21), while others have reported that oval cells can give rise to preneoplastic foci and nodules that can progress to cancer (14,15).

The present study describes the histopathology and changes in gene expression in the rat liver during short-term feeding of FB<sub>1</sub>.

### Materials and methods

#### Chemicals

FB<sub>1</sub> was purified from corn cultures of *F.moniliforme* strain MRC 826 as described previously (22). The purity as compared with an analytical standard by high performance liquid chromatography (23) was in the order of 92-95%. The monomethylester derivatives of FB<sub>1</sub>, which are artifacts of the purification procedure (22), constituted the remainder of the FB<sub>1</sub> preparation.

#### Animals and diet

Animal care was according to the criteria outlined in the guide for the care and use of laboratory animals, prepared by the National Academy of Sciences (24). Thirty male Fischer 344 rats weighing between 150 and 200 g were used in all the experiments. The animals were caged individually in a controlled environment at 23-24°C and 50% humidity with a 12 h artificial light cycle. Food and water were available *ad libitum*, and rats were weighed weekly. All the animals received the AIN-76 diet (25) with the following modifications: the corn starch was replaced with glucose/sucrose/corn starch (1:1:1) while sunflower oil was used instead of corn oil as a fat source. Corn products were excluded from the control diet in order to prevent any possibility of contamination by *F.moniliforme*. The cellulose was donated by Sappi Saicor, Umkomaas, Natal, South Africa.

#### Treatments

The FB<sub>1</sub>-containing diet (250 mg FB<sub>1</sub>/kg diet) was prepared as follows: FB<sub>1</sub> stock sample dissolved in methanol (50 ml) was evaporated onto a subsample (200 g) of the diet, and dried in a fume hood at room temperature for 12 h. Subsequently, the subsample was thoroughly mixed into the diet (6 kg) to

obtain the desired concentration of FB<sub>1</sub>. The control diet was treated in a similar way using only an equal volume of methanol. Each diet was prepared in 6 g quantities at a time and stored under nitrogen at 4°C until used.

#### Experimental

The 30 animals were randomly allocated into a group of 20 (treatment group) and a group of 10 (control group), and fed for up to 5 weeks with 250 mg FB<sub>1</sub>/kg or control AIN diet, respectively. Four rats from the treatment group and two rats from the control group were killed weekly till the end of 5 weeks. The livers were harvested, and slices of liver were fixed in 10% neutral buffered formalin for light microscopy and immunohistochemistry. The remaining liver was snap frozen in liquid nitrogen and stored at -70°C for mRNA analysis. Liver specimens from day 9 post partial hepatectomy combined with acetylaminofluorene (AAF-PH regimen) from another study (26) were included to serve as positive controls for oval cell proliferation. Maximal oval cell proliferation is known to occur at this time point post AAF-PH (27).

#### Light microscopy

For routine light microscopy, slices of liver 4–5 mm in thickness were immersion fixed in 10% neutral buffered formalin for 24 h before processing, embedding in paraffin wax and sectioning at 4 µm. Stains included haematoxylin and eosin (H&E), sirius red for collagen, and Gordon and Sweet's method for reticulin. Coded sections were examined for evidence of hepatocyte injury, apoptotic bodies, fatty change, mitoses, architectural distortion, fibrosis, regenerative nodules and oval cell proliferation. Cells undergoing apoptosis were detected *in situ* by TUNEL specific labeling of nuclear DNA strand breaks, as described by Gavrieli *et al.* (28).

#### Immunohistochemistry

Staining with Desmin D33 (Dako, Copenhagen, Denmark) for hepatic stellate (Ito) cells, and with rabbit polyclonal glutathione *S*-transferase  $\pi$  (GST $\pi$ ; Novacastra, Newcastle-Upon-Tyne, UK) for enzyme-altered hepatic foci and preneoplastic nodules was performed on paraffin sections. After sequential layering with biotinylated rabbit anti-mouse or swine anti-rabbit (Dako) 1:250 dilution as link antibodies, peroxidase conjugated Streptavidin (Dako) 1:500 was applied for 30 min at room temperature. The OV-6 mouse monoclonal antibody, which stains both oval cells and bile duct cells, was a generous gift from Professor Stewart Sell (Albany, NY). Acetone fixed cryostat sections were brought to room temperature and stained by means of a standard two-stage indirect peroxidase conjugated technique (Dako P161). Antibody to mature TGF- $\beta$ 1 protein was a generous gift from Dr K.Flanders (National Cancer Institute, Bethesda, MD). Immunohistochemical staining for transforming growth factor beta-1 (TGF- $\beta$ 1) protein was performed on 5 µm deparaffinized sections with an indirect immunoperoxidase antiserum detection protocol (Elite kit; Vector Laboratories). Mature TGF- $\beta$ 1 protein was detected by the rabbit polyclonal LC(1–30) antibody, as described previously (29).

#### Probes

Antisense riboprobes labeled with [<sup>32</sup>P]CTP were utilized for each of the following. A 429 bp piece of the 5' end rat  $\alpha$ -fetoprotein (AFP) cDNA subcloned into pGEM-4Z (kindly provided by Dr Thomas D.Sargent, National Institute of Child Health and Human Development, Bethesda, MD) was linearized by *Pst*I and transcribed by SP6 RNA polymerase (30). A 600 bp cDNA fragment encoding the 3' end of rat hepatocyte growth factor (HGF) subcloned into the pBluescript SK vector (kindly provided by Dr Brian Carr, University of Pittsburgh School of Medicine, Pittsburgh, PA) was linearized by *Hind*III and transcribed by T3 RNA polymerase. A 335 bp fragment of rat TGF- $\alpha$  cDNA was obtained by RT-PCR and cloned as described previously (31). *Eco*RV and SP6 RNA polymerase were used for its linearization and *in vitro* transcription. A 985 bp fragment of rat TGF- $\beta$ 1 cDNA cloned in pBluescript II KS+ vector (kindly provided by Dr Su Wen Qian, National Cancer Institute, Bethesda, MD) was linearized by *Xho*I and transcribed by T3 RNA polymerase. Mouse cDNA for *c-myc* subcloned into pGEM4 was linearized by *Eco*RI and transcribed by T7 RNA polymerase. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe labeled with [<sup>32</sup>P]dCTP by the random primer extension method was used as an internal control (32).

#### RNA isolation and northern blot analysis

RNA was extracted from rat liver with guanidium thiocyanate followed by centrifugation in cesium chloride solution. Poly(A)<sup>+</sup> RNA was selected by oligo(dT)-cellulose chromatography. Ten micrograms of poly(A) RNAs per lane were electrophoresed on 0.8% agarose gels containing 2.22 mol/l formaldehyde and were later transferred to nylon filters. After UV cross-linking, the filters were hybridized with riboprobes at 60°C and at 42°C with cDNA probes. Blots were washed twice each with 1× standard sodium citrate/0.1% sodium dodecyl sulfate (SSC/SDS) at room temperature, 0.1× SSC/SDS at room temperature, and 0.1× SSC/SDS at 60°C. Autoradiography was per-

formed on Kodak X-OMAT AR film (Rochester, NY) at -70°C using an intensifying screen.

## Results

### Light microscopy and immunohistochemistry

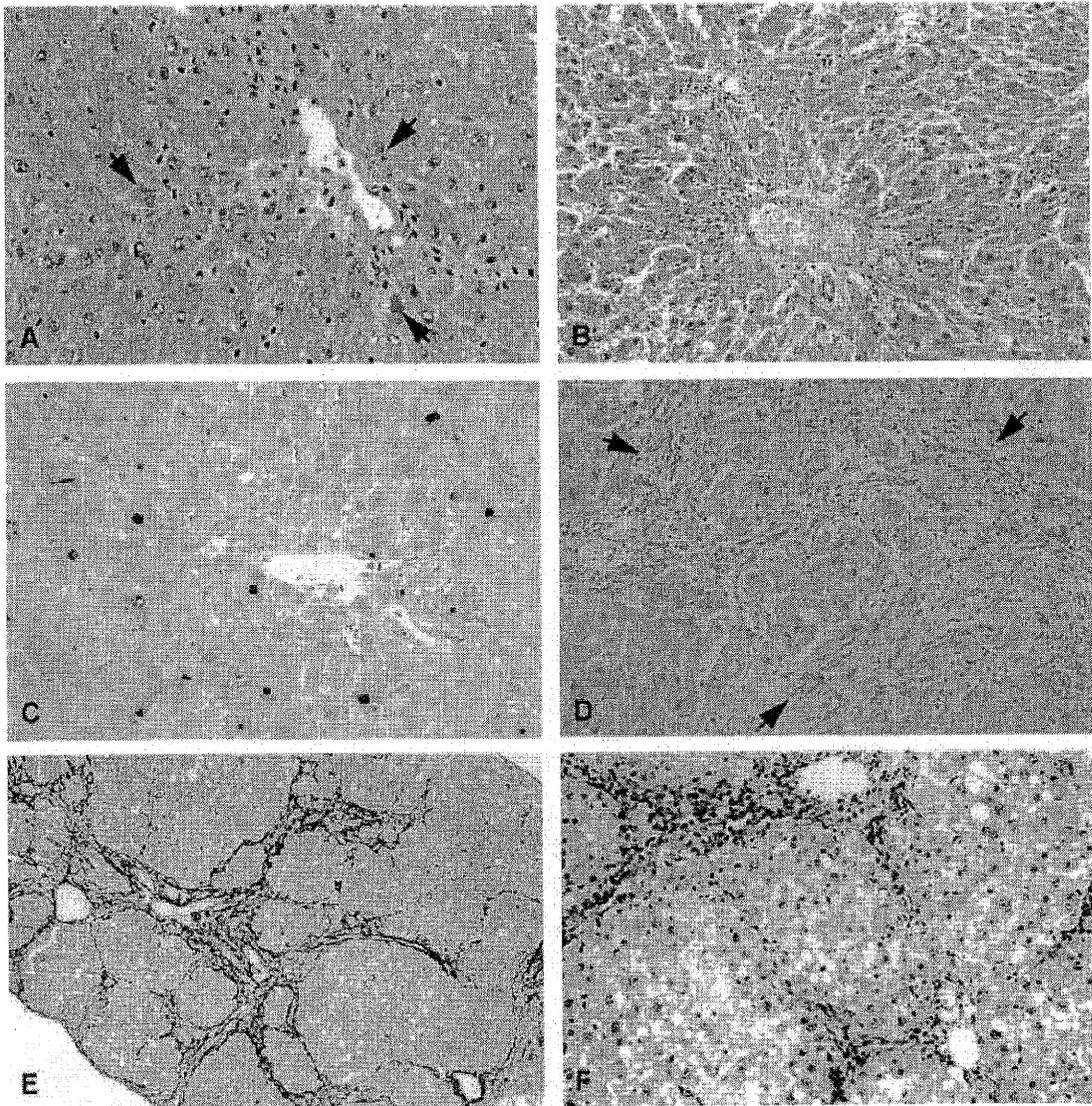
Staining with H&E showed that FB<sub>1</sub> 250 mg/kg diet caused a toxic injury in livers of all treated animals. At week 1, necroinflammatory lesions were confined to zone 3 of the liver acinus. Hepatocyte death was due to both necrosis and apoptosis, and numerous apoptotic bodies and a mild infiltrate of mononuclear cells were seen (Figure 1A). Loss of hepatocytes, due to fragmentation of the apoptotic cells, was accompanied by collapse of the reticulin framework. Scattered mitoses and mild to moderate macrovesicular fatty change in hepatocytes were also present. By week 2, oval cell proliferation was just discernible in the portal tracts (Figure 1B) and apoptosis was apparent in all zones of the liver together with mild and variable fatty change. Apoptosis continued to be seen in all zones of the liver at weeks 3, 4, and 5, and was confirmed by the TUNEL method (Figure 1C). Frequent hepatocyte mitoses were also seen in all zones at each time point. By week 5, marked oval cell proliferation, seen as single small epithelial cells, cords of cells and small ductules, was seen in all portal tracts with extension into the adjacent hepatic parenchyma (Figure 1D). By week 4, there was considerable distortion of the architecture due to the presence of bands of fibrous tissue linking terminal hepatic venules to mildly fibrotic portal tracts, and early regenerative nodule formation. The architectural distortion progressed and by week 5 the features were those of a developing cirrhosis (Figure 1E and F). Numerous desmin positive hepatic stellate cells were seen in the injured zone 3 regions at week 1 (Figure 2A). At all subsequent time points, stellate cells were seen in the portal tracts and were also scattered through zones 1, 2 and 3. However, hepatic stellate cell proliferation appeared to be maximal at 3 weeks, whereafter the overall numbers appeared to decrease, but with a predominance in the portal tracts and zone 1 (Figure 2B).

Positive staining with OV-6 confirmed that the small epithelial cells, seen as single cells, cords and ductules (Figure 1B) were oval cells (Figure 2C). Maximal proliferation of oval cells was seen during weeks 3–5 (Figure 2D). Initially, the oval cells stained with GST $\pi$ , but at 4 weeks following treatment with FB<sub>1</sub>, many oval cells did not express GST $\pi$  (Figure 2E).

After 1 week, single GST $\pi$ -positive hepatocytes were scattered throughout the acini, and by week 2 there were GST $\pi$ -positive enzyme-altered hepatic foci, varying from one to three per section of liver. The GST $\pi$ -positive hepatic foci increased in number and size from weeks 3 to 5. Some foci were close to the terminal hepatic venules while others abutted on the portal tracts. By weeks 4 and 5 some rats had developed large atypical nodules in the liver, which were readily seen in the H&E sections and confirmed by GST $\pi$  staining. Of note was the close relationship of some hepatic foci and nodules to portal tracts that contained the proliferating oval cells, and the presence of cells with morphological features of oval cells inside several of these atypical nodules (Figure 2F).

### Expression of AFP, HGF and TGF- $\alpha$

Transcripts for AFP were not detected in normal liver. Feeding with FB<sub>1</sub> 250 mg/kg resulted in a progressive increase in the expression of the 2.1 kb AFP transcripts from weeks 3 to 5.



**Fig. 1.** Liver pathology in the fumonisin-fed rat. (A) Liver at week 1 showing a terminal hepatic venule (center right), numerous apoptotic bodies (arrows), focal hepatocyte drop-out and a sparse infiltrate of mononuclear cells in zone 3. H&E, objective  $\times 20$ . (B) Liver at week 2 showing mild oval cell proliferation seen as a cord of small epithelial cells and a small ductule (center top) in a portal tract and a sparse infiltrate of mononuclear cells. H&E, objective  $\times 10$ . (C) Liver at week 3 showing a terminal hepatic venule (center) and numerous apoptotic hepatocytes scattered throughout the parenchyma. TUNEL method, objective  $\times 10$ . (D) Liver at week 5 showing moderately fibrotic portal tracts which are linked by bands of fibrous tissue, and numerous small, proliferating ductules (arrows). (E) Liver at week 5 showing loss of the normal acinar architecture due to the presence of regenerative nodules of hepatocytes which are partially or completely surrounded by bands of fibrous tissue. Gordon and Sweets stain for reticulin, objective  $\times 4$ . (F) Liver at week 5 showing the features of an early/developing cirrhosis. Sirius red, objective  $\times 10$ .

The peak level of AFP expression was, however, much less than that seen at day 9 post AAF-PH (Figure 3).

Transcripts for HGF were also not detected in normal liver. Feeding with FB<sub>1</sub> 250 mg/kg resulted in a moderate but sustained increase in expression of HGF from weeks 3 to 5. HGF expression in FB<sub>1</sub>-fed animals was much less than that seen at day 9 post AAF-PH (Figure 3).

There was no expression of TGF- $\alpha$  mRNA in normal liver. Short-term feeding with FB<sub>1</sub> 250 mg/kg resulted in a moderate and fluctuating increase in expression of TGF- $\alpha$  to levels that were similar to that seen at day 9 post AAF-PH (Figure 3).

#### *Expression of TGF- $\beta$ 1 and c-myc*

Some expression of TGF- $\beta$ 1 mRNA was detected in normal liver. Feeding with FB<sub>1</sub> 250 mg/kg resulted in a marked and

progressive increase in expression of TGF- $\beta$ 1 from weeks 3 to 5. The maximum level of expression of TGF- $\beta$ 1 in FB<sub>1</sub>-fed rats greatly exceeded that seen at day 9 post AAF-PH (Figure 4).

Transcripts for *c-myc* were detectable at a low level in normal liver. Feeding with FB<sub>1</sub> 250 mg/kg resulted in a marked increase in expression of *c-myc*, with maximum levels being expressed at week 4. This level of expression of *c-myc* was similar to that seen at day 9 post AAF-PH (Figure 4).

#### *Immunostaining for TGF- $\beta$ 1 protein*

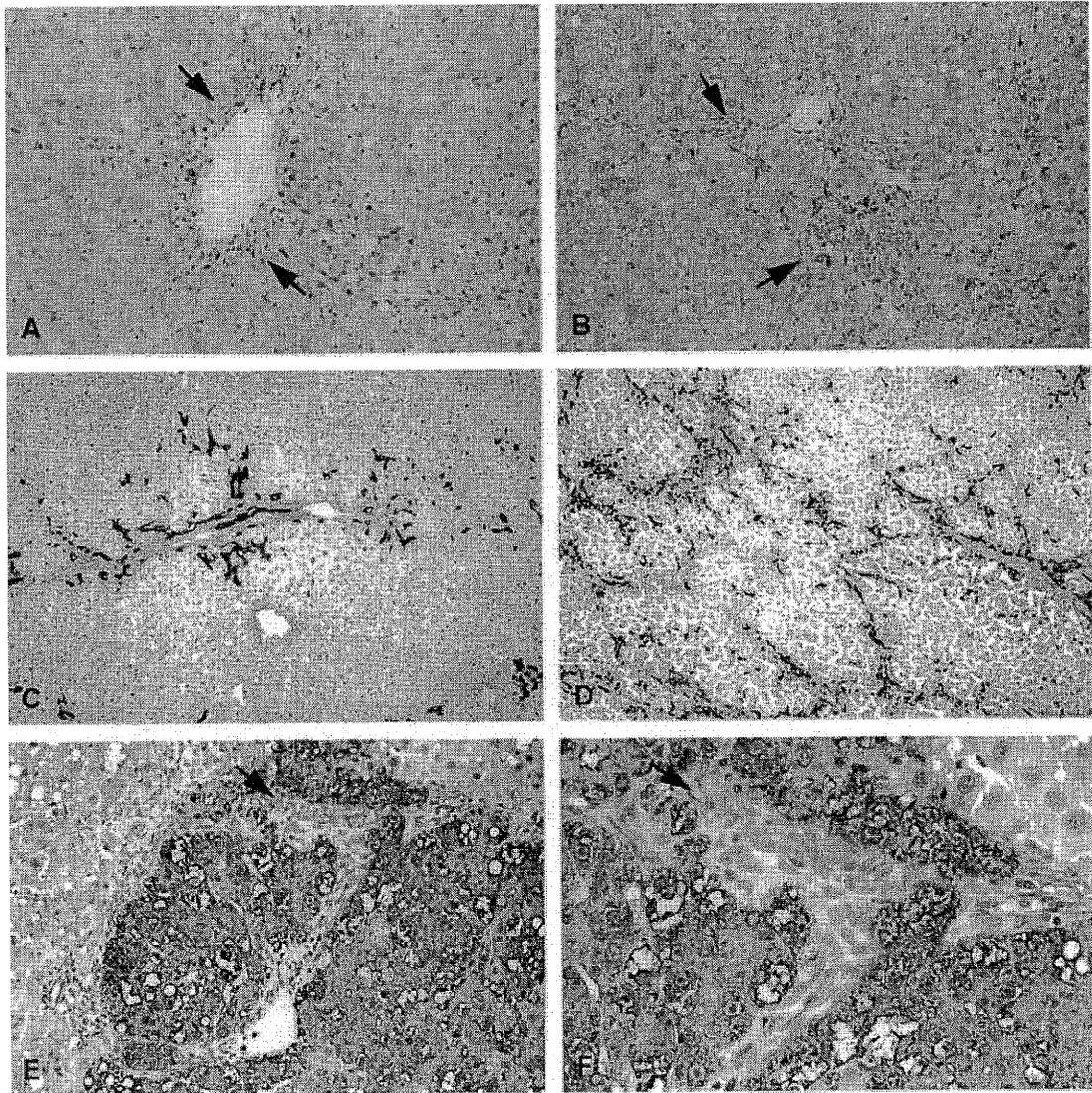
Staining with LC(1-30) antibody demonstrated a progressive increase in expression of mature TGF- $\beta$ 1 protein in the rat liver over the 5 week FB<sub>1</sub> feeding period (Figure 5B-D). Expression of TGF- $\beta$ 1 protein by hepatocytes was initially

focal (Figure 5B), but with continued feeding the protein was abundantly expressed in most hepatocytes (Figure 5C and D). TGF- $\beta$ 1 protein was not detected in zone 3 hepatocytes, which showed evidence of frequent mitoses and apoptosis. Oval cells, stellate cells and Kupffer cells were consistently negative for TGF- $\beta$ 1 protein.

### Discussion

FB<sub>1</sub> has been shown to be hepatotoxic (1) and hepatocarcinogenic (6) in rats. The principal pathological change in rats treated with FB<sub>1</sub> in the diet (1000 mg/kg) in short-term toxicity tests (21–33 days) is progressive toxic hepatitis

characterized by hepatocellular necrosis, bile duct proliferation ('hyperplasia') and fibrosis (1). During a chronic feeding study over a period of 26 months with FB<sub>1</sub> 50 mg/kg, animals developed a chronic toxic hepatitis that progressed to cirrhosis and cholangiofibrosis, and which terminated in hepatocellular carcinoma and cholangiocarcinoma, respectively (6). In a preliminary study, Voss *et al.* (7) reported that FB<sub>1</sub> was hepatotoxic in rats fed a diet containing 150 mg/kg for 4 weeks and also nephrotoxic at 15–50 mg/kg. Scattered single cell necrosis, bile duct proliferation and an increase in mitotic cells were described. The scattered single cell necrosis was subsequently confirmed as apoptosis (33).



**Fig. 2.** Immunohistochemical studies of livers from FB<sub>1</sub>-fed rats. (A) Liver at week 1 showing numerous desmin positive hepatic stellate cells which are located in the zone 3 region of the liver (arrows) with a similar distribution to the liver injury shown in Figure 1. P-A-P, objective  $\times 20$ . (B) Liver at week 3 showing numerous desmin positive hepatic stellate cells which are located in the portal tracts and zone 1 regions (arrows). Numerous proliferating oval cells are also seen in the portal tracts, and the hepatocytes show mild fatty change. Objective  $\times 10$ . (C) Liver at week 2 showing small numbers of OV-6 positive single cells and small ductules in the portal tracts. The OV-6 positive cells correspond to the small epithelial cells and ductules of the type seen in Figure 1B. Objective  $\times 10$ . (D) Liver at week 5 showing numerous proliferating oval cells, which are seen as single cells and small ductules that are OV-6 positive, located in the portal tracts and adjacent liver parenchyma. The OV-6 positive cells correspond to the small ductules seen in Figure 1D. Objective  $\times 4$ . (E) Liver at week 5 showing an atypical nodule composed of GST $\pi$ -positive hepatocytes which is located next to a portal tract (left). Proliferating oval cells, which are GST $\pi$  negative, are seen in the portal tract and within the nodule (arrow). Objective  $\times 20$ . (F) Higher magnification of the liver in Figure 2E showing oval cells (arrow) with a group of GST $\pi$ -positive hepatocytes. Objective  $\times 40$ .

In this short-term feeding study, FB<sub>1</sub> 250 mg/kg caused toxic liver injury, initially with apoptosis in zone 3, followed by apoptosis and mitoses in all zones of the liver, progressive hepatic fibrosis and regenerative nodule formation and development of enzyme-altered hepatic foci and atypical nodules. The bile duct proliferation (hyperplasia) previously described by Gelderblom *et al.* (1) was confirmed in this study as proliferation of OV-6 positive oval cells, radiating from portal tracts into the adjacent liver parenchyma. Oval cell proliferation occurred despite clear evidence of continued hepatocyte regeneration at all time points. Proliferation of adult hepatocytes has been noted in other models for oval cell activation, including galactosamine (34) and dipin (35). FB<sub>1</sub> has been reported to cause a dose-dependent inhibition of PH-induced incorporation of [<sup>3</sup>H]thymidine in hepatocytes (36), and bile ductular hyperplasia has been noted at FB<sub>1</sub> dosages of 50 mg/kg diet (unpublished data). FB<sub>1</sub>-induced oval cell proliferation does not appear to be an 'all-or-nothing' phenomenon, which occurs only when hepatocyte regeneration is absent. Thus, complete mitoinhibition of hepatocytes does not appear to be an absolute requirement for the activation of the progenitor cell compartment, and other factors (e.g. functional impairment of hepatocytes) may conceivably also play a role.

Immunohistochemical staining demonstrated marked proliferation of desmin positive hepatic stellate cells from week 2, which appeared to mirror the distribution of the hepatocyte injury as well as the development of hepatic fibrosis. Hepatic stellate cells are perisinusoidal non-parenchymal cells which in normal liver are non-proliferative and are the main storage site for vitamin A (37). Following liver injury of any

kind, stellate cells undergo 'activation' and transformation with loss of intracellular retinoid, enhanced production of extracellular matrix proteins, increased contractility and secretion of a variety of growth factors and cytokines which act in an autocrine and paracrine fashion on cells in the liver (27). Hepatic stellate cells thus play a major role in hepatic fibrogenesis (37).

There is currently no information on changes in hepatic gene expression during feeding of FB<sub>1</sub>. Feeding with FB<sub>1</sub> 250 mg/kg resulted in increased expression of HGF, TGF- $\alpha$  and AFP transcripts at weeks 3, 4 and 5, which coincided with desmin positive stellate cell and OV-6 positive oval cell proliferation. AFP expression was used as a marker for oval cell proliferation (38). HGF and TGF- $\alpha$  are important growth factors in the liver, and are involved in both normal liver regeneration (39,40) and activation/proliferation of the oval cell compartment (16,41). Expression of transcripts for HGF and TGF- $\alpha$  during feeding of FB<sub>1</sub> was less than that seen at day 9 post AAF-PH (maximal oval cell proliferation). The moderately increased but sustained expression of genes for liver growth factors during feeding with FB<sub>1</sub> presumably reflects ongoing hepatotoxicity. This contrasts with the AAF-PH model, in which massive but transient liver injury and growth factor expression occurs.

FB<sub>1</sub> feeding resulted in marked overexpression of TGF- $\beta$ 1 in rat liver. Contrary to what was anticipated, TGF- $\beta$ 1 protein was expressed almost exclusively by hepatocytes, while stellate cells and oval cells were persistently negative. Immunostaining demonstrated a progressive increase in the numbers of hepatocytes expressing mature TGF- $\beta$ 1 protein during FB<sub>1</sub> feeding, and by 3 weeks most hepatocytes in the liver lobule were stained by LC(1-30) antibody. However, zone 3 hepatocytes remained persistently negative for TGF- $\beta$ 1 protein, and many of these hepatocytes were undergoing mitosis or apoptosis. The markedly increased levels of TGF- $\beta$ 1 expression by hepatocytes may be responsible for the marked apoptosis and fibrosis seen in the FB<sub>1</sub>-fed rat.

The prominent pro-apoptotic effects of FB<sub>1</sub> in rat liver are intriguing, and contrast with the *in vitro* effects of this mycotoxin on chemotherapy-mediated tumor cell destruction (42). FB<sub>1</sub> has been found to have specific, potent activity as an inhibitor of sphingolipid biosynthesis by blocking the conversion of sphinganine to ceramide (43-45). Ceramide is a recently identified lipid second messenger that is believed to be one of the immediate signals for cell death generated in tumor cells treated with the chemotherapeutic agent, daunorubicin (46). The production of ceramide and the ensuing onset of apoptosis in murine leukemia cells cultured in the presence of daunorubicin is prevented by pretreatment with FB<sub>1</sub> (42). The molecular mechanisms of action of FB<sub>1</sub> are thus complex, and it appears that this fungal toxin may act as either an inhibitor or promoter of apoptosis, depending on the experimental situation (47).

At later time points in this study, we noted the close relationship of some 'pre-malignant' hepatic foci and nodules to portal tracts that contained proliferating oval cells and ductules, and the presence of oval cells and ductules inside several of these nodules. It is possible that some of these oval cells were in fact adjacent to ('swirling around') the pre-malignant nodules, depending on the plane of section of the liver specimens. These cells showed typical morphological features of oval cells, but did not express GST $\pi$ , thus aiding their recognition within the GST $\pi$ -positive nodules.

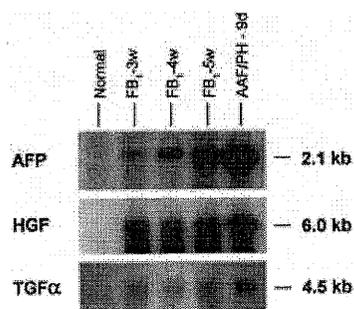


Fig. 3. Analysis of expression of poly(A)<sup>+</sup> RNA (10  $\mu$ g per lane) for AFP, HGF and TGF- $\alpha$  by northern blotting during weeks 3-5 of FB<sub>1</sub> feeding. Timed rat liver specimens from day 9 post AAF-PH served as positive controls.

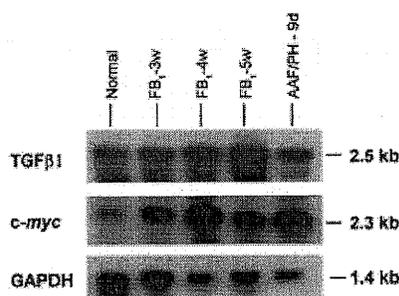


Fig. 4. Analysis of expression of poly(A)<sup>+</sup> RNA (10  $\mu$ g per lane) for TGF- $\beta$ 1 and *c-myc* by northern blotting during weeks 3-5 of FB<sub>1</sub> feeding. Timed rat liver specimens from day 9 post AAF-PH served as positive controls. GAPDH poly(A)<sup>+</sup> RNA was used as loading control.

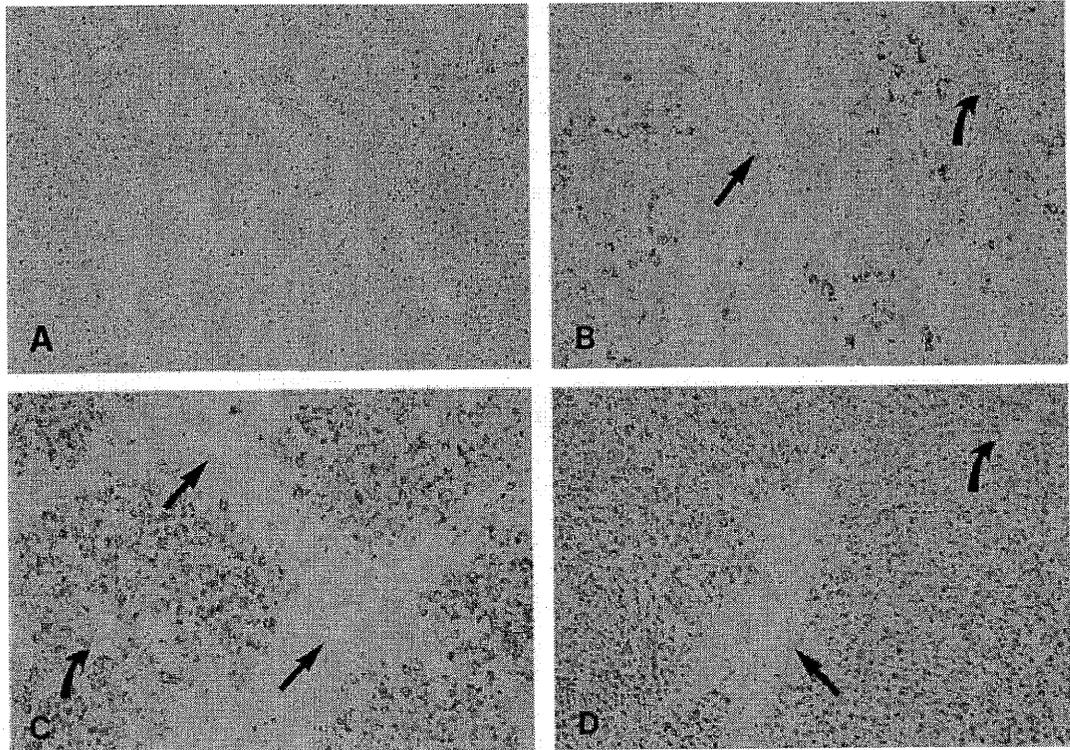


Fig. 5. Mature TGF- $\beta$ 1 protein in timed liver specimens from FB<sub>1</sub>-fed rats, detected by staining with LC(1–30) antibody. (A) Control, with omission of the primary antibody. (B) Liver at 1 week, showing patchy expression of TGF- $\beta$ 1 by hepatocytes. (C) Liver at 2 weeks, showing expression of TGF- $\beta$ 1 by most hepatocytes in zones 1 and 2. (D) Liver at 3 weeks, showing extensive expression of TGF- $\beta$ 1, but persistent lack of staining by zone 3 hepatocytes. DAB, objective  $\times 10$ . (Straight arrows indicate central veins; curved arrows indicate portal tracts.)

Feeding of FB<sub>1</sub> also resulted in overexpression of *c-myc*, an important nuclear oncogene. The *myc* oncoproteins act as sequence-specific transcription factors that regulate a variety of genes important in normal cellular growth and differentiation processes (48,49). Deregulation of *c-myc* expression is frequently observed in experimentally induced HCC in rodents, as well as in primary human tumors (50–53). Transgenic mice chronically overexpressing *c-myc* develop liver tumors, and this process is accelerated by concomitant overexpression of TGF- $\alpha$  (54). Mechanisms of oncogenesis in *c-myc*/TGF- $\alpha$  double transgenic mice appear to involve disruption of the Rb/E2F pathway and deregulation of cell cycle control (55). Both transgenes contribute to induction of cyclin D1 expression and resultant inactivation of Rb, and *c-myc* may directly induce E2F (55). Short-term feeding of FB<sub>1</sub> has recently been shown to cause overexpression of cyclin D1 (56), and it would be important in the future to study the co-expression of *c-myc*, cyclin D1 and E2F in rat liver during FB<sub>1</sub>-induced carcinogenesis.

### Conclusion

Short-term feeding with FB<sub>1</sub> causes a severe toxic liver injury characterized by hepatocyte death due to apoptosis and necrosis, hepatic stellate cell proliferation, fibrosis, oval cell proliferation and the appearance of premalignant hepatic foci and nodules. Oval cells closely related to foci and nodules appeared to be undergoing phenotypic changes, and long-term FB<sub>1</sub> feeding studies are required to determine the ultimate fate of these oval cells. The markedly increased expression of TGF- $\beta$ 1 by hepatocytes may be causally related to the prominent

apoptosis and fibrosis seen in FB<sub>1</sub>-induced liver injury, and overexpression of *c-myc* may be involved in the cancer promoting effects of FB<sub>1</sub>. The FB<sub>1</sub>-fed rat is an attractive model for the study of liver injury, apoptosis, fibrosis, oval cell proliferation and hepatocarcinogenesis.

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## A potential mechanism for fumonisin B<sub>1</sub>-mediated hepatocarcinogenesis: cyclin D1 stabilization associated with activation of Akt and inhibition of GSK-3 $\beta$ activity

Darica Ramljak<sup>7</sup>, Richard J. Calvert<sup>1</sup>,  
Paddy W. Wiesenfeld<sup>2</sup>, Bhalchandra A. Diwan<sup>3</sup>,  
Branimir Catipovic<sup>4</sup>, Walter F.O. Marasas<sup>5</sup>,  
Tommie C. Victor<sup>6</sup>, Lucy M. Anderson and  
Wentzel C.A. Gelderblom<sup>5</sup>

Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick Cancer Research and Development Center, Building 538, Room 205E, Frederick, MD 21702, <sup>1</sup>Clinical Research and Review Staff and <sup>2</sup>Division of Science and Applied Technology, Office of Special Nutritionals, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Laurel, MD 20708, <sup>3</sup>Intramural Research Support Program, SAIC Frederick, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702, <sup>4</sup>Asthma and Allergy Center, Johns Hopkins University, Baltimore, MD 21224, USA, <sup>5</sup>Research Institute for Nutritional Diseases, South African Medical Research Council, PO Box 70 and <sup>6</sup>University of Stellenbosch, Medical Research Council Center for Molecular and Cellular Biology, PO Box 19063, Tygerberg 7505, South Africa

<sup>7</sup>To whom correspondence should be addressed  
Email: ramljak@mail.ncifcrf.gov

**Fumonisin B<sub>1</sub> (FB<sub>1</sub>) is a worldwide corn contaminant and has been epidemiologically linked to the high incidence of human esophageal cancer in South Africa and China. FB<sub>1</sub> is hepatocarcinogenic in rats by an unknown mechanism. Inhibition of ceramide synthase and disruption of membrane phospholipids have been shown to be mechanisms of toxicity. Here we show overexpression of cyclin D1 protein in both preneoplastic and neoplastic liver specimens obtained from a long-term feeding study of FB<sub>1</sub> in rats. In rats fed FB<sub>1</sub> short-term, cyclin D1 protein levels in liver were increased up to five-fold in a dose-responsive manner. Northern blot analysis demonstrated no increase in mRNA levels of cyclin D1. 2D electrophoresis of cyclin D1 protein in FB<sub>1</sub>-treated samples showed a distinct pattern of migration (presence of less negatively charged form of the protein) that differed from controls. Recently, it has been shown that phosphorylation of cyclin D1 by glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) on a single threonine residue (Thr-286) positively regulates proteosomal degradation of cyclin D1. In FB<sub>1</sub>-treated samples we detected GSK-3 $\beta$  phosphorylated on serine 9; activated protein kinase B (Akt) appears to be responsible for this activity-inhibiting phosphorylation. These findings suggest that overexpression of cyclin D1 results from stabilization due to a lack of phosphorylation mediated by GSK-3 $\beta$ . We also observed an increase in cyclin dependent kinase 4 (Cdk4) complexes with cyclin D1 in FB<sub>1</sub>-treated samples; additionally, elevated Cdk4 activity was shown by increased phosphorylation of the retinoblastoma protein. In summary, the activation of Akt leads to increased survival, inhibition of GSK-3 $\beta$  activity**

**Abbreviations:** Akt, protein kinase B; Cdk4, cyclin dependent kinase 4; DEN, *N*-nitrosodiethylamine; FB<sub>1</sub>, fumonisin B<sub>1</sub>; GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; HCC, hepatocellular carcinoma; IEF, isoelectric focusing; IHC, immunohistochemistry; MAPK, mitogen-activated protein kinase; PB, phenobarbital; PCNA, proliferating cellular nuclear antigen; PI3K, phosphatidylinositol-3-OH kinase; pRb, retinoblastoma protein; Ser<sup>9</sup>, serine 9; SSCP, single-strand conformation polymorphism.

and post-translational stabilization of cyclin D1, all events responsible for disruption of the cell cycle G<sub>1</sub>/S restriction point in hepatocytes. This is the first report suggesting the mechanism by which FB<sub>1</sub> acts as a carcinogen.

### Introduction

The mycotoxin fumonisin B<sub>1</sub> (FB<sub>1</sub>) is carcinogenic in rat liver, causing primary hepatocellular carcinomas (HCCs) when fed chronically at 50 mg/kg of diet (1). It is produced by the ubiquitous fungus, *Fusarium moniliforme*, a worldwide contaminant of major grain crops, especially abundant in corn and corn-based products for both animal and human consumption (2). FB<sub>1</sub> may be a contributing factor in the development of esophageal cancer in people of South Africa and China (3). It has been shown to be present in corn-based food products in the USA at levels up to 2.7 p.p.m., and in some cases is present together with the hepatocarcinogenic aflatoxin (4).

A mechanism for FB<sub>1</sub> toxicity involves disruption of sphingolipid metabolism by inhibition of the enzyme *N*-acyltransferase (ceramide synthase), responsible for the conversion of sphinganine to sphingosine. This enzymatic inhibition leads to a decrease in cellular ceramide levels (5). *In vitro* studies in primary hepatocytes (6) and *in vivo* studies in rat liver (7) suggest that the disruption of phospholipid, cholesterol and fatty acid metabolic pathways might also be important factors in effecting FB<sub>1</sub>-induced hepatotoxicity. Intracellular effects of FB<sub>1</sub> have been studied mainly in non-hepatocyte cell culture and tissue slice models and have included alterations in cell morphology (8), cell–cell interaction (9), mitogen-activated protein kinase (MAPK) activity (10), protein kinase C expression (4) and apoptosis (11). In a cancer target tissue, rat hepatocytes, FB<sub>1</sub> was found to inhibit proliferation via G<sub>1</sub> arrest and cause apoptosis in the majority of normal hepatocytes, whereas a small minority of hepatocytes survived by escaping from G<sub>1</sub> arrest (12). It has been hypothesized that tumor development could be a result of FB<sub>1</sub> mimicking genotoxic carcinogens in inducing hepatotoxicity, resulting in compensatory cell proliferation (survival). Clonal outgrowth of initiated cells resistant to the cytotoxic effects of FB<sub>1</sub> would then occur amidst the non-proliferative background of surrounding normal cells (13).

Progression through the cell cycle from G<sub>1</sub> to S phase is controlled by a restriction point (R) which is limited by the activity of several proteins: the tumor suppressors retinoblastoma (pRb) and p53, the growth factor TGF $\beta$ , the cyclins D, E and A, their corresponding kinases Cdk2, Cdk4, Cdk5 and Cdk6, and the inhibitors of these cyclin-dependent kinases: the Ink4 family (p15, p16, p18, p19) and Waf/Kip family (p21, p27, p57) (14). The majority of human cancers have been reported to have alterations in the function of one or more of these cell cycle regulatory proteins. Cyclin D1 is a key regulatory protein in the Rb pathway, which controls transition through the restriction point, and is responsible, together with

its main catalytic partners, Cdk4/Cdk6, for pRb phosphorylation (15). This process is negatively regulated by the Cdk inhibitors p16<sup>INK4</sup>, p27<sup>KIP1</sup> and p21<sup>WAF1</sup>. Cyclin D1 is a highly responsive sensor of the growth factor environment of the cell and it is targeted for degradation when mitogenic factors are absent in the cell's milieu (16). When overexpressed due to gene amplification, gene rearrangement, protein stabilization or other mechanisms, cyclin D1 acts as an oncogene by enhancing cell transformation, either alone (17) or in combination with activated *ras* (18), thereby shortening the G<sub>1</sub> phase of the cell cycle. Deregulated function of cyclin D1, often resulting from overexpression of the protein, has been documented in numerous human cancers, including HCC (19,20). Recent evidence indicates that cyclin D1 may also be regulated through the p21 *ras*/mitogen activated protein kinase (MAPK) pathway (21,22). Interestingly, it has been demonstrated in only a few cell types (e.g. human mammary epithelial cells) that overexpression of cyclin D1 can inhibit cell-cycle progression rather than stimulate growth (23). Cyclin D1 protein expression is regulated during the cell cycle both transcriptionally and post-transcriptionally with differences being cell-type-dependent. Unlike other cyclins (E, A and B) which are regulated by a ubiquitin-dependent proteosomal pathway (24), the regulation of cyclin D1 protein level/activity as a function of cell cycle phase has not yet been clearly defined.

Recently, it was reported that glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) phosphorylates cyclin D1 on Thr-286, thereby triggering rapid cyclin D1 turnover (25). It was also shown that a mutant cyclin D1 protein refractory to phosphorylation by GSK-3 $\beta$  remained present in the nucleus throughout the entire cell cycle. GSK-3 $\beta$  is a ubiquitously expressed protein-serine/threonine kinase whose activity is inhibited upon phosphorylation of serine 9 (Ser<sup>9</sup>) by Akt (protein kinase B) (26). Akt is a part of the anti-apoptotic phosphatidylinositol 3 kinase (PI3K)/Akt cell survival pathway (27). It can be activated by different stimuli involved in cellular survival (such as insulin, growth factors, cytokines) or inhibited by the pro-apoptotic lipid molecule ceramide (28,29). Akt is activated by phosphorylation predominantly at two regulatory sites (Thr308 and Ser473) (30). The mechanism by which ceramide inhibits Akt phosphorylation remains unclear.

Although several mechanisms for FB<sub>1</sub>-associated liver toxicity have been proposed, very little supportive data exist to explain its carcinogenic effects. This investigation was undertaken to determine whether the overexpression of cyclin D1, which disrupts the G<sub>1</sub> checkpoint in hepatocytes, may be an early event and a part of the mechanism(s) responsible for FB<sub>1</sub>-induced hepatocarcinogenesis. Our data indicate that activation of Akt and consequent inhibition of GSK-3 $\beta$  activity could be responsible for the detected overexpression and post-translational modification of cyclin D1. These molecular events may play a major role in FB<sub>1</sub>-induced carcinogenesis and provide possible links between the toxic and carcinogenic effects of FB<sub>1</sub>.

## Materials and methods

### Short-term FB<sub>1</sub> study

F344/NCr rats were fed 0, 50, 100 or 250 mg FB<sub>1</sub>/kg diet over a period of 21 days using a purified (AIN 76) diet as described previously (31). Half of each liver sample was kept at -80°C prior to analyses, while the other half was paraffin-embedded. A total of 14 paraffin-embedded livers were analyzed by immunohistochemistry (IHC) (nine FB<sub>1</sub>-treated and five controls), whereas a total of eight liver protein lysates were analyzed by both western analysis

and immunoprecipitation (six FB<sub>1</sub>-treated and two controls) and an additional nine liver protein samples were lysed in kinase buffer (six FB<sub>1</sub>-treated and three controls) for GSK-3 $\beta$  and Akt kinase activity assays.

### Long-term carcinogenic studies

Tumors were generated by chronically feeding FB<sub>1</sub> (50 mg/kg of diet) to BD IX rats (1) and nitroglycerin (32) or *N*-nitrosodiethylamine (DEN)/phenobarbital (PB) to F344/NCr rats (33). A total of 15 paraffin-embedded FB<sub>1</sub>-treated rat hepatic specimens (10 carcinomas, three adenomas and two livers with preneoplastic foci), seven control livers and 12 HCCs induced by the other two carcinogens were analyzed.

### Genomic DNA, RNA and protein isolation

DNA was recovered from seven paraffin-embedded controls and 15 liver specimens from FB<sub>1</sub> long-term treated rats as described previously (34), with the exception that only a single wash with acetone was used following octane extraction. One-half of each of the frozen livers from the short-term FB<sub>1</sub>-feeding study was used for RNA analysis following RNA extraction with TRIZOL (Gibco BRL, Gaithersburg, MD), and protein samples were prepared from the other half of the same livers. For protein analysis (western blot analysis, immunoprecipitations and kinase assays), tissues were frozen in liquid nitrogen. Powders of frozen tissue samples for analysis by both western blot and immunoprecipitation were dissolved in lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.25 TIU/ml aprotinin and 1 mM sodium orthovanadate). Liver tissue samples used for both GSK-3 $\beta$  and Akt kinase analysis were lysed in a kinase buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 2.5 mM EGTA, 1 mM EDTA, 0.1% Tween-20 and protease and phosphatase inhibitors as described above). The Bio-Rad (Richmond, CA) protein assay was used for protein quantification.

### K-ras and H-ras mutation analysis

Genomic DNA, from liver specimens from the long-term study with FB<sub>1</sub>, was examined for mutations in the *K-ras* and *H-ras* genes (exons 1 and 2) by single-strand conformation polymorphism (SSCP) analysis, restriction fragment length polymorphism analysis and sequencing, as described previously (32,35). The PCR conditions for *H-ras* exon 1 were 92, 58 and 72°C for 30 s each (30–35 cycles) and for *H-ras* exon 2 were 95, 53 and 72°C for 30 s each (30–35 cycles). The exon 1 primers were: 5'-GCA ACC CCT GTA GAA GC-3' and 5'-TCA TAC TCG TCC ACA AAA TG-3'. The exon 2 primers were: 5'-CCC TTA AGC TGT GTT CTT TTG-3' and 5'-CTG TGC GCA TGT ACT GGT-3'. The sequencing primer for *H-ras* exon 2 was 5'-CAG GTA GTC ATT GAT GGG GA-3'. 'Cold SSCP' analysis for *H-ras* exon 1 mutation detection was conducted as described (36). The denaturant methyl mercury hydroxide (MeHgOH) was omitted for exon 2 analysis, and additional PCR primers were included (2  $\mu$ l of 2  $\mu$ M stock per final 20  $\mu$ l reaction volume) to allow visualization of mutant mobility shifts caused by primer-single-strand PCR product heteroduplexes. The mixture was heated to 95°C, then the primers were allowed to anneal to the single strands at room temperature for 5 min prior to gel loading. Gels were electrophoresed at 300 V for 3.5 h at 8°C (buffer temperature). Positive mutant controls were used to optimize conditions for mutation detection. In all cases, confirmation of SSCP analyses was made by repeating PCR from the original template for PCR products with mobility shifts on initial SSCP. Mutations were identified by cycle sequencing using  $\alpha$ -<sup>32</sup>P-labeled dideoxynucleotides (Thermo Sequenase Kit; Amersham Pharmacia Biotech, Cleveland, OH).

### Immunohistochemical staining

Paraffin-embedded liver sections (5  $\mu$ m) from both short-term (nine FB<sub>1</sub>-treated and five control livers) and long-term FB<sub>1</sub>-treated rats (10 carcinomas, three adenomas and two livers with neoplastic nodules), and from seven normal livers (corresponding control rats), were used for immunohistochemical analysis. In addition, kidney, prostate, heart, thyroid, intestine, salivary gland, adrenal gland, lung, brain, spleen and testes from the same animals treated with FB<sub>1</sub> and from the corresponding control animals were examined. A total of 12 HCCs induced by two other carcinogens (nitroglycerin and DEN/phenobarbital) were also included in this IHC analysis. Freshly cut tissue sections were microwaved twice in 10 mM sodium citrate (pH 6.0) for 5 min to expose the antigen. Goat serum was used to suppress non-specific binding. Tissue sections were incubated at 4°C overnight with a 1:1000 dilution of rabbit polyclonal anti-human cyclin D antibody which recognizes only form [a] of cyclin D1 and has some cross-reactivity with cyclin D2 (Upstate Biotechnology, Lake Placid, NY) (37,38). The sections were then washed in buffer and incubated with biotinylated anti-rabbit secondary antibody for 30 min. Sections for PCNA staining were microwaved in water, blocked with horse serum and incubated with a 1:1600 dilution of mouse monoclonal anti-PCNA antibody (clone PC10, Dako Corp., Carpinteria, CA), followed by anti-mouse IgG. Diaminobenzidine from the Vectastain Elite ABC kit (Vector

Laboratories, Burlingame, CA) was used for final detection. As a staining control, primary antibodies were omitted on one slide from each staining series. All the liver tissues were evaluated by a blinded observer. Nuclear staining was graded as follows: zero to very few cells staining (0), weak (1), moderately positive (2) and very strongly positive (3) compared with normal tissues. The tissues were also evaluated for cyclin D1 cytoplasmic staining.

#### Western blot

An aliquot of 100 µg of total liver protein was used for each analysis. The samples were run on 12% SDS-PAGE gels for analysis of cyclin D, Cdk2 and cyclin E. The samples for pRb analysis were run on 8% SDS-PAGE gels. Proteins were electroblotted to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA). The membranes were then stained with Ponceau protein stain (Sigma, St Louis, MO) to confirm equal sample loading. Primary antibodies used were: anti-human cyclin D rabbit polyclonal IgG (UBI), rabbit polyclonal cyclin E (M-20), Cdk4 (C-22; Santa Cruz Biotechnology, Santa Cruz, CA), Cdk2 (UBI) and anti-human pRb (PMG3-245) mouse monoclonal (kind gift of Dr Wen-Hwa Lee, University of Texas, San Antonio, TX). The positive control used for cyclin D1 western blot analysis was EGF-stimulated A431 cell lysate (UBI). Membranes first probed for cyclin D were subsequently stripped and probed for Cdk4 (C-22). The secondary antibodies used were horseradish peroxidase-labeled anti-mouse or anti-rabbit antibodies (Amersham Co., Arlington Heights, IL), with an enhanced chemiluminescence detection kit and X-ray film (Amersham) used for detection.

#### Isoelectric focusing (IEF) and 2D electrophoresis

IEF was performed by using IEF gels pH 3–10 (pI performance range of 3.5–8.5; Novex, San Diego, CA). A total of 100 µg of protein lysate, as used for cyclin D1 expression analysis by western blotting, from all control and FB<sub>1</sub>-treated livers (short-term experiment) was mixed (1:2) with IEF sample buffer (Novex) and loaded on the gels. Gels were run according to the manufacturer's instructions. Before running in the second dimension, gels were stained with Coomassie blue dye and later destained. IEF gel slices were cut and loaded on 12% 2D gels (Novex) in such a way that in both control and experimental samples the upper part of the IEF gel (more basic pH) was positioned toward the left part of the 2D gel next to the well for the marker. The more acidic part of the slice was positioned toward the right side. The protein marker used was prestained Kaleidoscope marker (Bio-Rad, Richmond, CA). Samples were run in a way that the gel with one FB<sub>1</sub>-treated protein sample was always parallel to the gel with one control sample as a sandwich in the gel box (Novex) and gels were run according to instructions provided by the manufacturer (Novex). After electroblotting, membranes (Immobilon-P) were blocked with 2% BSA, washed three times with PBST, and probed with a monoclonal antibody (clone DCS-6; Neomarkers, Fremont, CA) that recognizes both forms [a] and [b] of cyclin D1 (38). The secondary antibody used was horseradish peroxidase-labeled anti-mouse (Amersham). Protein detection was performed using an enhanced chemiluminescence kit and X-ray film (Amersham).

#### Determination of GSK-3β and Akt kinase activity

Western blots of 25 µg of total protein were performed as described above. The samples were run on 8% SDS-PAGE gels (Novex) for Akt, and 10% SDS-PAGE gels for GSK-3β. For Akt analysis we used phospho- and non-phospho cell extracts from NIH 3T3 cells prepared following PDGF treatment at 50 ng/ml for 20 min (New England Biolabs, Beverly, MA); these lysates were loaded on Akt gels and served as both positive and negative controls. In order to determine the expression and phosphorylation status/activity of Akt, membranes were probed with a primary antibody recognizing Akt independent of its phosphorylation status, and an antibody which detects Thr308 phosphorylated Akt protein (one of the residues of Akt targeted by activating phosphorylation). Both Akt antibodies were purchased from New England Biolabs. For detection of GSK-3β protein levels, mouse monoclonal antibody (0011-A) was used (Santa Cruz). The antibody which specifically recognizes the serine 9 phosphorylated form of GSK-3β, which is the site at which Akt phosphorylation inactivates GSK-3β, was used to detect the phosphorylated GSK-3β protein (BioSource International, Camarillo, CA). The secondary antibodies used were horseradish peroxidase-labeled anti-mouse or anti-rabbit IgG (Amersham). Protein detection was performed using an enhanced chemiluminescence kit and X-ray film (Amersham).

#### Immunoprecipitation and immunoblotting analysis of cyclin D–Cdk4 complexes

An aliquot of 5 µg of anti-cyclin D (UBI) was incubated with 500 µg of total liver protein lysate at 4°C overnight. The immune complexes were then captured with 50 µl of packed protein A-agarose beads (Boehringer Mannheim, Indianapolis, IN) while rocking at 4°C for 2 h. After three washes with cold lysis buffer, the beads were resuspended in 20 µl of 2× Laemmli sample buffer. Samples were run on 12% SDS-PAGE gels and transferred to membranes, which were blocked with 2% BSA and probed with primary

antibody for Cdk4 (C-22), followed by secondary anti-rabbit polyclonal antibody (Amersham). Detection was performed as described above.

#### Cdk4 kinase assay

A total of 500 µg of total protein was immunoprecipitated with 2 µg of Cdk4 antibody (C-22) and kinase activity assay was performed as described previously (39). The plasmid for the fusion protein GST-Rb (amino acids 379–928) was the generous gift of Dr Mark E. Ewan (Dana Farber Cancer Institute, Boston, MA). Labeled products were separated on denaturing 8% polyacrylamide gels (Novex). Gels were fixed in 15% methanol/10% acetic acid, dried and bands with phosphorylated GST-pRb were visualized by autoradiography. In addition, western blot analysis was performed to confirm Cdk4 protein levels using 20 µl of the two controls and two samples from each animal treated with 250 p.p.m. of FB<sub>1</sub> from the same aliquots used to assess Cdk4 kinase activity.

#### Northern blot analysis

An aliquot of 20 µg of total RNA was electrophoresed on a 1% agarose/formaldehyde gel and transferred to a Gene Screen Plus nylon membrane (Du Pont NEN Research Products, Boston, MA). A 1.3 kb fragment of the mouse cyclin D1 cDNA (the kind gift of Dr Charles J. Sherr, St Jude Children's Research Hospital, Memphis, TN), labeled by random priming with [ $\alpha$ -<sup>32</sup>P]dATP (3000 Ci/mmol) (Lofstrand Labs Limited, Gaithersburg, MD), was used to probe the membranes. After stripping, the membranes were probed with a rat 18S ribosomal RNA probe (DNA oligomer, RAT 18SHP) (40). The intensity of the bands detected by both northern and western blot analysis was quantified using a Molecular Dynamics enhanced-laser densitometer.

## Results

### Early cyclin D1 overexpression caused by short-term exposure to FB<sub>1</sub>

We hypothesized that FB<sub>1</sub> acts as a carcinogen by disrupting the G<sub>1</sub>/S checkpoint in rat hepatocytes resistant to its mitoinhibitory effects, due to an overexpression of cyclin D1 protein. To test whether cyclin D1 overexpression is an early event during FB<sub>1</sub> hepatocarcinogenesis, rats were fed FB<sub>1</sub> doses for 21 days. Half of each liver was frozen for immunoblot analysis, while the other half was paraffin-embedded for IHC. Cyclin D1 was found to be overexpressed in FB<sub>1</sub>-treated livers, as detected by IHC, compared with control livers where little staining was observed (Figure 1). About two-thirds of all hepatocyte nuclei in treated rats showed some staining for cyclin D1, whereas ~5–10% were intensively stained. Consecutive sections from the same blocks were stained for PCNA, a marker for proliferating cells. In control liver sections, only a few cells were stained, whereas in experimental liver sections the number of PCNA positive cells exceeded those positive for cyclin D1 (data not shown). In addition, we confirmed the overexpression of cyclin D1 by western analysis, detecting an overexpression of ~36 kDa cyclin D1 protein in a FB<sub>1</sub> dose-dependent manner (up to 5-fold) in experimental total protein lysates compared with control (Figure 2). Furthermore, in western analysis a 34 kDa protein was detected and was considered to be cyclin D2, since the antibody used for detection of cyclin D1 cross reacts with cyclin D2 and this molecular weight has been reported previously for cyclin D2 (37). Cyclin D2 levels were somewhat higher in most FB<sub>1</sub> samples compared with non-treated controls, but did not show a dose-response.

Other proteins of the pRb pathway (Cdk4, pRb, cyclin E and Cdk2) were analyzed by immunoblot (Figure 2). Levels of Cdk4 were not altered by FB<sub>1</sub> treatment. The pRb antibody recognized several bands ranging from ~105 to ~115 kDa. pRb was present in a slower migrating (hyperphosphorylated) form in some of the livers from animals treated with high doses of FB<sub>1</sub> (100 or 250 p.p.m.); it is noteworthy that these were livers in which levels of cyclin D1 were especially high. Levels of cyclin E and Cdk2 were not changed between control

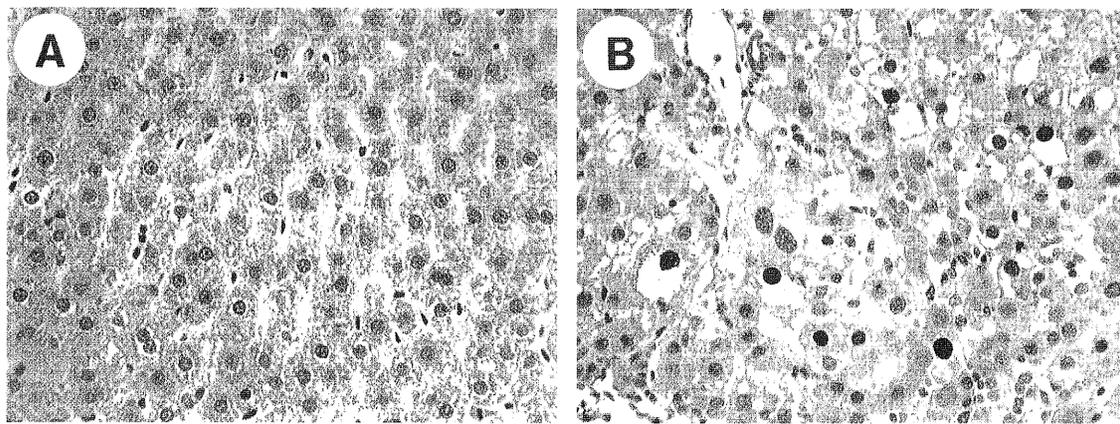


Fig. 1. Immunohistochemical staining for cyclin D1 in control and FB<sub>1</sub>-treated livers from short-term exposure study. In control livers only a few cells were stained (A), whereas in FB<sub>1</sub>-treated (100 p.p.m.) livers many nuclei were stained (B).

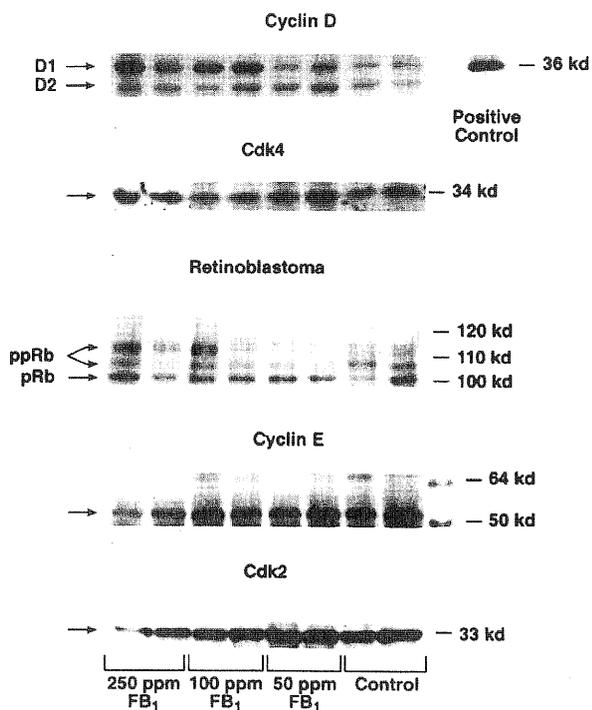


Fig. 2. Western immunoblot analysis for cyclins D1, D2 and E, Cdk4, Cdk2 and pRb in livers from control rats and rats fed FB<sub>1</sub> for 21 days. The loading sequence for all gels was, in each case (right to left): two controls, two at 50 p.p.m., two at 100 p.p.m. and two at 250 p.p.m. FB<sub>1</sub> (see label at bottom of figure). The positions of relevant molecular weight markers, in kDa, are indicated at the right and the positions of bands of interest with arrows at the left. These results are representative of three identical, independent assays using the same lysates.

and FB<sub>1</sub>-treated livers with the exception of a single sample (250 mg/kg of diet) in which a decrease in both proteins was detected.

*Cyclin D1 protein stability as a post-translational event*

Northern blot analysis was performed in order to test whether the levels of the mRNA were increased in the FB<sub>1</sub>-treated samples in which the overexpression of cyclin D1 protein was detected. Both RNA and protein samples were taken from the same liver lobe. An ~4 kb cyclin D1 transcript was detected in all samples with no consistent change in cyclin D1 mRNA

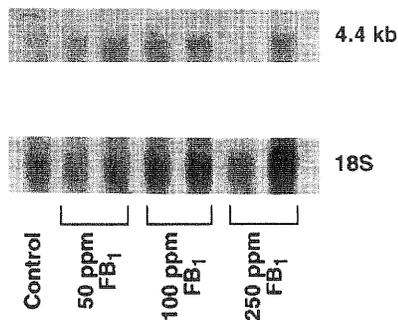
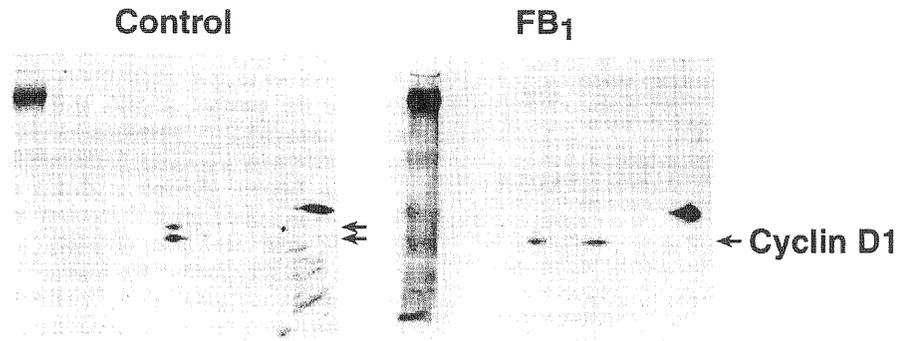


Fig. 3. Northern blot analysis for cyclin D1 mRNA from FB<sub>1</sub>-treated and control livers. The blot was probed with a 1.3 kb mouse cyclin D1 cDNA probe, then stripped and probed with a rat cDNA probe for 18S ribosomal RNA as an internal control for RNA loading. Data are representative of two identical assays.

expression between control and experimental samples (Figure 3). To test whether the overexpression of cyclin D1 protein is due to post-translational events, the same protein lysates in which its stabilization was detected (Figure 2) were analyzed by IEF and 2D electrophoresis followed by probing of membranes with a monoclonal antibody for cyclin D1 (DCS-6). Although this antibody is directed against human cyclin D1, the epitope of the DCS-6 antibody is at the C-terminal portion of the cyclin box, an area with 100% conservation between rat and human cyclin D1 (41). 2D gels from control samples showed two distinct closely-running spots in the middle of the membranes at ~36 kDa, corresponding to the expected size for the cyclin D1 protein (Figure 4, left panel). These represent the two forms of the cyclin D1 [a] and [b] recently reported to be detected by the DCS-6 antibody (Neomarkers) (38). This monoclonal antibody does not possess cross-reactivity to cyclin D2. On membranes from gels for all six protein samples from FB<sub>1</sub>-treated livers (representative blot, Figure 4, right panel), we detected one form of cyclin D1 in the same position as the lower spot in the control membranes. In the FB<sub>1</sub>-treated samples, the upper spot (detected in controls) was not seen; instead, an additional spot lying toward the right portion of the membrane was detected. Clearly, in the experimental samples, the upper form [a] of cyclin D1 became less negatively charged and was shifted to the right. In contrast, two spots were also found for cyclin E, but these did not vary in position in control compared with FB<sub>1</sub>-treated livers (data not shown).



**Fig. 4.** Representative 2D gel electrophoresis of cyclin D1. One-hundred micrograms of the same protein lysates used for determination of cyclin D1 expression levels (Figure 2) were separated via IEF on gels run according to the manufacturer's instructions (Novex). Gel slices containing the separated proteins were positioned for running in the second dimension, with the top (containing the more negatively charged, rapidly migrating proteins in the first separation) positioned next to the protein marker (left of each panel). After the second electrophoresis, the proteins were electroblotted to Immobilon-P membranes and probed with a monoclonal antibody to cyclin D1. Data are representative of two identical assays.

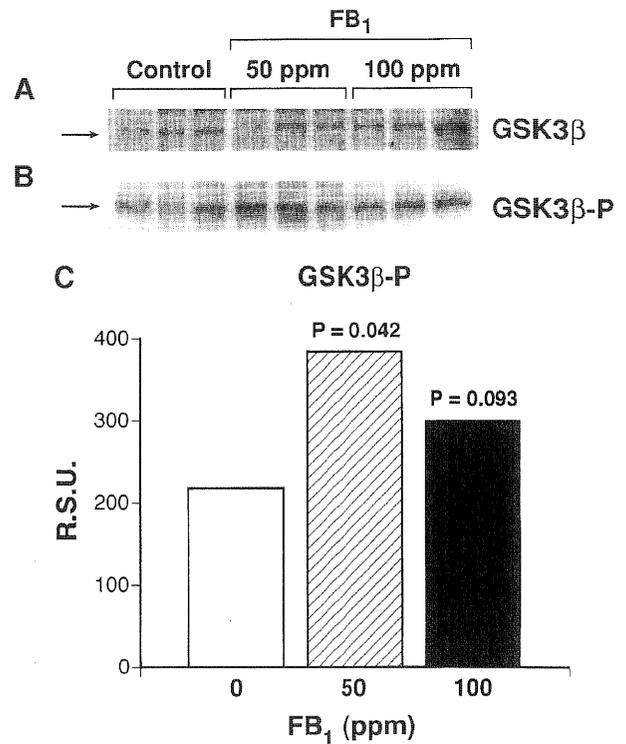
Also, 2D electrophoresis of the same samples did not reveal any differences in the migration pattern of the Cdk4 protein (data not shown), thus confirming the specific effects of FB<sub>1</sub> treatment on post-translational modification of cyclin D1.

*Inhibition of GSK-3β by activated Akt in FB<sub>1</sub>-treated livers*

Recently, GSK-3β has been shown to be a specific kinase that phosphorylates cyclin D1 on Thr-286, thus triggering its rapid degradation (25). Upon phosphorylation by activated Akt on Ser<sup>9</sup>, GSK-3β kinase activity is inhibited (26). In order to test if the detected post-translational modification of cyclin D1 documented by the presence of a less negatively charged form of cyclin D1 (possibly a form with fewer phosphorous groups) in FB<sub>1</sub>-treated samples is linked to inhibition of GSK-3β, the same liver specimens were analyzed for both the expression level and phosphorylation status of GSK-3β. We detected ~50 kDa GSK-3β protein expressed in both control and FB<sub>1</sub>-treated samples to an equal extent (Figure 5A). However, in FB<sub>1</sub>-treated samples, the protein migrated more slowly compared with control samples. In addition, more phosphorylated GSK-3β protein was detected (~2-fold increase in rats fed 50 p.p.m. of FB<sub>1</sub>; ~1.5-fold increase in rats fed 100 p.p.m.), indicative of its possible inactivation (Figure 5B). However, we did not detect a dose-response-related phosphorylation of GSK-3β (Figure 5C), possibly due to the limitations of the analysis (it is difficult to evaluate the linearity of kinase activation by using this approach and further complication arises when using protein from tissue samples). Because Akt has been shown to inhibit GSK-3β by phosphorylation, we predicted that FB<sub>1</sub> would activate Akt, possibly due to its toxic action on both sphingolipids and phospholipids. This could lead to alterations of the signaling molecules involved in the control of Akt activation (29). Because GSK-3β is a target of activated Akt, we analyzed the same lysates for both expression and phosphorylation status of Akt. Akt protein was detected at ~60 kDa and total levels of Akt were similar in both FB<sub>1</sub>-treated livers and controls (Figure 6A); however, in FB<sub>1</sub>-treated livers, a slower migrating Akt was detected. Furthermore, more phosphorylated Akt (on Thr 308) was detected in FB<sub>1</sub>-treated samples (4-fold increase in rats fed 50 p.p.m. and 7-fold increase in rats fed 100 p.p.m.) (Figure 6B), indicative of dose-dependent activation of Akt kinase.

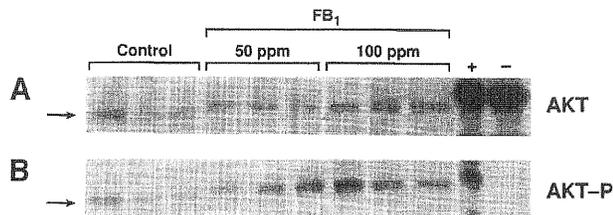
*Increased cyclin D-Cdk4 complex formation and Cdk4 activity in FB<sub>1</sub>-treated livers*

Cyclin D-containing complexes were immunoprecipitated and immunoblots were then probed with an antibody against Cdk4.

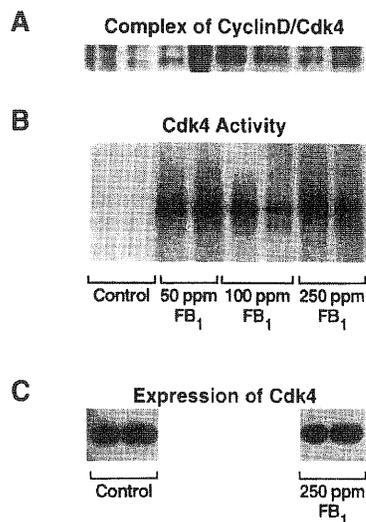


**Fig. 5.** Western immunoblot analysis for expression levels and activity of total GSK-3β in livers from control rats and rats fed FB<sub>1</sub> (50 p.p.m. and 100 p.p.m.) for 21 days. The band at ~50 kDa represents total GSK-3β detected using an antibody recognizing GSK-3β protein independent of its phosphorylation status (A). More highly phosphorylated (inactivated by phosphorylation on Ser<sup>9</sup>) GSK-3β protein was detected in FB<sub>1</sub>-treated samples (B). In rats fed 50 p.p.m. of FB<sub>1</sub> the increase in phosphorylated GSK-3β was ~2-fold, whereas in rats fed 100 p.p.m. of FB<sub>1</sub>, the increase was only 1.5-fold (C). Data are representative of two identical assays. R.S.U., relative scan units.

Cyclin D-Cdk4 complexes were more prominent in the FB<sub>1</sub>-treated samples than in control samples (Figure 7A). This increase was due to overexpression of cyclin D1, because the total levels of the Cdk4 were not changed in the FB<sub>1</sub>-treated livers (Figure 2). To investigate whether the increased levels of cyclin D1 upregulated Cdk4 activity, we performed an *in vitro* kinase assay for Cdk4 activity using recombinant GST-pRb fusion protein as the kinase substrate. Cdk4 from FB<sub>1</sub>-



**Fig. 6.** Western immunoblot analysis for expression and phosphorylation status of Akt in livers from controls and FB<sub>1</sub>-treated rats. (A) Akt protein was detected at ~60 kDa in controls, whereas a slower migrating protein form was detected in FB<sub>1</sub>-treated livers. (B) More highly phosphorylated Akt (Thr 308; one of the Akt activation sites *in vivo*) was detected in livers from rats treated with 50 p.p.m. (4-fold) and 100 p.p.m. (7-fold) FB<sub>1</sub> as compared with controls. Data are representative of two identical assays.



**Fig. 7.** Complex formation, activity and amounts of Cdk4. (A) Cyclin D1 was immunoprecipitated from the protein lysate, separated by gel electrophoresis, blotted to membrane and the membrane was probed with an antibody to Cdk4. (B) Cdk4 kinase activity in the liver lysates was measured by immunoprecipitating Cdk4 from the lysates, incubating with fusion protein (GST-pRb) in the presence of <sup>32</sup>P, separating the products in denaturing gels, and detecting phosphorylated Rb by autoradiography. (C) The Cdk4 immunoprecipitates used in the kinase activity assay were additionally analyzed by western blot and equal levels of Cdk4 expression in two control and high-dose FB<sub>1</sub> samples were observed. All data represent two identical assays.

treated livers had much higher kinase activity compared with Cdk4 in control livers where no visible GST-pRb phosphorylation could be detected (Figure 7B), in spite of the presence of small amounts of cyclin D/Cdk4 complexes and equal levels of Cdk4 protein in both control and experimental samples (Figures 2 and 7C). The reason why Cdk4 activity does not parallel the increase in cyclin D1 dose-dependent overexpression is not clear. It is possible that linearity of the assay was exceeded. In other words, the assay is in the area of the curve where it is impossible to see a dose-response. The absence of a detectable level of kinase activity in control samples could be predicted, since it is likely that the activation of cyclin D1/Cdk4 complexes is very critically restricted in normal, quiescent adult rat hepatocytes.

**Overexpression of cyclin D1 protein in FB<sub>1</sub>-induced tumors**  
Immunohistochemical staining was performed in order to analyze the level of cyclin D1 expression in tumors from a long-term FB<sub>1</sub>-feeding study and to determine both the

liver cell type overexpressing cyclin D1 as well as its subcellular localization. Staining in normal livers was limited to the nuclei of a few hepatocytes (Figure 8A), while an increase in the number of cells with weak nuclear staining (grade 1) was seen in preneoplastic foci which previously stained positive for  $\gamma$ -glutamyltranspeptidase (Figure 8B) (1). All benign tumors showed evidence of grade 2 and all HCCs showed strong grade 3 nuclear staining for cyclin D (Figure 8C and D). The intensity of the staining was correlated with the grade of the lesion. These findings suggest that cyclin D1 deregulation is involved in development of early lesions as well as in malignant conversion. Highly cirrhotic and necrotic areas within the HCCs and focal aggregates of lymphocytes, common within the connective tissue septa, were both found to be negative for cyclin D1. Cyclin D1 overexpression was detected in the epithelial cells of the bile ducts in two tumors with lesions typical of cholangiofibrosis (data not shown).

#### *Absence of cyclin D1 overexpression in other tissues*

Alteration in cyclin D1 expression was a liver-specific finding as all other tissues examined lacked cyclin D1 overexpression with the possible exception of the kidney (data not shown). Kidney showed some positive nuclear staining in the proximal tubules in both untreated and treated rats. This finding must be interpreted with caution due to the tendency of the proximal tubules to stain non-specifically in IHC. Because chronic interstitial nephritis was present in the kidneys and FB<sub>1</sub> has been shown to have toxic effects in rat kidneys (42), this finding may also reflect an actual role of cyclin D1 in this pathology.

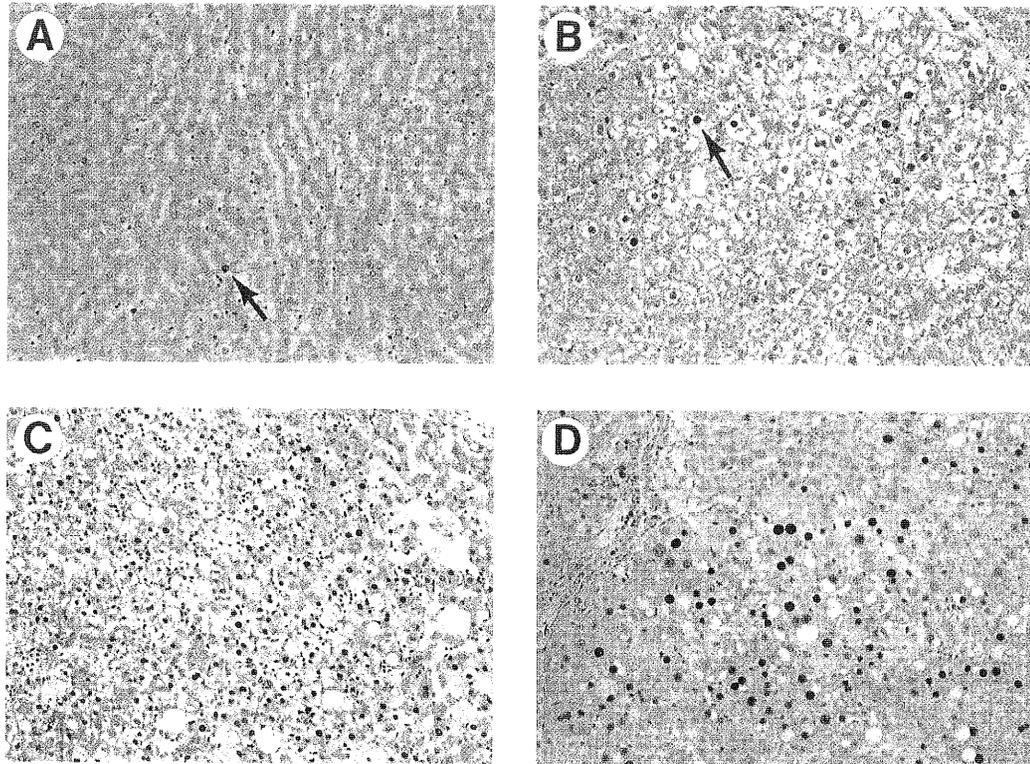
#### *Specificity of the FB<sub>1</sub> effect on cyclin D1 in hepatocytes*

To test whether the overexpression of cyclin D1 was a common property of rat HCCs induced by other carcinogens, liver sections from paraffin-embedded rat HCC caused by nitroglycerin or DEN/PB (32,33) were compared with those induced by FB<sub>1</sub>. Tissues from all three studies were processed identically. The cyclin D1 overexpression, which was characteristic of FB<sub>1</sub>-induced HCC (Figure 9A), was not seen in HCC caused by either DEN/PB (Figure 9B) or nitroglycerin (Figure 9C).

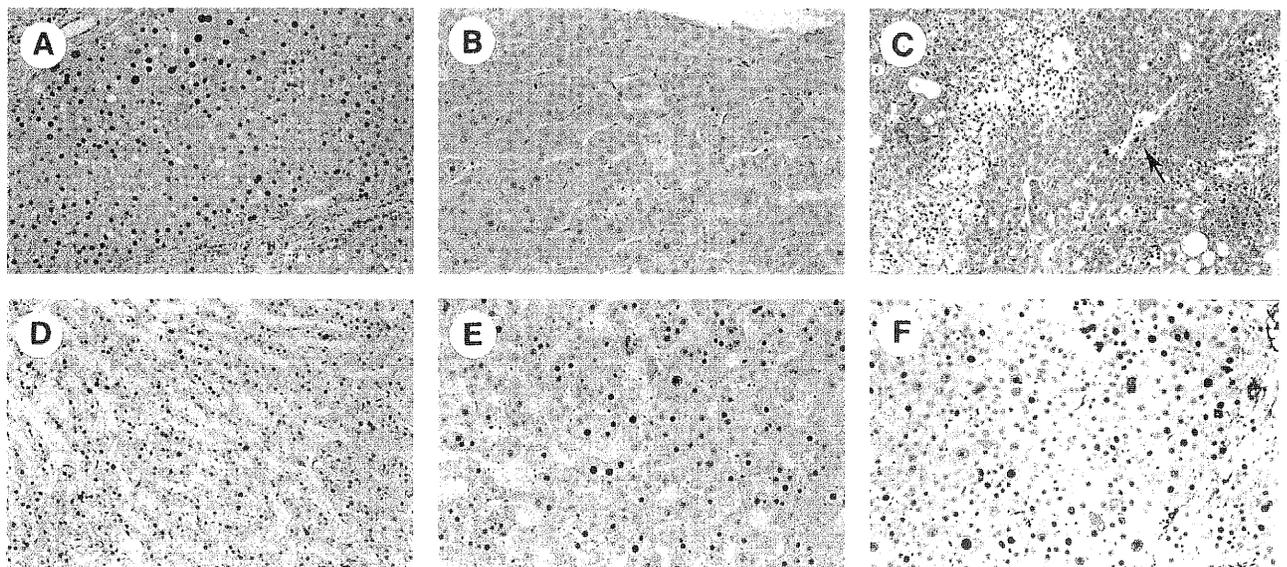
To test whether increased cyclin D1 expression was merely a reflection of the rate of cell proliferation, the tissues were stained for PCNA and cyclin D1 in consecutive sections for all experimental and control livers. In control livers, staining for both antigens was detected in only a few cells (graded as 0). In preneoplastic foci in the FB<sub>1</sub>-treated livers, the PCNA-positive cells exceeded the number of those positively staining for cyclin D1 by ~2-fold; in benign and malignant FB<sub>1</sub>-induced tumors, the same areas stained positively for both antigens, with cyclin D1 slightly exceeding the PCNA-positive staining cells (data not shown). HCC induced by nitroglycerin or DEN/PB showed PCNA staining rates similar to those in the FB<sub>1</sub> tumors (Figure 9D-F). Thus, although a high rate of cell proliferation is a common property of the HCCs induced in each of the three different induction models, only FB<sub>1</sub>-induced HCCs exhibited overexpression of cyclin D1.

#### *Absence of K-ras and H-ras activating mutations*

To test whether mutations in *ras* oncogenes could be driving the cyclin D1 protein overexpression, we analyzed all experimental and control samples for K-*ras* and H-*ras* mutations.



**Fig. 8.** Immunohistochemical staining for cyclin D1 in control and FB<sub>1</sub>-exposed rat livers. (A) In control livers, only occasional nuclei (arrow) show light staining for cyclin D1. (B) A preneoplastic focus in the center of the picture shows increased numbers of nuclei with moderate cyclin D1 staining (arrow). (C) A liver adenoma with most nuclei stained for cyclin D1. (D) In HCCs, the majority of the nuclei stain with greater intensity (dark brown) than liver adenomas.



**Fig. 9.** Comparison of cyclin D1 staining in tumors from FB<sub>1</sub>-treated livers versus tumors from other treatments (includes analysis of levels of PCNA in these livers). (A) Strong nuclear staining is apparent in a FB<sub>1</sub>-caused rat liver tumor, whereas staining was absent from tumors caused by DEN/PB (B) and only a few nuclei (arrow) stained in carcinomas induced by nitroglycerin (C). The intensity of staining for PCNA in the HCCs induced by the three carcinogens: FB<sub>1</sub> (D), DEN/PB (E) and nitroglycerin (F) is very similar.

Only two samples with third position A→T transversions were found in codon 61 of exon 2 of *H-ras* (data not shown), thus ruling out activating mutations in *ras* as a major contributor to the observed cyclin D1 overexpression.

## Discussion

To date, fumonisin B<sub>1</sub> hepatocarcinogenicity in rats occurs by an unknown mechanism. The present study reveals that FB<sub>1</sub> treatment results in cyclin D1 overexpression via protein stabilization due to post-translational modification(s) without effect on cyclin D1 mRNA levels. In addition to cyclin D1 stabilization, we detected Akt activation resulting in phosphorylation and inhibition of GSK-3β. These findings indicate that the activation of a major survival molecule (Akt) and its downstream effectors leads to alteration of cell cycle progression in hepatocytes; therefore, this could prove to be a mechanism or part of a mechanism responsible for FB<sub>1</sub> carcinogenesis.

Our 2D gels electrophoresis patterns indicated that a less negatively charged form of cyclin D1 predominated in FB<sub>1</sub>-treated livers as compared with control livers from a short-term FB<sub>1</sub> feeding study where both forms detected were of similar charge. It is likely that the two spots detected were [a] and [b] forms of cyclin D1, as reported by Sawa *et al.* (38). Cyclin D1 form [a] associates in a ternary complex with PCNA, WAF-1 and Cdk4. It has been known for some time that cyclin D1 contains a C-terminal PEST sequence (24). Removal of the PEST sequence from yeast G<sub>1</sub> cyclins prolongs their half-life (43). Cyclin D1 form [a], not form [b], contains a PEST-rich region which is probably responsible for its rapid turnover (38). One interpretation of our findings is that the cyclin D1 spot, which has an altered position by 2D gels electrophoresis after FB<sub>1</sub> treatment, is the form [a] which recently has been implicated in controlling cell cycle entry (38). It appears that FB<sub>1</sub> treatment causes post-translational modification of cyclin D1 form [a] in such a way as to make it less negatively charged (possibly less phosphorylated), thus leading to an increase in its stability. Efforts to identify the exact nature of this post-translational change are currently in progress. Also, additional confirmation of the involvement of form [a] is derived by detection of overexpressed cyclin D1 in FB<sub>1</sub>-treated samples by western blotting (Figure 2) using an anti-cyclin D1 polyclonal Ab (UBI) reported to recognize only form [a] of cyclin D1 (38). We used the same antibody (UBI) in the analysis of binding between cyclin D1 and Cdk4; therefore, it appears that the [a] form of cyclin D1 is responsible for cyclin D1/Cdk4 binding in our studies and is responsible for the pRb phosphorylation we detected.

A lack of correlation between cyclin D1 protein and mRNA expression has been reported in some human cell lines and tumors (20,44). There is frequently a correlation between cyclin D1 gene amplification and cyclin D1 protein overexpression in human hepatic, esophageal and head and neck cancer, whereas in breast cancer, amplification is detected in only 13% of the tumors despite evidence that >50% exhibit cyclin D1 protein overexpression. Similar findings have been reported in sarcomas, colon cancers and melanomas (20), further implicating post-translational modification of cyclin D1 as a mechanism for overexpression.

Recently, cyclin D1 has been shown to be regulated post-transcriptionally by GSK-3β (25,45), calpain (46) and retinoic acid (47). GSK-3β has been shown to phosphorylate cyclin

D1 specifically on Thr-286, thus triggering rapid turnover of the protein. Defective GSK-3β kinase activity could be responsible for the high stability of cyclin D1 (which remains in the nucleus throughout the cell cycle) (25). Because the activity of GSK-3β can be inhibited by signalling through a pathway that involves Ras/PI3K/Akt (25) (a major survival pathway in cells), the involvement of some of these molecules in the regulation of GSK-3β activity and cyclin D1 stability was analyzed. We detected more phosphorylated and activated Akt in FB<sub>1</sub>-treated livers as compared with control livers, and have reasoned that this alteration is responsible for the inhibition of GSK-3β as well as the less negatively charged state of the [a] form of cyclin D1 (possibly less phosphorylated).

Also, because cyclin D1 protein overexpression has been linked to both the ras/MAPK (21,22) and PI3K pathways (25), and *K-ras* is frequently mutated in rat liver tumors (48), we tested our samples for mutations in *ras* oncogene. Only two mutations in codon 61 of exon 2 of the *H-ras* oncogene were found; therefore, a potential role for *ras*-activating mutations in the overexpression of cyclin D1 in our FB<sub>1</sub> experimental samples is very limited. This is in agreement with previous reports involving human esophageal cell lines and primary esophageal cancers where cyclin D1 overexpression is detected frequently (49,50), whereas activating mutations in *ras* oncogenes are rare to non-existent (51).

Recent evidence suggests that cyclin D1 expression from a heterologous promoter can lead to apoptosis in serum-starved rat fibroblasts; potentially, this outcome may involve phosphorylation of pRb (52). FB<sub>1</sub> has been reported to be capable of inducing apoptosis in rat liver cells (11). It appears that the absolute level of cyclin D1 expression can determine whether cells will undergo transformation (moderate overexpression) or apoptosis (high overexpression).

In the majority of cell types, activated Akt has been shown to inhibit apoptosis through inhibition of pro-apoptotic molecules: Bad, caspase 9, caspase 3 and Fas/CD95 (53,54). Activated Akt detected in FB<sub>1</sub>-treated liver could potentially be responsible for the inhibition of apoptosis in those hepatocytes that are resistant to FB<sub>1</sub> toxicity (those that probably give rise to tumor development), whereas the majority of hepatocytes are sensitive to FB<sub>1</sub>-induced apoptosis due to its toxicity. Although it is not completely clear which molecules are participating in its activation, Akt has been reported to be negatively regulated by the tumor suppressor gene PTEN (55), and the lipid signalling molecule ceramide (29). FB<sub>1</sub> has been shown to inhibit ceramide synthase both *in vitro* and *in vivo*, therefore leading to a decrease in cellular ceramide levels and an increase in intracellular sphinganine (5). These events could potentially contribute to the detected activation of Akt in our study. Additionally, due to the effects of FB<sub>1</sub> on total phospholipids and fatty acids in hepatocytes (6,7), it is possible that FB<sub>1</sub> is altering molecules involved in the activation/deactivation of Akt (PI3K, PDK1, PDK2 or tumor suppressor gene PTEN) (53,55).

The ability of FB<sub>1</sub> to cause a relatively early dose-dependent increase in cyclin D1 protein expression was shown by both IHC and immunoblot analysis of livers from rats exposed to FB<sub>1</sub> for only 3 weeks. Although levels of Cdk4 protein were not increased by FB<sub>1</sub> treatment, the amount of cyclin D1-Cdk4 complex formed as well as Cdk4 kinase activity were greatly elevated as shown by increased phosphorylation of a GST-pRb substrate. These findings are in agreement with previous reports in which the expression level of cyclin D1,

regardless of Cdk4 level, is rate limiting for complex formation and is responsible for Cdk4 kinase activity (49). In addition, more hyperphosphorylated forms of pRb were detected *in vivo* in some samples with high cyclin D1 levels. The key role of cyclin D1 was further underscored by lack of alteration in the level of proteins involved in control of the late G<sub>1</sub> phase of the cell cycle (cyclin E and Cdk2). Although cyclin E is sometimes overexpressed in rodent and human tumors (56,57), the overexpression of cyclin D1 and consequent hyperphosphorylation of pRb occur more frequently (20).

The effects of FB<sub>1</sub> on cyclin D1 during short term exposure were further substantiated by results from FB<sub>1</sub> chronic treatment: clear association of its nuclear overexpression with liver neoplasia (especially in progression to carcinoma). This was evidenced by immunohistochemical analysis of pre-neoplastic foci, adenomas and carcinomas in the livers of rats chronically fed FB<sub>1</sub> (50 mg/kg diet) over a period of 20–26 months (1). The overexpression of cyclin D1 was specific to the liver as a targeted organ, with few to no cells stained in the control livers nor in the 12 other tissues analyzed from the same rats (with the possible exception of kidney which is also a target for the toxic effects of FB<sub>1</sub>). In the majority of the experimental rats, proximal tubules of the kidney stained intensively for cyclin D1. However, it is difficult to interpret this finding, because in some of the control rats, we detected a similar staining, although less intense.

Notably, rat HCCs induced by two other carcinogens failed to show overexpression of the protein when stained for cyclin D1 in parallel with the FB<sub>1</sub> tumors despite similar cell proliferation as indicated by PCNA staining. Thus, the effect on cyclin D1 was unique to FB<sub>1</sub>. There are a few chemical carcinogens reported to have an effect on cyclin D1 overexpression in rodent tumors similar to that of FB<sub>1</sub>: in mouse skin tumors, dimethylbenz[*a*]anthracene was used as an initiator followed by 12-*O*-tetradecanoylphorbol-13-acetate as the promoter (56,58) and *N*-methyl-*N*-nitrosourea in rat mammary carcinomas (59). However, to our knowledge, there are no other reports of a chemical liver carcinogen affecting cyclin D1 post-translationally.

We hypothesize that there are several possible mechanisms by which FB<sub>1</sub> is causing cyclin D1 post-translational modification(s). (i) Although there are no activating mutations in the *ras* oncogenes, it is possible that FB<sub>1</sub>, by affecting the metabolism of sphingolipids (5), phospholipids and fatty acids (7), important constituents of cellular membranes, could increase p21 ras membrane association or signaling through the PI3K pathway (ultimately impacting on Akt and GSK-3 $\beta$ , signaling molecules involved in cyclin D1 degradation). In addition to these effects, it is possible that FB<sub>1</sub> is affecting the Raf/MEK/MAPK pathway and possibly influencing signals involved in cyclin D1 synthesis and assembly. (ii) Independent of *ras*, FB<sub>1</sub> might alter the function of some proteins and signalling molecules in the control of the activation or inactivation of Akt. (iii) In addition to its effects on cyclin D1 protein stability/degradation or post-translational modifications due to the inhibition of GSK3- $\beta$ , it is possible that FB<sub>1</sub> can affect some additional proteins responsible for cyclin D1 degradation and translation. Currently, we are investigating all of these possibilities.

In conclusion, dietary FB<sub>1</sub> specifically causes activation of Akt, inhibition of GSK-3 $\beta$  and overexpression of cyclin D1 related to post-translational modification(s). This causes a consequent increase in Cdk4 kinase activity, resulting in

hyperphosphorylation of pRb and alteration in cell-cycle progression (G<sub>1</sub>/S) of rat hepatocytes. Our data contribute significantly to the understanding of the carcinogenic mechanism of FB<sub>1</sub>, a common environmental contaminant, recently shown to be carcinogenic in both rats and mice by the National Center for Toxicological Research of the US Food and Drug Administration (60).

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# Inhibition of Sphingolipid Biosynthesis in Rat Primary Hepatocyte Cultures by Fumonisin B<sub>1</sub> and Other Structurally Related Compounds

L. van der WESTHUIZEN<sup>1</sup>\*, G. S. SHEPHARD<sup>1</sup>, S. D. SNYMAN<sup>1</sup>,  
S. ABEL<sup>1</sup>, S. SWANEVELDER<sup>2</sup> and W. C. A. GELDERBLOM<sup>1</sup>

<sup>1</sup>Programme on Mycotoxins and Experimental Carcinogenesis, <sup>2</sup>Centre for Epidemiological Research in South Africa, Medical Research Council, PO Box 19070, Tygerberg 7505, South Africa

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**Abstract**—The fumonisins and toxins produced by *Alternaria alternata* f. sp. *lycopersici* (AAL toxins) are structurally related mycotoxins that disrupt sphingolipid biosynthesis by inhibiting the rate-limiting enzyme, ceramide synthase. Rat primary hepatocytes were exposed to fumonisin B<sub>1</sub> (FB<sub>1</sub>), its *N*-acetyl analogue, FA<sub>1</sub>, its fully hydrolysed analogue, AP<sub>1</sub> and the AAL toxins (TA and TB) at concentrations of 1 μM for 40 hr in culture. The extent to which these compounds disrupt sphingolipid biosynthesis in hepatocytes *in vitro* was investigated by analysing the sphingosine (So) and sphinganine (Sa) levels by HPLC. The inhibition of ceramide synthase was irreversible as the Sa:So ratio was maximally increased by FB<sub>1</sub> after 24 hr of exposure and the subsequent removal of FB<sub>1</sub> had no effect on the ratio as compared with the 40-hr incubation period in the presence of FB<sub>1</sub>. The Sa concentration was significantly ( $P < 0.01$ ) increased in all the cultures treated with the different structurally related compounds, while only AP<sub>1</sub> increased the So concentration significantly ( $P < 0.05$ ) above the control. As AP<sub>1</sub> was found to be less effective in disrupting sphingolipid biosynthesis it would appear that the tricarballic (TCA) moiety is required for maximal inhibition of ceramide synthase. The presence of an amino group appears not to be a requisite for activity, since FA<sub>1</sub> increased the Sa:So ratio to the same extent as FB<sub>1</sub>. The AAL toxins TA and TB increased the Sa concentration significantly ( $P < 0.01$ ) above that of FB<sub>1</sub> and FA<sub>1</sub>, while the Sa:So ratios were altered to the same extent. The structural requirements for the induction of cytotoxicity differ from those required for ceramide synthase inhibition as TA and TB were significantly ( $P < 0.05$  to  $P < 0.01$ ) less toxic to primary hepatocytes than FB<sub>1</sub> at all the concentrations tested. © 1998 Elsevier Science Ltd. All rights reserved

**Abbreviations:** AP<sub>1</sub> = minopentol; DMSO = dimethyl sulfoxide; EGF = epidermal growth factor; LDH = lactate dehydrogenase; FA<sub>1</sub> = fumonisin A<sub>1</sub>; FB<sub>1</sub> = fumonisin B<sub>1</sub>; FB<sub>2</sub> = fumonisin B<sub>2</sub>; OPA = *o*-phthalaldehyde; Sa = sphinganine; So = sphingosine; TCA = tricarballic acid.

## INTRODUCTION

Fumonisin is a mycotoxin produced by *Fusarium moniliforme* Sheldon, a fungus that occurs worldwide on maize (Shephard *et al.*, 1996). Fumonisin B<sub>1</sub> (FB<sub>1</sub>) (Fig. 1) is the most abundant of the various analogues that have been isolated (Shephard *et al.*, 1996). FB<sub>1</sub> causes various syndromes in different animal species: leukoencephalomalacia in horses (Kellerman *et al.*, 1990; Marasas *et al.*, 1988), pulmonary oedema in pigs (Harrison *et al.*, 1990) and hepatocarcinoma in rats (Gelderblom *et al.*, 1991). Although the occurrence of the fumonisins has been statistically associated with a high incidence of human oesophageal cancer in southern

Africa (Rheeder *et al.*, 1992), evidence for a contributory role in the development of this disease in experimental animals is lacking. The AAL toxins, of which TA and TB are the main forms (Fig. 1), are structurally related phytotoxins produced by *Alternaria alternata* (Fr.) Keissler f. sp. *lycopersici*, a fungus that causes stem canker disease in certain susceptible tomato cultivars (Bottini *et al.*, 1981; Gilchrist and Grogan, 1976; Shephard *et al.*, 1993). AAL toxin (TA) is cytotoxic to certain rat and dog tissue culture cells, whereas its *N*-acetylated analogue is not (Mirocha *et al.*, 1992). At present it is not known whether biological effects similar to those occurring with the fumonisins could be induced *in vivo* by the AAL toxins in the different animal species (Abbas *et al.*, 1994). Certain *in vitro*

\*Author for correspondence.

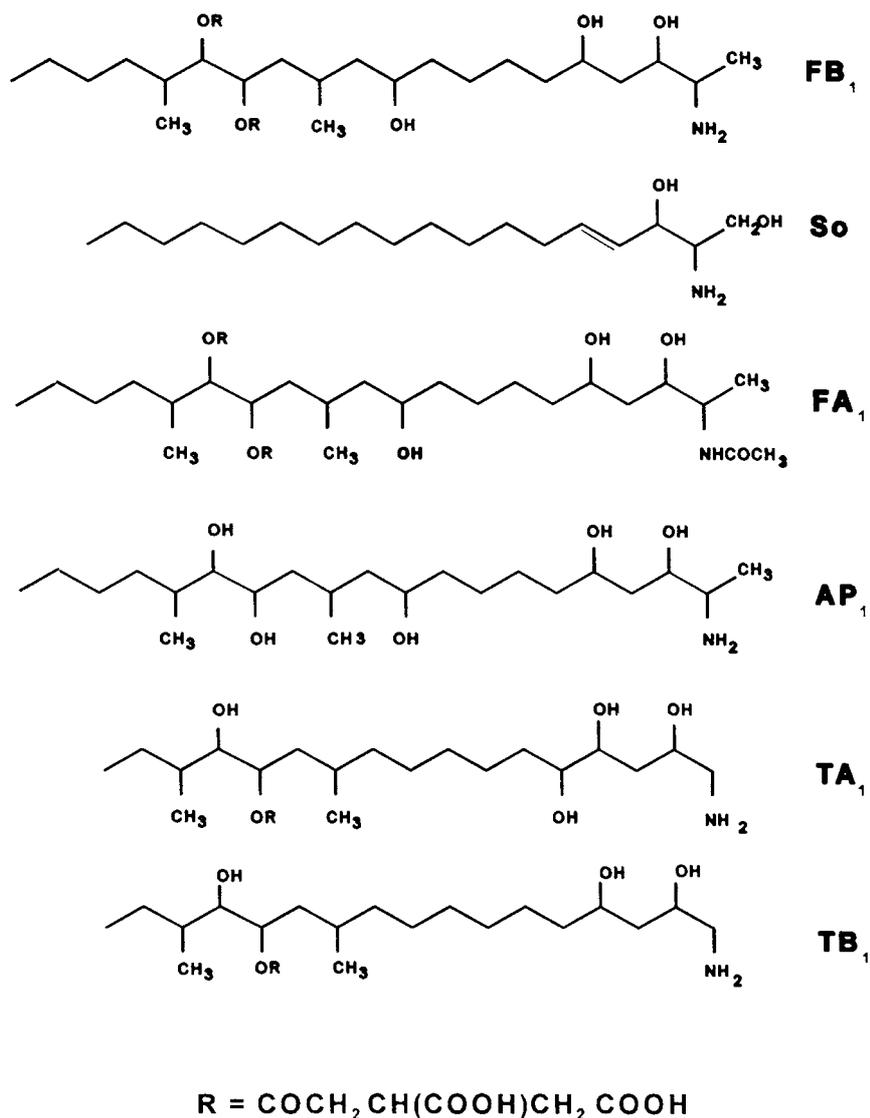


Fig. 1. Chemical structures of fumonisin B<sub>1</sub>, sphingosine, fumonisin A<sub>1</sub>, the hydrolysis product of FB<sub>1</sub>, AP<sub>1</sub> and the individual isomers of the AAL toxins, designated TA<sub>1</sub> and TB<sub>1</sub>. The isomers esterified at C-14 are called TA<sub>2</sub> and TB<sub>2</sub> (see Materials and Methods).

biological effects such as cytotoxicity and phytotoxicity are known to be similar.

Structural differences within the fumonisin group of mycotoxins have been utilized to study structure-activity relationships with respect to specific biological effects (Abbas *et al.*, 1993 and 1994; Gelderblom *et al.*, 1993; Shier *et al.*, 1991). FB<sub>1</sub>, as well as TA and TB, have been shown to be cytotoxic to certain mammalian cell lines. There were variations in sensitivities to fumonisins and AAL toxins among cell lines tested, depending on the tissue of origin and possibly the degree of differentiation (Shier *et al.*, 1991). When monitoring the inhibitory effect on cell proliferation in Madin-Darby canine kidney (MDCK) cells and a rat hepatoma cell line (H4TG), FA<sub>1</sub> (*N*-acetyl derivative of FB<sub>1</sub>) exhibited little or no activity while AP<sub>1</sub> (a hydrolysis product of FB<sub>1</sub>) showed similar or greater

effects than FB<sub>1</sub>, fumonisin B<sub>2</sub> (FB<sub>2</sub>) and fumonisin B<sub>3</sub> (FB<sub>3</sub>) (Abbas *et al.*, 1993). In primary hepatocytes, FA<sub>1</sub> exhibited a lower and AP<sub>1</sub> a higher cytotoxicity than FB<sub>1</sub> and FB<sub>2</sub> (Gelderblom *et al.*, 1993). In plants, FB<sub>1</sub> and TA caused higher leaf necrotizing activity on detached tomato leaves than FA<sub>1</sub> and AP<sub>1</sub> (Lamprecht *et al.*, 1994). FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub> exhibited cancer-initiating activity in an *in vivo* cancer initiating/promoting model in rat liver, while AP<sub>1</sub> and FA<sub>1</sub> lack activity (Gelderblom *et al.*, 1993).

Fumonisin (Wang *et al.*, 1991) and AAL toxins (Merrill *et al.*, 1993b) inhibit sphingosine *N*-acetyltransferase (ceramide synthase), a key enzyme in the sphingolipid biosynthetic pathway in animal cells. The inhibition of this enzyme leads to an elevation of sphingosine (So) and sphinganine (Sa) levels in cells, although Sa levels rise to a much

greater extent than the So levels, thus resulting in an increase in the Sa:So ratio (Riley *et al.*, 1994). FB<sub>1</sub> disrupted the sphingolipid profiles in the following cell culture systems: rat primary hepatocytes (Gelderblom *et al.*, 1995; Wang *et al.*, 1991), a renal epithelial cell line, LLC-PK<sub>1</sub> (Yoo *et al.*, 1992), Swiss 3T3 fibroblasts (Schroeder *et al.*, 1994) and mouse cerebellar neurons (Merrill *et al.*, 1993a). AAL toxin and FB<sub>1</sub> also disrupt sphingolipid biosynthesis in plants (Abbas *et al.*, 1995).

In the present study the structural requirements for ceramide synthase inhibition was investigated by comparing the effect of structurally related compounds (FB<sub>1</sub>, FA<sub>1</sub>, AP<sub>1</sub>, TA and TB) on the Sa:So ratio as well as the Sa and So concentrations in rat primary hepatocyte cultures. The extent to which sphingolipid biosynthesis was affected was correlated with the respective cytotoxicities of the structural analogues *in vitro*. The reversibility of the inhibitory effect was investigated to obtain more information about the biological significance of sphingolipid inhibition in primary hepatocytes.

## MATERIALS AND METHODS

### *Mycotoxin standards and chemicals*

FB<sub>1</sub>, FA<sub>1</sub> and AP<sub>1</sub> were purified as described previously by Cawood *et al.* (1991) and Gelderblom *et al.* (1993). The chemical purity of the structural analogues were determined by <sup>13</sup>C-NMR, HPLC and TLC as described by Cawood *et al.* (1991). TA and TB were purified as described by Shephard *et al.* (1993) and, in solution, consisted of a natural equilibrium of two isomers where either the C-13 (TA<sub>1</sub> and TB<sub>1</sub>) or the C-14 (TA<sub>2</sub> and TB<sub>2</sub>) hydroxyl group is esterified. Solutions of the individual toxins were prepared either in saline for FB<sub>1</sub>, TA and TB or in dimethyl sulfoxide (DMSO):saline (1:1) for AP<sub>1</sub> and FA<sub>1</sub>. Sa and So were obtained from Sigma Chemical Company (St Louis, MO, USA). C<sub>20</sub>-Sa was a generous gift from Professor A.H. Merrill, Jr, Department of Biochemistry, Emory University School of Medicine, Atlanta, GA, USA.

### *Preparation of hepatocyte cultures*

Primary hepatocytes were prepared from male Fischer 344 rats, weighing 150–200 g, by a collagenase perfusion technique (Hayes *et al.*, 1984). The viability of the hepatocyte preparations varied between 90 and 95% when using trypan blue exclusion. The cells were seeded ( $6 \times 10^5$  cells/plate; 60 mm) in collagen-coated dishes in triplicate for 3 hr in modified Williams' E 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). The cells were washed with Hanks' buffer solution and supplemented with serum-free, modified Williams' E medium containing L-proline (2 mM) and sodium

pyruvate (10 mM) and incubated at 37°C. The treated plates and the controls (except the 0 hr control) were incubated for 40 hr.

### *Sphinganine:sphingosine ratios in rat primary hepatocyte cultures*

The plated cells were washed with ice-cold saline (2 ml; three times) prior to being harvested (0.5 ml saline; three times) by scraping with a rubber policeman. An aliquot (0.1 ml) was removed for protein determination (Kaushal and Barnes, 1986). The Sa and So concentrations were determined by HPLC with C<sub>20</sub>-Sa as an internal standard, according to the method of Riley *et al.* (1994) with minor modifications. The lipids were extracted from the remainder of the hepatocyte cell suspension by incubation with methanol:chloroform (2:1) (containing 0.01% butylated hydroxytoluene as an antioxidant) under nitrogen at 37°C for 1 hr. Thereafter the mixture was washed twice with alkaline water, the phases separated by centrifugation and the chloroform fraction dried under nitrogen gas below 40°C. The residue was hydrolysed to release the free So by redissolving it in 0.1 M methanolic potassium hydroxide:chloroform (4:1), and incubated at 37°C for 1 hr. After washing with alkaline water, the chloroform phase was dried under nitrogen below 40°C.

### *HPLC quantification*

The dried residues were stored at –20°C overnight. Prior to analysis, the residues were redissolved in 250 µl methanol, sonicated and derivatized with 50 µl *o*-phthalaldehyde (OPA) reagent as previously described (Riley *et al.*, 1994). A 25–75 µl aliquot was injected into the HPLC which consisted of a Waters (Milford, MA, USA) Model 510 solvent delivery system, Waters U6K injector, Waters Radial-Pak<sup>TM</sup> cartridge packed with Nova-Pak C<sub>18</sub> (4 µm, 100 × 8 mm), Autochröm APEX Integration Chromatography Workstation and Perkin-Elmer (Norwalk, CT, USA) 650 S fluorescence detector (excitation 335 nm and emission 440 nm). The isocratic mobile phase of methanol:0.005 M potassium phosphate buffer, pH 7.0 (91:9) was pumped at a flow rate of 2 ml/min.

### *Treatment of hepatocyte cultures*

FB<sub>1</sub>. The hepatocyte control and treated cultures were incubated in triplicate. The control cultures, 0 hr (harvested before incubation) and 40 hr, were incubated with media containing saline without FB<sub>1</sub>. The treatment groups, 12 hr and 40 hr, were incubated as described above with media containing 1 µM FB<sub>1</sub> for 40 hr. After 12 hr and 24 hr, respectively, the media of the corresponding cultures containing the FB<sub>1</sub> were replaced with media without FB<sub>1</sub>.

Table 1. The effect of FB<sub>1</sub> exposure, for various time intervals, on Sa and So concentrations in rat primary hepatocyte cultures after a 40-hr incubation period

Treatment	Sphinganine (Sa) (pmol/mg protein)	Sphingosine (So) (pmol/mg protein)	Ratio (Sa:So)
Control (40 hr)	1.73 ± 0.27 a	16.5 ± 1.8 a	0.11 ± 0.02 a
Control (0 hr)	0.56 ± 0.08 B	12.7 ± 0.4 b	0.04 ± 0.01 b
FB <sub>1</sub> (12 hr)	199.3 ± 9.9 C	11.7 ± 0.8 C	17.1 ± 0.7 C
FB <sub>1</sub> (24 hr)	249.7 ± 14.2 D	11.6 ± 1.0 C	21.8 ± 3.0 D
FB <sub>1</sub> (40 hr)	224.7 ± 3.1 C	13.1 ± 1.2 c	17.4 ± 1.5 C

Values represent means ± SD of triplicate determinations. Control (40 hr) was separately compared with control (0 hr) and with the individual FB<sub>1</sub> treatment groups (12 hr, 24 hr and 40 hr). Values in a column followed by different letters (lower and upper case) differ significantly ( $P < 0.05$ ) from the control (40 hr); if both the letters and the cases differ, then  $P < 0.01$ . Values followed by the same letter (lower or upper case) do not differ significantly ( $P > 0.05$ ).

*Structurally related compounds.* The hepatocyte control and treated cultures were incubated in triplicate. The hepatocyte control cultures were incubated in media with either saline or DMSO:saline (1:1). The primary hepatocytes were incubated in media containing 1 μM each of the mycotoxins FB<sub>1</sub>, FA<sub>1</sub>, AP<sub>1</sub>, TA and TB for 40 hr.

#### Cytotoxicity determination

Detailed studies on the comparative cytotoxicity of AP<sub>1</sub>, FA<sub>1</sub>, FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub> have been published elsewhere (Gelderblom *et al.*, 1993). In the present study the cytotoxicity of different concentrations (75, 250 and 500 μM) of FB<sub>1</sub>, TA and TB were compared over a 40-hr incubation period. The release of lactate dehydrogenase (LDH) in the culture medium was monitored by the method of Hayes *et al.* (1984). Cytotoxicities of the compounds were expressed as LDH release in the medium calculated as a percentage of the total LDH release in the control cells after treatment with Triton X100.

#### Statistical analysis

All the data were subjected to analysis of variance (ANOVA; one-way), while the Tukey studentized range test was used to determine the statistical differences between means of the different treatment groups.

## RESULTS

### *The effect of FB<sub>1</sub> on sphingolipid biosynthesis (Table 1)*

Relatively small, but statistically significant, increases in the Sa concentration ( $P < 0.01$ ), So concentration ( $P < 0.05$ ) and Sa:So ratio ( $P < 0.05$ ) were observed between the 0 hr and 40 hr control hepatocyte cultures. In the hepatocytes incubated with FB<sub>1</sub> for 12, 24 and 40 hr the Sa level increased significantly ( $P < 0.01$ ) over the controls with a maximum accumulated at 24 hr. The corresponding So levels were significantly decreased ( $P < 0.01$  at 12 and 24 hr;  $P < 0.05$  at 40 hr) and therefore the Sa:So ratios were significantly ( $P < 0.01$ ) increased over the control after 40 hr of incubation. Within the treated groups the maximal change in the Sa:So ratio ( $P < 0.05$ , compared with 12 and 40 hr period) was obtained at 24 hr, while there was no significant difference in the ratios between the 12 and 40 hr exposure treatments.

### *The effect of structurally related compounds (Table 2)*

There were no marked differences in the So concentrations and Sa:So ratio profiles of the control hepatocyte cultures incubated for 40 hr with the carrier solvents, saline or DMSO:saline (1:1), while the Sa levels decreased significantly ( $P < 0.01$ )

Table 2. The effect of structurally related compounds on sphingolipid profiles

Treatment	Sphinganine (Sa) (pmol/mg protein)	Sphingosine (So) (pmol/mg protein)	Ratio (Sa:So)
<i>Hepatocytes incubated with saline</i>			
Control (saline)	1.73 ± 0.27 a	16.5 ± 1.8 a	0.11 ± 0.02 a
FB <sub>1</sub>	224.7 ± 3.1 b	13.1 ± 1.2 a	17.4 ± 1.5 b
TA	312.4 ± 9.7 c	19.0 ± 0.8 a	16.6 ± 0.2 b
TB	357.9 ± 10.3 d	25.2 ± 11.2 a	16.0 ± 5.7 b
<i>Hepatocytes incubated with DMSO:saline (1:1)</i>			
Control (DMSO:saline)	0.96 ± 0.03 c	13.6 ± 1.2 b	0.07 ± 0.01 c
FA <sub>1</sub>	305.1 ± 10.2 dA	23.1 ± 0.1 b	13.3 ± 0.5 d
AP <sub>1</sub>	153.5 ± 18.8 eB	37.8 ± 10.5 c	4.2 ± 0.6 e

Values represent means ± SD of triplicate determinations. Statistical comparisons between control and individual toxins were made within the different groups using saline and DMSO:saline as the control solvents. Values in a column followed by the same letter are not significantly different from the control ( $P > 0.05$ ), if the letter differs (upper cases) then  $P < 0.05$ , if the letters (lower case) differ then  $P < 0.01$ .

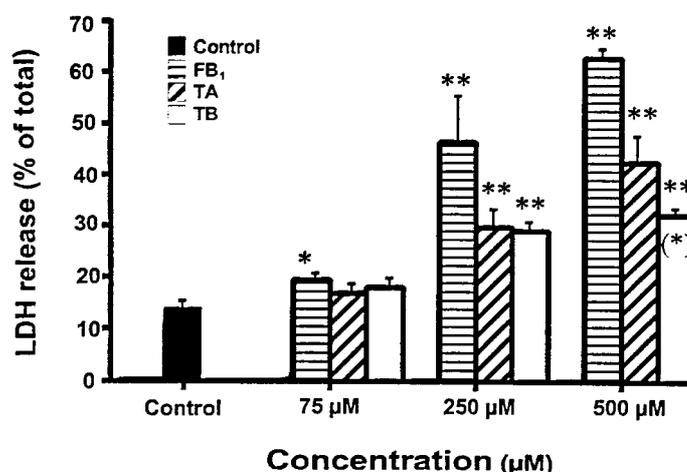


Fig. 2. The cytotoxic effect of FB<sub>1</sub>, TA and TB on rat primary hepatocyte cultures expressed as the amount of LDH released (% of total). Values represent means  $\pm$  SD of triplicate determinations. Values differ significantly from the control treatment: \* $P < 0.05$ ; \*\* $P < 0.01$ . The cytotoxicity of TA and TB differs significantly (\*\* $P < 0.01$ ) from FB<sub>1</sub> at 250 and 500  $\mu$ M while TB was significantly (\* $P < 0.05$ ) lower than TA at 500  $\mu$ M.

with the DMSO:saline (1:1) as the carrier solvent. In comparing the different compounds where saline was the solvent, the Sa levels increased significantly ( $P < 0.01$ ) over the control value in the order TB > TA > FB<sub>1</sub> ( $P < 0.01$ , between the individual treatment groups). There were no significant differences ( $P > 0.05$ ) in the So level in the saline group. The resulting Sa:So ratio increased significantly ( $P < 0.01$ ) over the control with no significant difference between the ratios for FB<sub>1</sub>, TA and TB, due to the increase in the corresponding So concentrations. Within the group of analogues (AP<sub>1</sub> and FA<sub>1</sub>) with DMSO:saline (1:1) as solvent, a significant ( $P < 0.01$ ) increase in the concentration of So was obtained with AP<sub>1</sub>, and the Sa concentration and Sa:So ratio of AP<sub>1</sub> and FA<sub>1</sub> increased significantly ( $P < 0.01$ ) over the control.

In comparing the AP<sub>1</sub> and FA<sub>1</sub> data with the results of the other analogues, FA<sub>1</sub> exhibits a similar effect on the Sa levels as compared with FB<sub>1</sub>, TA and TB, although it was significantly higher than FB<sub>1</sub> ( $P < 0.01$ ) and lower ( $P < 0.01$ ) than TB. The Sa level of AP<sub>1</sub> was significantly lower ( $P < 0.01$ ) as compared with the other compounds, while the So level of AP<sub>1</sub> increased significantly ( $P < 0.05$ ) above those of the other treatments. Hence, the mean Sa:So ratio increased from the average baseline value of 0.1 to 4.2 after exposure to AP<sub>1</sub>, which was significantly ( $P < 0.01$ ) lower as compared with the mean value of 15.8 obtained after exposure to FB<sub>1</sub>, FA<sub>1</sub>, TA and TB.

#### Comparative cytotoxicity of FB<sub>1</sub>, TA and TB (Fig. 2)

A typical dose-response effect was obtained with all the toxins. FB<sub>1</sub> exhibited the highest cytotoxicity

at concentrations of 75 ( $P < 0.05$ ), 250 ( $P < 0.01$ ) and 500  $\mu$ M ( $P < 0.01$ ) as compared with the control. TA and TB exhibited similar cytotoxicities at 250  $\mu$ M ( $P < 0.01$ ) as compared with the control, while TA tended to be slightly ( $P < 0.05$ ) more toxic than TB at 500  $\mu$ M.

#### DISCUSSION

FB<sub>1</sub> concentrations from 5 to 500  $\mu$ M did not increase the Sa:So ratio in hepatocyte cultures significantly above that which was achieved with 1  $\mu$ M FB<sub>1</sub> (Gelderblom *et al.*, 1995). Wang *et al.* (1991) found that the Sa concentration increased 110-fold in rat hepatocyte cultures after incubation with 1  $\mu$ M FB<sub>1</sub> for 4 days. In the present study, a maximum increase in the Sa concentration was observed after 12 to 24 hr after exposure to 1  $\mu$ M FB<sub>1</sub> that represent 115- to 144-fold increase as compared with the control value. The removal of FB<sub>1</sub> from the incubation media, even after 12 hr, did not result in a decrease in the Sa concentration and hence in the Sa:So ratio compared with that of the cells exposed to FB<sub>1</sub> for 40 hr (Table 1). Therefore, the inhibition of ceramide synthesis is either persistent or the Sa does not easily diffuse out of the cells (Merrill *et al.*, 1993b). It seems that the inhibition of sphingolipid biosynthesis by the fumonisins is an early event that cannot solely be associated with the adverse biological effects induced by FB<sub>1</sub> in primary hepatocytes. This can be deduced from the finding that the inhibitory effect of FB<sub>1</sub> on the epidermal growth factor (EGF) mitogenic response in primary hepatocyte cultures is reversible on removal of the toxin (Gelderblom *et al.*, 1995). In addition, no direct involvement of the sphingolipids, sphinganine or sphingosine on the EGF response in primary

hepatocytes was noted. The inhibitory effect of the EGF response in primary hepatocytes is a common property of many liver cancer promoters, including FB<sub>1</sub> (Gelderblom *et al.*, 1996a). The present finding concerning the irreversibility of ceramide synthase inhibition further supports the hypothesis that the disruption of sphingolipid biosynthesis seems not to be a key event in the inhibition of growth stimulatory effects in primary hepatocytes. A similar type of response was noticed in LLC-PK<sub>1</sub> cells, a pig renal epithelial cell line, where FB<sub>1</sub> (35  $\mu$ M) inhibited cell proliferation, as measured by protein content, and increased the Sa:So ratio over a 48-hr period (Yoo *et al.*, 1992). Those LLC-PK<sub>1</sub> cells, which survived FB<sub>1</sub> exposure, resumed normal cell growth after removal of the FB<sub>1</sub>, indicating that the FB<sub>1</sub>-induced inhibition of cell proliferation is also reversible in these cells.

FB<sub>1</sub> is not cytotoxic to primary hepatocytes when exposed for 4 days to concentrations of 1  $\mu$ M (Wang *et al.*, 1991). Even at higher concentrations (50 to 250  $\mu$ M), fumonisins exhibit a low to moderate cytotoxicity in primary hepatocytes as measured by LDH release (Gelderblom *et al.*, 1993). As FA<sub>1</sub> is less cytotoxic than FB<sub>1</sub> at concentrations of 125–1000  $\mu$ M, it was suggested that the free amino-group plays a role in the *in vitro* cytotoxicity (Gelderblom *et al.*, 1993). On the other hand, AP<sub>1</sub> is known to be more cytotoxic to primary rat hepatocytes than FB<sub>1</sub> (Gelderblom *et al.*, 1993). In the present study, AP<sub>1</sub> increased the Sa level and the Sa:So ratio to a much lesser extent than FB<sub>1</sub>. This indicated that the tricarballic (TCA) moieties are required for maximal inhibition of ceramide synthase. TA and TB, which are also less toxic than FB<sub>1</sub>, significantly ( $P < 0.01$ ) increased the Sa concentration above that of FB<sub>1</sub> although the Sa:So ratios were very similar due to variations in the So concentrations. It would therefore appear that a single TCA group is also sufficient for maximal ceramide synthase inhibition under the present conditions. Despite the significant ( $P < 0.01$ ) increases in Sa levels induced by FA<sub>1</sub> when compared with FB<sub>1</sub>, the Sa:So ratio is of the same order due to variations in the So concentration. As compared with the presence of the TCA groups, it seems that the presence of a free amino-group is not a requisite for enzyme inhibition. Although the structural basis for the inhibition of ceramide synthase is not known, two possible modes of inhibition have been postulated (Merrill *et al.*, 1993b). The structural similarity in the head group of the toxins and the sphingoid bases (Fig. 1) allows the enzyme to recognize them as substrates or, alternatively, the tricarballic acid moieties interact with the binding site for the fatty acid moiety. The inhibition occurring with AP<sub>1</sub> indicates that the former is the most probable mode, although the presence of a tricarballic acid moiety seems to further enhance the interactions with the enzyme.

Regarding FB<sub>1</sub> and AP<sub>1</sub>, the absence of the TCA moieties increased the cytotoxic effect in primary hepatocytes, possibly due to a decrease in the polarity of the molecule (Gelderblom *et al.*, 1993). In the case of TA and TB the presence of a single TCA moiety seems not to correlate with cytotoxicity as TA is more cytotoxic than TB while both toxins are less cytotoxic than FB<sub>1</sub>. In this regard, other structural differences between the fumonisins and TA and TB also could play a contributing role. The contrasting results between cytotoxicity and the elevation of Sa levels and Sa:So ratios in primary hepatocytes indicate that the cytotoxicity of these compounds is not solely due to inhibition of ceramide synthase and the subsequent changes in sphingoid base concentrations. The lack of direct association of cytotoxicity and the inhibition of sphingolipid biosynthesis in rat hepatocytes has previously been reported. Hepatocytes exposed to both toxic and non-toxic concentrations of FB<sub>1</sub> interrupt sphingolipid biosynthesis to the same extent as the concentration (1  $\mu$ M) used in the present study (Gelderblom *et al.*, 1995 and 1996b). It would appear that the structural requirements for cytotoxicity and inhibition of ceramide synthase differ in primary hepatocytes. In contrast to this, studies on LLC-PK<sub>1</sub> cells have shown a direct correlation between FB<sub>1</sub>-induced cytotoxicity and inhibition of sphingolipid biosynthesis (Yoo *et al.*, 1992). These differences may be related to the lower cytotoxicity and greater sensitivity to sphingolipid disruption by FB<sub>1</sub> in primary hepatocytes as compared to the LLC-PK<sub>1</sub> renal cells.

*In vivo* studies on the cancer-initiating potential of the structural analogues, AP<sub>1</sub>, and FB<sub>1</sub>, FB<sub>2</sub> and FB<sub>3</sub> indicated that only the fumonisin B mycotoxins exhibited activity in a short-term carcinogenesis model in rat liver (Gelderblom *et al.*, 1993). The present investigation showed that differences exist in the structural requirements for the induction of cytotoxicity in primary hepatocytes and the inhibition of ceramide synthase. Future studies into the mechanism of action of the fumonisins and AAL toxins concerning their biological effect on and role in ceramide synthase inhibition might enhance current knowledge.

#### REFERENCES

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