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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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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Disruption of sphingolipid biosynthesis in hepatocyte nodules: selective proliferative stimulus induced by fumonisin B_1

Liana van der Westhuizen [a,*], Wentzel C.A. Gelderblom [a], Gordon S. Shephard [a], Sonja Swanevelder [b]

[a] *Programme on Mycotoxins and Experimental Carcinogenesis Unit, Medical Research Council, P.O. Box 19070, Tygerberg 7505, South Africa*

[b] *Centre for Epidemiological Research in South Africa, Medical Research Council, P.O. Box 19070, Tygerberg, South Africa*

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Abstract

In order to investigate the role of sphingolipid disruption in the cancer promoting potential of fumonisin B_1 (FB_1) in the development of hepatocyte nodules, male Fischer 344 rats were subjected to cancer initiation (FB_1 containing diet or diethylnitrosamine (DEN) by i.p. injection) and promotion (2-acetylaminofluorene with partial hepatectomy, 2-AAF/PH) treatments followed by a secondary FB_1 dietary regimen. Sphinganine (Sa) and sphingosine (So) levels were measured by high performance liquid chromatography in control, surrounding and nodular liver tissues of the rats. The disruption of sphingolipid biosynthesis by the secondary FB_1 treatment in the control rats was significantly ($P < 0.05$) enhanced by the 2-AAF/PH cancer promotion treatment. The nodular and surrounding Sa levels returned to baseline following FB_1 initiation and 2-AAF/PH promotion. When comparing the groups subjected to the secondary FB_1 treatment, the initiation effected by FB_1 was less ($P < 0.01$) sensitive to the accumulation of Sa in the nodular and surrounding tissues than DEN initiation and the 2-AAF/PH control treatment. In contrast, the So level of FB_1 initiation was marginally increased in the nodules compared to the surrounding liver after 2-AAF/PH promotion and significantly ($P < 0.05$) higher with the secondary FB_1 treatment. Although, the FB_1-induced hepatocyte nodules were not resistant to the disruption of sphingolipid biosynthesis, the nodular So levels were increased and might provide a selective growth stimulus possibly induced by bio-active sphingoid intermediates such as sphingosine 1-phosphate (S1P).
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1. Introduction

Fumonisin B_1 (FB_1) is the major mycotoxin predominantly produced by *Fusarium verticillioides* occurring ubiquitously on corn (Shephard et al., 1996). Ingestion of fumonisin-contaminated feed results in various animal diseases (Shephard, 2001). High incidences of human esophageal cancer (Rheeder et al.,

Abbreviations: 2-AAF, 2-acetylaminofluorene; FB_1, fumonisin B_1; DEN, diethylnitrosamine; PH, partial hepatectomy; So, sphingosine; S1P, sphingosine 1-phosphate; Sa, sphinganine

* Corresponding author. Tel.: +27-21-938-0521; fax: +27-21-938-0260.

E-mail address: liana.van.der.westhuizen@mrc.ac.za (L. van der Westhuizen).

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1992; Chu and Li, 1994; Yoshizawa et al., 1994) and liver cancer (Ueno et al., 1997) have been associated with the consumption of fumonisin-contaminated corn. Fumonisins are not mutagenic (Gelderblom et al., 1991; Knasmuller et al., 1997) nor genotoxic in primary rat hepatocytes (Norred et al., 1992), however FB_1 exhibits clastogenesis (Ehrlich et al., 2002) and epigenetic properties (Mobio et al., 2000) in cell cultures. FB_1 is hepatocarcinogenic in male BD IX rats (Gelderblom et al., 1991, 2001) and in $B6C3F_1$ female mice and nephrocarcinogenic in male Fischer 344 rats (Howard et al., 2001).

FB_1 inhibits ceramide synthase, a key enzyme in de novo sphingolipid biosynthesis, preventing the conversion of sphinganine (Sa) to dihydroceramide and the reacylation of sphingosine (So) to ceramide (Riley et al., 1994; Wang et al., 1991). The disruption of the sphingolipid biosynthetic pathway elevates sphingoid bases and their 1-phosphate levels and decrease ceramide and more complex sphingolipids, such as sphingomyelin and gangliosides, and their intermediates (Riley et al., 2001; Merrill et al., 2001). However, an increase in the sphingoid bases can only occur once the capacity of sphingosine kinase to metabolize these bases to their 1-phosphates has been exceeded (Riley et al., 2001). Sphingolipids are predominantly found in cellular membranes and are critical for the maintenance of the membrane structure, while complex sphingolipids function as precursors for second messengers and are important in sustaining cellular growth and differentiation (Merrill et al., 2001). FB_1 inhibits cell proliferation in various cell culture systems as well as in rat liver and kidney (Gelderblom et al., 1996; Riley et al., 2001; Yoo et al., 1992). FB_1-induced disruption of sphingolipid biosynthesis can either induce or prevent apoptosis, depending on the cell type and the relative amounts of the bio-active sphingolipid molecules generated (Desai et al., 2002; Tolleson et al., 1996). The impairment of apoptotic pathways during liver cancer promotion results in an imbalance between cell death and proliferation and thus the outgrowth of hepatocyte nodules in the presence of a promoter (Schulte-Hermann et al., 1993). In this regard cells with decreased ceramide and increased So 1-phosphate (S1P) levels might be selected to survive and proliferate, provided that increased sphingoid bases are not growth inhibitory in these cells (Riley et al., 2001). Hence, the disruption of sphingolipid biosynthesis has been implicated in the carcinogenic activity of FB_1 (Riley et al., 2001; Voss et al., 2002).

Cancer initiation by chemicals in rat liver is generally characterized by the appearance of phenotypically altered resistant hepatocytes (Solt et al., 1980). These resistant hepatocytes escape the mitoinhibitory effects of FB_1 on normal hepatocyte growth and selectively proliferate into hepatocyte nodules (Gelderblom et al., 1995, 2001). The exact mechanism involved in the selection of initiated cells by FB_1 is unknown. The purpose of this study was to determine whether hepatocyte nodules are resistant to the inhibitory effect of FB_1 on ceramide synthase, resulting in a growth differential which could selectively stimulate their outgrowth.

2. Materials and methods

2.1. Chemicals

FB_1 was purified as described previously by Cawood et al. (1991). Diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), Sa and So were obtained from Sigma Chemical Company (St. Louis, MO, USA). C_{20}-Sa was a generous gift from Prof. A.H. Merrill Jr. All other chemicals and solvents were analytical grade from Merck (Darmstadt, Germany).

2.2. Animals

Male Fischer (F344) rats were bred and maintained on the AIN76 diet (AIN, 1977) at the Primate Unit of the MRC Diabetes Research Group in a controlled environment of 23–25 °C and 12 h light/dark cycles. During the experimental period the rats were caged individually with normal access to feed and water. The experimental protocol was approved by the Ethics Committee for Research on Animals of the Medical Research Council, Tygerberg, South Africa.

2.3. Experimental procedures

Experimental male Fischer 344 rat (150–200 g) groups were subjected to various cancer initiation and promotion regimens, whereas control rat groups were subjected only to the promotion regimens (Table 1). Initiation treatment consisted of either a

Table 1
Treatment protocols of male Fischer rats to determine whether hepatocyte nodules are resistant to the inhibitory effect of FB_1 on ceramide synthase

Group number and treatment code	Initiation[a] (21 days)	Recovery (14 days)	Promotion[b] (4 days)	Recovery (14 days)	Secondary treatment (14 days)
Control groups					
1 Control	Control diet	Control diet	–	Control diet	Control diet
2 Control/FB_1	Control diet	Control diet	–	Control diet	250 mgFB_1/kg diet
3 2-AAF/PH	Control diet	Control diet	2-AAF/PH	Control diet	Control diet
4 2-AAF/PH/FB_1	Control diet	Control diet	2-AAF/PH	Control diet	250 mgFB_1/kg diet
Experimental groups					
5 FB_1/2-AAF/PH	500 mgFB_1/kg diet	Control diet	2-AAF/PH	Control diet	Control diet
6 FB_1/2-AAF/PH/FB_1	500 mgFB_1/kg diet	Control diet	2-AAF/PH	Control diet	250 mgFB_1/kg diet
7 DEN/2-AAF/PH	DEN/control diet	Control diet	2-AAF/PH	Control diet	Control diet
8 DEN/2-AAF/PH/FB_1	DEN/control diet	Control diet	2-AAF/PH	Control diet	250 mgFB_1/kg diet

[a] Initiation treatment consisted of either a 3-week FB_1 (500 mg/kg) dietary treatment (groups 5 and 6) or a single i.p. injection (200 mg/kg) of diethylnitrosamine (DEN, groups 7 and 8).
[b] Promotion treatment consisted of 2-acetylaminofluorene (2-AAF, 20 mg/kg) gavage doses on 3 consecutive days followed by partial hepatectomy (PH).

3-week FB_1 dietary treatment (500 mg/kg feed) or a single i.p. injection of diethylnitrosamine (DEN, 200 mg/kg body weight). Promotion treatment followed 2 weeks after the initiation treatment and consisted of 2-acetylaminofluorene (2-AAF, 20 mg/kg body weight) gavage doses on 3 consecutive days followed by partial hepatectomy (PH) (Semple-Roberts et al., 1987). The rats were anaesthetized with 2–3% fluothane (95% O_2) and received 5% glucose supplementation in their drinking water for 12 h post-operative. After initiation and promotion all the rats received control diets for a 2-week recovery period. Subsequently, 50% of all the groups were subjected to a secondary 2-week FB_1 dietary treatment (250 mg/kg feed). At the end of the experimental period all the rats were sacrificed (sagatal anaesthesia) and the macroscopically distinguishable encapsulated hyperplastic nodules were separated by scooping the nodules from the surrounding liver tissue. The nodular, surrounding and control liver tissues were collected, frozen on dry ice and stored at −80 °C.

2.4. Sphingolipid analyses

Homogenized liver extracts were prepared from all the liver tissues in phosphate buffer (Van der Westhuizen et al., 2001a) and the levels of the sphingolipid bases, Sa and So, quantified by reversed-phase HPLC as fluorescent derivatives (Van der Westhuizen et al., 2001b). Protein content of the liver extracts was determined by a modified Lowry method (Markwell et al., 1978).

2.5. Statistical analysis

The data were tested for normality, using the Kolmogorov–Smirnov test, as well as for equality of variances. *T*-tests were used to test for group differences (two groups), using the Pooled Method when variances were equal, and the Satterthwaite Method when variances were unequal.

3. Results

3.1. Control treatments

The liver Sa ($P < 0.001$) and So ($P < 0.05$) levels, as well as the Sa/So ratio ($P < 0.01$), were significantly increased in rats subjected to the secondary FB_1 treatment compared to the baseline levels of the untreated control rats (Table 2). Although both the Sa and So levels were significantly ($P < 0.05$) increased in the liver of the rats subjected to the 2-AAF/PH promo-

Table 2
Sphinganine (Sa) and sphingosine (So) levels and Sa/So ratios of the livers of male Fischer rats in control, surrounding and nodular tissues[a]

Group number and treatment code	n	Sphinganine (pmol/mg protein)	Sphingosine (pmol/mg protein)	Sa/So ratio
Control				
1 Control	4	1.13 ± 0.15 (1.03–1.35) a	14.7 ± 4.32 (9.57–19.2) a	0.08 ± 0.02 (0.06–0.11) a
2 Control FB_1	4	16.2 ± 2.04 (14.4–18.6) B	27.2 ± 6.75 (17.2–31.6) b	0.63 ± 0.17 (0.46–0.86) B
3 2-AAF/PH	4	1.91 ± 0.46 (1.29–2.39) b	28.3 ± 7.34 (17.9–34.1) b	0.07 ± 0.03 (0.05–0.11) a
4 2-AAF/PH/FB_1	6	46.3 ± 25.7 (20.3–90.0) B	36.8 ± 20.3 (11.8–59.0) b	1.38 ± 0.39 (0.78–1.79) B
Surrounding				
5 FB_1/2-AAF/PH	4	1.22 ± 0.30 (1.01–1.65) a	20.5 ± 6.32 (11.7–26.6) a	0.06 ± 0.02 (0.04–0.09) a
6 FB_1/2-AAF/PH/FB_1	4	13.0 ± 3.13 (9.00–16.1) B	32.7 ± 4.49 (28.8–38.9) B	0.39 ± 0.06 (0.31–0.45) B
7 DEN/2-AAF/PH	6	1.11 ± 0.64 (0.59–2.11) a	17.0 ± 10.4 (5.80–32.7) a	0.08 ± 0.03 (0.04–0.12) a
8 DEN/2-AAF/PH/FB_1	4	28.6 ± 7.44 (19.4–36.6) B	48.4 ± 17.4 (25.2–67.2) b	0.62 ± 0.12 (0.50–0.77) B
Nodules				
5 FB_1/2-AAF/PH	4	2.59 ± 1.33 (1.29–3.98) a	36.8 ± 13.9 (25.7–55.3) b	0.06 ± 0.03 (0.03–0.09) a
6 FB_1/2-AAF/PH/FB_1	4	15.4 ± 6.58 (9.55–24.4) b	47.2 ± 10.8 (33.3–57.0) B	0.32 ± 0.10 (0.22–0.45) b
7 DEN/2-AAF/PH	5	1.15 ± 0.34 (0.60–1.51) a	21.8 ± 10.4 (9.82–34.6) a	0.06 ± 0.02 (0.04–0.07) a
8 DEN/2-AAF/PH/FB_1	6	27.6 ± 8.09 (16.8–38.5) B	50.4 ± 10.5 (38.1–65.3) B	0.55 ± 0.12 (0.37–0.68) B

[a] Values represent mean ± standard deviation with the range in parentheses. Values in a column followed by the same letter are not significantly different from the control, if the letter differs then $P < 0.05$, if the cases differ then $P < 0.01$.

tion regimen, the Sa/So ratio was similar to the baseline ratio. The So level in the 2-AAF/PH treated rats was similar to the rats subjected to the secondary FB_1 treatment. The secondary FB_1 treatment significantly enhanced the accumulation of Sa ($P < 0.05$) and the Sa/So ratio ($P < 0.01$) induced by the 2-AAF/PH promoting regimen.

3.2. Initiation protocols using FB_1 and DEN regimens

3.2.1. Feeding the control diet during the secondary treatment period

Hepatocyte nodules were observed in the livers of all the rats, which were subjected to either FB_1 or DEN initiation treatment prior to the 2-AAF/PH promotion regimen as reported previously (Gelderblom et al., 1992, 1996). Histological features of the nodules have been described previously (Gelderblom et al., 2002). The nodules were scattered randomly throughout the liver and sharply demarcated from the surrounding liver and showed increased mitotic figures, mixed eosinophilic and clear cell changes. Oval cells were observed forming a rim around the nodules (Fig. 1). The DEN cancer initiation regimen followed by the 2-AAF/PH promotion did not significantly affect either the Sa or So levels in both the nodular and surrounding tissues compared to the baseline levels. When FB_1 was used as the cancer initiator, both the Sa and So levels and the Sa/So ratio in the surrounding liver tissue returned to baseline levels. However, the nodular So level was significantly ($P < 0.1$) increased above the baseline level, whereas neither the Sa level nor the Sa/So ratio were affected.

3.2.2. Feeding the FB_1-containing diet during the secondary treatment period

The FB_1 cancer initiation treatment significantly increased the Sa levels to a similar extent in the surrounding ($P < 0.01$) and nodular ($P < 0.05$) tissues when compared to the rats that received a control diet during the secondary treatment period. The Sa levels in the nodular and surrounding tissues were similar to the control rats treated with the secondary FB_1, but were significantly ($P < 0.05$) lower than the control rats subjected to the combined 2-AAF/PH promotion and secondary FB_1 regimens. The nodular So level was significantly ($P < 0.05$) higher compared to the surrounding tissue, but was similar to the control rats subjected to the combined 2-AAF/PH promotion and secondary FB_1 regimens. The Sa/So ratios in both the surrounding ($P < 0.001$) and nodular ($P < 0.05$) tissues were significantly increased above the baseline ratio, but were significantly lower ($P < 0.05$) than the ratios observed in both the control and 2-AAF/PH groups subjected to the secondary FB_1 treatment. When DEN

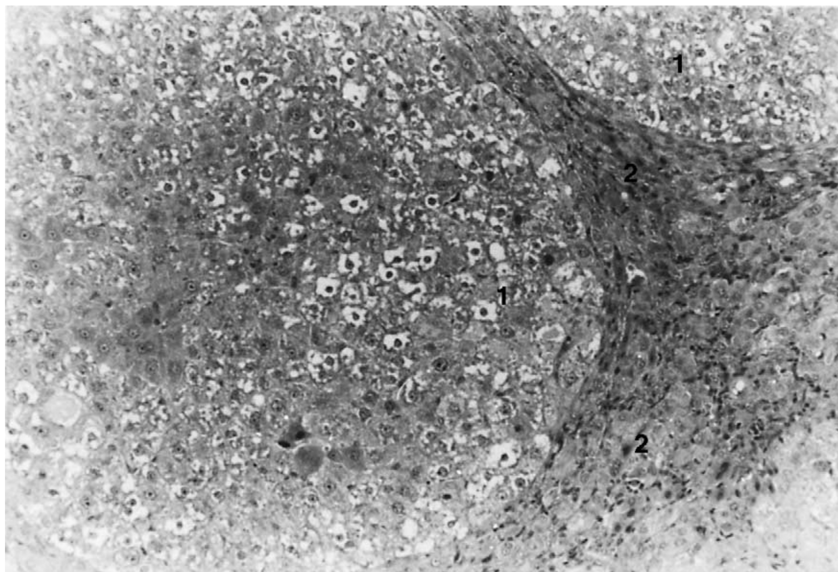

Fig. 1. Hepatocyte nodules in a rat from experimental group 6. Note the hepatocyte nodules (1) and proliferating oval cells in the surrounding tissue (2) (H&E × 100).

was used as cancer initiator the Sa and So levels were similarly increased in both the surrounding and nodular tissues compared to the rats fed a control diet during the secondary treatment period. The Sa levels in the surrounding ($P < 0.01$) and nodular ($P < 0.05$) tissues were significantly higher than when FB_1 was used as a cancer initiator. Both the surrounding and nodular Sa levels were significantly increased ($P < 0.01$) over the baseline level and markedly (not significantly) lower than the 2-AAF/PH treated rats subjected to the secondary FB_1 treatment. A similar effect was noticed for the So level, except that the So levels were markedly higher in comparison to the 2-AAF/PH rats, subjected to the secondary treatment. The Sa/So ratio was similar in the nodular and surrounding tissues and in the control rats subjected to the secondary FB_1 treatment, but was significantly ($P < 0.01$) lower than the 2-AAF/PH subjected to the secondary FB_1 treatment.

4. Discussion

In normal regenerating liver disruption of sphingolipid biosynthesis induced by FB_1 enhanced the accumulation of Sa (Li et al., 2000). In the present study the 2-AAF/PH promotion regimen significantly sensitized the liver to the accumulation of Sa by the 2-week secondary FB_1 treatment. Additionally, the significant increase in So, 4 weeks post 2-AAF/PH treatment, could have enhanced the S1P levels and led to stimulation of cell proliferation and suppression of apoptosis (Desai et al., 2002). As in previous studies, hepatocyte nodules developed in the livers of all the rats subjected to the cancer initiation (FB_1 or DEN) treatment followed by the 2-AAF/PH promotion regimen (Gelderblom et al., 1992, 1996). In the absence of the secondary FB_1 treatment, the Sa levels returned to baseline in both the nodules and surrounding tissue 6 weeks after cessation of FB_1 initiation treatment followed by the 2-AAF/PH promoting regimen. This reversibility of ceramide synthase inhibition was also apparent upon removal of FB_1-contaminated diet in animal studies or when FB_1 is removed from the media in primary as well as transformed cell culture systems (Enongene et al., 2002; Gelderblom et al., 1995; Wang et al., 1999; Yoo et al., 1992). In the presence of the secondary FB_1 treatment, the Sa level was enhanced to a similar extent in nodular and surrounding liver, comparable with the control rats treated with the secondary FB_1. However, in the absence of the secondary FB_1 treatment, the nodular So in the FB_1 initi-

ated rats did not return to baseline level, but remained increased similar to the control group subjected to both the 2-AAF/PH and the secondary FB_1 regimens. The increased nodular So might be attributed to an increase in cell proliferation induced by the 2-AAF/PH promotion regimen. In the presence of the secondary FB_1 treatment, the So level was selectively further increased above the surrounding tissue, especially in the FB_1-induced initiated cell population. It would appear that nodules induced by FB_1 are sensitized to accumulate So, which could selectively support cell proliferation of initiated cells through the production of S1P.

When utilising DEN as the cancer initiator model, in the absence of the secondary FB_1 treatment, sphingolipid biosynthesis was not disrupted in the nodular or surrounding tissue. However, DEN initiation, followed by the secondary FB_1 treatment, disrupted sphingolipid biosynthesis significantly in the nodules and surrounding liver tissue. Both the nodular and surrounding So were significantly increased to a similar level as in the FB_1-induced nodules subjected to the secondary FB_1 treatment. It would appear that DEN sensitized nodules and surrounding liver treated with the secondary FB_1 regimen, accumulated Sa to a higher extend than FB_1-initiated liver. However, the Sa level was still lower than in the control rats subjected to the combined 2-AAF/PH promotion and secondary FB_1 regimen. The increased sensitivity in DEN rats towards the disruption of sphingolipid metabolism by FB_1, implies that FB_1 pre-treatment rendered the liver more resistant to the accumulation of Sa, but not So, which tended to selectively accumulate in the FB_1-induced hepatocyte nodules. This resulted in a significantly lower Sa/So ratio in the FB_1-induced nodules compared to the DEN-induced nodules.

The present study confirmed that normal proliferating hepatocytes are more sensitive to the disruption of sphingolipid biosynthesis by FB_1 than quiescent hepatocytes. The inhibitory effect of FB_1 on ceramide synthase was reversible in hepatocyte nodules, although an apparent delayed effect on the reversal of So was observed. The FB_1-induced hepatocyte nodules were not resistant to the disruption of sphingolipid biosynthesis, implying that it might not be a major growth stimulus in their outgrowth. However, the delayed recovery effect of the So levels in the FB_1-induced nodules compared to the surrounding tissue, and the sensitization of So accumulation in the nodules upon subsequent FB_1 exposure, could provide a selective growth stimulus resulting in their selective outgrowth.

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CHAPTER V

RISK ASSESSMENT OF THE FUMONISINS

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RISK ASSESSMENT OF THE FUMONISINS

5.1 Involvement in human diseases

The association between the consumption of *Fusarium verticillioides* infected maize and oesophageal cancer (OC) development was first established in the former Transkei region of the Eastern Cape Province, South Africa (Marasas *et al*., 1979, 1981). A detailed survey on the occurrence of *Fusarium* mycotoxins on home-grown maize showed high levels of the fumonisins with a typical geographical distribution depending on the environmental conditions that influence the colonisation and fumonisin production by *Fusarium verticillioides* (Sydenham *et al*., 1990a, 1990b; Rheeder *et al*., 1992; Shephard *et al*., 1996a). Although clear fumonisin distribution patterns were noticed between high and low OC incidence regions, recent cancer registry data showed very few differences in the patterns of the disease between former low and high incidence regions (Somdyala *et al*., 2003). In general it would appear that the high consumption of maize in this region increased the risk of the population to be exposed to a cocktail of mycotoxins such as fusarin C (Gelderblom *et al*., 1984), deoxynivalenol, nivalenol, zearalenone, moniliformin (Sydenhan *et al*., 1990a), fumonisins (Rheeder *et al*., 1992) and other unknown toxic principles (Bever et al., 2000). Similar findings regarding the co-contamination of maize with fumonisins and different other *Fusarium* mycotoxins have been reported in high-risk areas for OC in China (Luo *et al*., 1990; Yoshizawa *et al*., 1994; Gao and Yoshizawa, 1997; Zhang *et al*., 1997). Studies on the fungal and mycotoxin contamination of maize and the possible involvement with OC have been described in other countries such as the Republic of Iran, northern Italy and Brazil (Bolger *et al*., 2001). A recent study in China showed high contamination rates of FB_1 in maize samples collected from high incidence areas of oesophageal and liver cancer suggesting a possible contributing role in the development of these cancers (Ueno *et al*., 1997; Sun *et al*., 2007).

5.1.1 Maize as a risk factor for OC

Maize seed entered Africa during 1500 AD and gradually replaced sorghum and millet as the dominant crop in southern Africa specifically along the eastern coast in the so-called maize belt, including countries such as South Africa, Zimbabwe, Zambia, Kenia and Ethiopia (Mcann, 2005). A significant correlation exists between

the incidence of OC and the maize supply in these regions while no association was obtained between the supply of sorghum and millet (Viljoen, 2003; Isaacson, 2005). An increase in OC patterns in the former Transkei region of the Eastern Cape Province was first noticed by Burrell (1962) and Rose (1973) in 1940 to 1950. The consumption of maize meal and home-grown maize has been identified as a risk factor for the development of OC in case-control studies (Van Rensburg 1985; Sammon 1992; Sammon and Iputo, 2006; Sewram, 2006). Additional risk factors for the development of OC associated with an underlying nutritional deficiency due to the consumption of maize as a major dietary staple have been suggested. These include deficiencies of vitamins, selenium, folate, magnesium and molybdenum (Van Rensburg *et al*., 1983, 1985; Van Helden *et al*., 1987; Jaskiewicz *et al*., 1988). A specific dietary pattern has been identified in a hospital-based case-control study by principal component analyses, which include a diet of maize, imifino and dry beans as a risk factor for OC development (Sewram, 2006). Two other diet patterns were shown to have a protective effect including dietary pattern 1: sorghum, potted vegetables, fruit, meat and green leafy vegetables and dietary pattern 3: which is mainly wheat based. However, components of the latter two protective diets, specifically wheat are not known to feature as major dietary staples while sorghum is not utilised as a dietary staple in the low and high incidence rural districts (Centane, Butterworth, Lusikisiki and Bizana) of the former Transkei region (Beyers *et al*., 1979). As the study showed that rural residents were more likely to develop OC the implication of specific dietary patterns associated with OC is confounded by the fact that the study only reflected differences in dietary patterns between the urban and rural populations. This is of particular interest when using a food frequency questionnaire in conducting retrospective nutritional surveys to assess the intake patterns of specific dietary components, which is subjected to recall bias. As the case-control study reflected a high proportion of urban residents [patients (62%) and controls (70%)], extrapolation of findings to OC cancer patterns in rural areas seems inappropriate and findings are subjected to analyses bias and confounders known to exist when conducting hospital-based studies (Sutton-Tyrrel, 2007). At present all the studies conducted in this region have numerous methodological errors due to the type of dietary assessment methods used. The use of culture specific and validated methods would have been more appropriate in assessing specific dietary patterns associated with OC (Wolmarans and Wentzel-Viljoen, 2008).

The identification of specific dietary patterns in a population by Sewram (2006) without quantifying food intake also could provide erroneous information, especially in a population where sorghum and wheat do not form part of a rural diet (Beyers *et al*., 1979). Therefore, the validity of the food frequency questionnaire used in the study is questionable. This is further emphasised regarding the finding that bean consumption protects against OC development in males but not females, which again could be related to portion sizes consumed. It also contrasted the finding of Sammon (1998) that the consumption of beans is associated with OC development. The finding by Sewram (2006) that home grown maize is a risk factor has been reported previously (Van Rensburg *et al*., 1985). This strengthened the association of OC with fumonisin contamination of maize and the possible modulating role it could play in the development of the disease (Rheeder *et al*., 1992; Shephard *et al*., 2005).

5.1.2 Mouldy maize as a risk factor for OC

The consumption of mouldy maize was found not to be a risk factor in the PhD dissertation by Sewram (2006) although this finding is clouded by numerous confounding factors. Several aspects should be considered when trying to assess the level of mouldy maize consumption in the Transkeian population. As females prepare the food, including the sorting and washing of the home grown maize, it is difficult for the males to assess whether they consumed mouldy maize or not. The sorting and washing procedures will also differ from household to household as no standardisation has been implemented, which could have a important effect on the level of consumption of the fumonisins. It will therefore be difficult to assess the intake of mouldy maize and any attempt to use visualised *Fusarium* infected cobs to determine intake will lead to erroneous data as mouldy maize is selected from home-grown maize prior to preparation of the meal and is not consumed as such. The infected kernels are subsequently used for animal feed or beer making. The study also shown that although 84% of the cases and 78% of the controls consumed traditional beer prepared from mouldy maize although only 10% of the participants exclusively used home-grown maize that could be used to select mouldy maize for beer making. These contradictory findings make it difficult to evaluate the possible role of mouldy maize beer as a risk factor in the development of OC. The study, however, showed that maize and maize-beer are important risk factors for the development of the disease, however only the frequency of exposure was monitored.

When adjusted for alcohol, maize beer was not a risk factor although a synergistic interaction with carcinogens could not be evaluated. In this regard it has been suggested that alcohol, which is not a carcinogen *per se* exhibits a solvent effect on tobacco and other environmental carcinogens (Tuyns, 1979) which include the fumonisins, one of the major carcinogens present in maize and maize beer (Shephard *et al*., 2003, 2005). The carcinogen levels in food can only be assessed as a risk factor if the actual level of exposure is monitored using validated biomarkers. The low proportion of the study population consuming home-grown maize, the urban/rural distribution of the study population and the lack of actual intake levels are major weaknesses to assess the possible role of mouldy maize and therefore the fumonisins in the development of the disease.

5.1.3 Other diseases

Apart from a possible involvement in OC, fumonisins have also been implicated in the development of liver cancer in studies in China where the levels in maize were monitored over a period on three years (Chu *et al*., 1994; Ueno *et al*., 1997). As in the case of OC the interaction between mycotoxins including aflatoxin B_1, deoxynivalenol, and the algal toxins were considered. An interaction between AFB_1 and FB_1 in the development of liver carcinogenesis in experimental animals has been shown (Gelderblom *et al*., 2002; Carlson *et al*., 2001). The association between the fumonisins with neural tube defects (NTD) became of interest as these mycotoxins disrupt the folate receptor in cells (Hendricks, 1999; Marasas *et al*., 2004; Stevens and Tang, 1997). The role of sphingolipid, cholesterol, major constituent of lipid rafts associated with the folate receptor is critical for the early embryonic development (Piedrahita *et al*., 1999). Experimental models showed that FB_1-induced NTD by disrupting sphingolipid metabolism and reduction of folate level, which was partly prevented by folate supplementation (Gelineau-van Waes *et al*., 2005). A recent study showed that fumonisin exposure increases the risk of NTD in humans (Missmer *et al*., 2006). The high incidence of NTD closely mimics that of the OC incidence, when considering the incidence levels in Transkei and China (Marasas *et al*., 2004).

5.2 Interactive mechanistic and biological approaches

5.2.1 Biomarker studies

During the carcinogenic evaluation of the fumonisins as human carcinogens compelling evidence was found that FB_1 is carcinogenic in animals (IARC, 2002). Adequate epidemiological studies in humans are, however, hampered by the lack of a suitable biomarker in order to reflect a similar mechanism prevailing in humans and to accurately measure exposure. A suitable biomarker to accurately measure exposure in animals has been developed by utilising the sphingoid base ratios [sphinganine to sphingosine (Sa:So)] in urine and blood (Wang *et al*., 1992). In non-human primates the sphingolipid biomarker was used in the blood and urine to assess the exposure to different levels of culture material of *F. verticillioides* (Shephard *et al*., 1996b). Subchronic exposure at fumonisin levels over a period of 13.4 yrs showed that the Sa/So marker was significantly increased in the blood and urine. The presence of FB_1 in hair of the non human primates was also used as a biomarker for the fumonisin exposure (Sewram *et al*., 2001). Both biomarkers could effectively be related to changes in the specific matrixes as a result of fumonisin exposure. However, the presence of FB_1 in hair could not be related to the actual time and level of exposure due to its persistence in the hair over a longer period of time. The presence of fumonisin has also been detected in human hair and faeces although these biomarkers still needs to be validated to accurately monitor the level of exposure (Chulele *et al*., 2000; Sewram *et al*., 2003). Studies in humans using the Sa/So biomarker, however, provided conflicting results suggesting that it is not a suitable marker to monitor fumonisin exposure in humans. Studies thus far have failed to indicate changes in the Sa/So urinary marker in human populations known to be exposed to fumonisins (Van der Westhuizen *et al*., 1999, 2008; Solfrizzo *et al*., 2004; Abnet *et al*., 2001). A recent study showed that the presence of FB_1 in urine could be detected and correlated with the amount of tortillas consumed in a Mexican population (Gong *et al*., 2008). The utilization of this biomarker to assess the fumonisin exposure in other human populations is currently in progress.

5.2.2 Threshold effects related to non-genotoxic and synergistic effects

Separate parameters are used to distinguish between genotoxic and nongenotoxic chemicals when assessing risk to humans (Bolt *et al*., 2004). For non-genotoxic

carcinogens a threshold level exist which permits the derivation of a no-observed-effect-level (NOEL). With the introduction of a safety factor a permissible exposure level is derived at which no relevant human risk is anticipated. For genotoxins it is generally accepted that a non-threshold level exists although a whole array of threshold effects has been suggested depending on the type of mechanism that prevails (Streffer *et al*., 2004). Four basic types of thresholds have been distinguished including:

(i) a linear non-threshold (LNT) model for genotoxic carcinogens,
(ii) genotoxic carcinogens where a LNT model is used as a default when the precise nature of the dose response has not been established,
(iii) genotoxic carcinogens where a practical threshold is likely based on the mechanisms involved, and
(iv) non-genotoxic carcinogens where a perfect threshold exist which is associated with a NOEL as described above.

However, the application of LNT models has been questioned based on mechanistic arguments implying the existence for biological meaningful threshold dose-response effects for both DNA and non-DNA chemicals. It is suggested that a diversity of methods for carcinogenic risk extrapolation to low doses should be applied based on the mode of action (Kirsch-Volders *et al*., 2000). One exception is when the interaction between the carcinogen and cellular target, such as the DNA, represents a single event and in such a case a non-threshold theoretically does exist. However, when a single hit–single target is shown to exist in *in vitro* mutagenicity testing it is generally assumed in principle that a no-effect level *in vivo* also exists. However, major differences between *in vitro* and *in vivo* responses exist and aspects such as bioavailability metabolic activation/inactivation, DNA repair and differential sensitivity to the chemical and responses to different cell survival parameters including cell proliferation and apoptosis provide major difficulties in the interpretation of threshold type effects in risk assessment. As linearity between exposure and tumor frequency is assumed when considering the single hit hypothesis it becomes evident that as cancer development is a multistage process with a minimum of two steps, thresholds should be considered, even for genotoxic carcinogens. This would imply that a no-threshold effect is considered on the basis of the interaction with DNA while a

threshold exists for the ultimate adverse biological effect. This hypothesis was further developed when extrapolating data to estimate cancer risk for an exposed population that further complicates a decision about a safe threshold level. As individuals in a population vary widely in their susceptibility, altered cells may exist at different stages of carcinogenesis due to the presence of other cancer causing agents and/or dietary constituents. In this regard a carcinogen may pose some degree of risk to the population at any dose by exerting carcinogenic effects that are additive or synergistic.

When considering the carcinogenic properties of the fumonisins different scenarios have to be considered regarding threshold effects. It is evident that, although the fumonisins lack direct DNA reactivity in various *in vitro* genotoxicity assay systems, it induces the different stages of cancer development in the liver very similar to genotoxic carcinogens in both long-term and short-term cancer bioassays (Chapter IV). *In vitro* genotoxicity assays also showed that FB_1 induced clastogenic effects, presumably via the disruption of cellular oxidative pathways. Major emphasis on fumonisin-induced carcinogenesis is placed on the cancer promoting properties of the compound that, with respect to risk assessment, has adopted a threshold type of approach. However, fumonisins cause a wide spectrum of cellular effects that may act separately, additively and/or synergistically with other underlying factors or carcinogenic principles. These so-called epigenetic and synergistic events complicate, as mentioned above, a strict threshold type of approach in establishing risk of fumonisin exposure in humans.

5.2.3 Underlying interactive mechanisms during cancer promotion

FB_1 disrupts lipid metabolism in the cell involving cholesterol, phospholipid, sphingolipid and fatty acid biosynthesis (Section IV). The role of these changes in the cancer promoting properties in the liver and kidneys has also been debated as the proposed mechanisms were derived from different schools of thought. Irrespective of the actual mechanism involved the interaction between the underlying biochemical events involved in determining cell survival have been discussed in detail (Gelderblom et al 1996, 2001, 2008) and subsequently outlined in Section IV. The key determinants in the survival of early preneoplastic cells are ceramide and arachidonic acid (C20:4n-6), known to be important regulators of cell proliferation and

apoptosis. Underlying to these mechanisms FB_1 also disrupt the oxidative status of cells that are also important modulators of apoptosis. When considering the liver cancer hypothesis based on the resistant hepatocytes model, the modulation of these events is responsible for the selective proliferation of the resistant hepatocytes. It was shown that FB_1 closely mimics events that prevail in these preneoplastic events that are associated with their selective outgrowth and development in neoplasia. Similar events induced in normal cells resulted in the inhibition of growth and the subsequent induction of apoptosis whereby it creates the selective stimulus during cancer promotion. This hypothesis was studied in detail in the liver whereas the early events associated with the development of neoplastic lesions in the kidney are not well characterised.

When considering the underlying mechanisms associated with cancer promotion of the fumonisins, the disruption of biochemical pathways associated with different cell growth parameters and aspects regarding the recently derived epigenetic mechanisms of cancer development should also be considered. This theme also links closely to the biological threshold hypothesis discussed above and the current paradigm utilised for risk determination of the fumonisins. Epigenetic mechanisms of cancer development have been recognized almost 3 decades and are unrelated to genetic variation or mutations generally associated with genotoxic carcinogens (Farber and Rubin 1991). It is currently recognised that epigenetic events are perhaps more common than genetic changes and play an important role in the modulation of functional pathways that are key to neoplastic development. These mechanisms include promoter DNA methylation, histone modification and RNA interference to name a few and provide new opportunities for cancer prevention. It would appear that epigenetic events occur very early in neoplasia and that many tumor-suppressor genes in human neoplasia are inactivated by epigenetic mechanisms (Jones and Baylin, 2007; Issa, 2008). Aberrant methylation has been associated with many cancers including colon, oesophageal, liver and lung. The disruption of the folate receptor by FB_1 and the subsequent aberrant folate metabolism has been shown to induce NTD in mice (Gelineau-van Waes *et al*, 2005). Studies in cell cultures showed that FB_1 modifies the expression of folate receptor and folate carrier in HepG2 (Abdel Nour *et a*l., 2007) and folate uptake in Caco-2 cells (Stevens *et al*., 1997). The disruption of DNA methylation due to folate

deficiency and FB_1-induced carcinogenesis therefore should be investigated as a possible epigenetic mode of action that should impact on the current risk assessment paradigm for fumonisins

5.3 Risk assessment of f*umonisins as food contaminants in South Africa*

Differences exist in the implementing of risk assessment parameters of toxins and carcinogens between developed and developing countries, especially in remote microenvironments in developing countries where certain foods are used as a sole dietary staple. The health issues associated with these microenvironments are largely ignored when considering global trade between industrialized countries although they could negatively impact on health risk issues regarding foodborne toxins and carcinogens. In South Africa, differences exist in the health risk posed by the fumonisins and are determined by differences in the maize consumption patterns, which vary, not only between the different ethnic groups, but also between black South Africans living in rural areas compared to urbanized populations (Thiel *et al*., 1992). Different scenarios therefore exist with respect to the maize production and consumption patterns in different regions as well as between population groups residing in rural and urban areas. An interactive model (Table 1) has been developed to compare the impact of maize intake and fumonisin contamination levels on the provisional maximum tolerable daily intake (PMTDI) (Gelderblom *et al*., 2008; Marasas *et al*, 2008). With respect to the maize availability to the different population groups the dietary intake patterns of commercial and home-grown maize vs imported maize need to be considered:

(i) Home-grown maize

Fumonisin contamination of home-grown maize has been linked to the development of OC (Rheeder *et al*., 1992) and more recently to the development of NTD (Marasas *et al*., 2004) in population groups using maize as a monocereal staple diet. Fumonisin exposure in rural settings in South Africa reaches levels that are far above the PMTDI (2 μg/kg bw/day) level set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2001). The major determinant, however, appears to be the maize consumption patterns in different so-called “hotspots” of exposure. Most of the epidemiological studies focused on these areas to assess possible interactions of fumonisin exposure to a specific disease pattern in humans. In this context

Table 1. Interactive association between maize intake, FB contamination levels and the resultant PDI ((µg FB/kg bw/day) values.

B contamination (ppm)	Maize intake [g/person (60kg)/day]						
	10	50	100	150	200	400	600
0.2	0	0.2	0.3	0.5	0.7	1.4	2.1
0.5	0.1	0.4	0.8	1.3	1.7	3.4	5.1
1	0.2	0.8	1.7	2.5	3.3	6.6	9.9
2	0.3	1.7	3.3	5.0	6.7	13.4	20.1
3	0.5	**2.5**	**5.0**	**7.5**	**10**	**20**	**30**
4	0.7	**3.3**	**6.7**	**10**	**13.3**	**26.6**	**39.9**
5	0.8	**4.2**	**8.3**	**12.5**	**16.7**	**33.4**	**50.1**
10	1.7	**8.3**	**16.7**	**25.0**	**33.3**	**66.6**	**99.9**
	PDI (µg FB/kg bw/day)						

PMTDI = 2 µg/kg bw/day (nephrotoxicity); PMTDI = 0.7/0.8 µg/kg bw/day (nephro-/hepatocarcinogenicity). *Adapted from Marasas *et al*., 2008; Gelderblom *et al*., 2008.

established risk assessment parameters failed to adhere to the exact principles for which they have been established, i.e. to safeguard humans to the adverse effects of these mycotoxins. Most of the populations in developing countries consuming maize as a monocereal staple diet also lack sufficient levels of micronutrients that may enhance their susceptibility to adverse effects of the fumonisins. These aspects are

not included when setting risk assessment parameters and apparent PDI levels of the fumonisins in these regions and values of 5-10 times above the PMTDI are common. It is not known at present what effects the long-term exposure to fumonisins at these levels would have on the health status of specific populations at risk. The need to perform studies to quantify the exposure patterns as well as determining the nutritional status of the people at risk is self-evident. Proper intervention measures to reduce exposure to match the established risk assessment parameters is therefore of critical importance in these regions. The importance of setting new PMTDI levels to safeguard human population groups in developing countries is clearly indicated. A MTL of 0.2 mg FB/kg maize as suggested by Marasas (1997) appears to be remarkably close to a safe contamination level of maize in a South African setting where adults may consume up to 500 g and more maize per day. In children, however, the situation is far worse and intake varies between 169 to 541 g/child/day according to a recent survey in South Africa (http://www.sahealthinfo.org/nutrition/foodconsumption.htm) resulting in PDI levels (Table 2) that even further exceed the PMTDI level proposed by JECFA (JECFA, 2001).

(ii) Commercial vs imported maize

The commercial production of white maize, normally used as food, varies between 1.25 to 6.37 million tons per annum in South Africa (Viljoen and Marasas, 2003). A survey of 6 crop years (1989 to 1994) of FB contamination levels in white maize cultivated in the commercial maize production areas in South Africa recorded mean levels of 0.25 to 0.70 mg/kg maize. Imported maize from the USA contained considerably higher FB levels for the 1991 and 1992 crop years and reported to be 1.13 and 1.05 mg FB/kg, respectively [levels reported incorrectly due to a printing error in Viljoen and Marasas, (2003)]. If such imported maize enters the human food chain PDI levels will increase by a factor of 2-3 fold as compared to the consumption of South African maize. This is of particular importance during dry seasons when large quantities of maize need to be imported, some of which is channeled into the human food chain. As commercial South African maize contains low levels of FB compared to maize supplied on the international markets, imported maize could exaggerate the risk due to FB intake in the local population. When considering impoverished rural societies depending on maize as a sole dietary staple and a

Table 2: Maize consumption patterns of various population groups in selected countries.

Country	Corp year	Commercial maize/maize meal FB (mg/kg)	Homegrown maize FB (mg/kg)	Maize intake profiles (g/person/day)	PDI (µg/kg bw) (60 kg) Commercial	Homegrown	Ref
South Africa **Adults** Blacks Children (1-9 yrs) (bw - 25 kg) Eastern Cape Northern Province Western Cape	1989/94	0.25-0.70	0.58 (healthy) 4.8 (moldy)	460 (rural) 267 (urban) 435 541 169	1.91-5.3 1.1-3.1 4.35 - 12.1 5.41 - 15.1 1.69 - 4.7	4.4(healthy) 36.8 (moldy) - - -	Viljoen & Marasas (2003) V/d Westhuizen *et al.* (1999) Rheeder *et al.* (1992) http://www.sahealthinfo.org/nutrition
Botswana	1996/97	0.247	-	200g	-	0.8	Siame *et al.* 1998
China	1993/95 1989	0.1-4.2 (Penlai) 3.3-7.2 (Haimen) 3.46 (Linxian) 3.39 (Shanqiu) Guangxi Province	– 0.7 (moldy)	80 200	5.6 9.6 4.6 4.5	 2.3	Ueno *et al.*, 1997 Gao & Yoshizawa, 1997 Li *et al.* (1999)
Brazil	1999	2.87		2-12g (urban) 11-39g (rural)	0.09 - 0.50 0.52 – 1.8		van der Westhuizen *et al.* (2003) Machinski & Soares, 2000

Argentina Adults (78 kg) Children (1-5 yrs; 14 kg)	1997	0.79	-	250g 200g	2.5 11.3	-	Solovey et al. (1999)
Guatemala	1995	0.85 –2.2 (Tortillas)	-	400g (females) 600g (males)	5 – 14.6 8.5 – 22	-	Meredith et al. (1999)
				PDI			
Netherlands	National estimates (processed maize)			0.06-0.1		JECFA 2000	
UK	National estimates (processed maize)			0.03		JECFA 2000	
Canada	National estimates (processed maize)			0.02		JECFA 2000	
USA	National estimates (processed maize)			0.08		JECFA 2000	
Middle east	GEMS/Food regional diets (unprocessed maize)			1.1		JECFA 2000	
Far East	GEMS/Food regional diets (unprocessed maize)			0.7		JECFA 2000	
African	GEMS/Food regional diets (unprocessed maize)			2.4		JECFA 2000	
Latin American	GEMS/Food regional diets (unprocessed maize)			1.0		JECFA 2000	
European	GEMS/Food regional diets (unprocessed maize)			0.2		JECFA 2000	

PDI = probable daily intake. FB = fumonisins. JECFA, Joint FAO/WHO Expert Committee on Food Additives; GEMS = Global Environment Monitoring System.

reduced intake of micronutrients a “catch-22” situation arises, on the one hand with people facing starvation while on the other hand consuming maize containing FB levels that could have adverse effects on their health. Once again children appear to be the most vulnerable group of the population and PDI values of 3-5 fold above those calculated for adults are obtained (Table 2).

5.4 *Risk assessment parameters for the fumonisins*

Different models exist to determine the risk of toxins and carcinogens present in a food crop intended for human consumption. These include the no-observed-effect levels (NOEL)/safety factor approach (EHC 70, 1987) and computerized models such as the two-dimensional Monte-Carlo simulation (Humphreys *et al*., 2001) proposed recently, the Moolgavkar-Venzon-Knudson (MVK) two-stage, clonal expansion model (Kodell, *et al*., 2001) or dose-response models for tumour incidence (JECFA, 2001). The WHO has adapted the NOEL/Safety factor approach to determine the provisional maximum tolerable daily intake (PMTDI) for natural toxicants (EHC 70, 1987). For toxicants a safety factor of 100 is normally adopted which consists of a factor 10 for the extrapolation between animal to humans and 10 to account for the difference in sensitivity within the human population with respect to a specific biological adverse effect. The numerical size of the safety factor used may differ depending whether a compound is classified as having toxic effects or upon long term exposure could result in the induction of a carcinogenic response. In general larger safety factors are used for carcinogenic compounds depending on the mechanism of cancer induction related to epigenetic (non-genotoxic compounds) or genotoxic mechanisms (Kuiper-Goodman, 1990).

A recent joint meeting of JECFA on the fumonisin B mycotoxins mainly considered the toxicological effects in experimental animals, possible associations with respect to human diseases as well as food contamination levels in different countries worldwide (JECFA, 2001). A PMTDI level of 2µg/FB/kg body weight/day was calculated based on the NOEL of nephrotoxicity

in male Fischer rats using a safety factor of 100. The NOEL for nephrotoxicity was obtained from a long-term chronic feeding study (2 yr) in male Fischer rats by the National Toxicology Program (NTP) in the United States and was equivalent to a dietary level of 5 mg FB_1/kg diet resulting in a FB_1 intake of 200 µg/kg body weight/day (NTP, 2000). A 90-day study in male Fischer rats also provided a similar NOEL (Voss *et al*., 1995). However, when considering the carcinogenicity of FB_1 the NOEL for nephrotoxicity of 200 µg/kg bw/day is below the no-effect threshold for carcinogenicity (700µg/kg bw/day) for the induction of adenoma and carcinoma in the kidneys. The NOEL for nephrocarcinogenicity and the hepatocarcinogenicity are very similar and vary between an FB_1 intake of 700 µg to 800µg/kg bw/day, respectively. A tolerable daily intake (TDI) of 0.8µg FB/kg bw/day, using the NOEL for hepatocarcinogenicity and a safety factor of a 1000 has been proposed (Gelderblom *et al*., 1996) which is very similar to the 0.7 µg/kg bw/day using the NOEL for nephrotoxicity and a similar safety factor. The latter approach resulted in a TDI that is in the order of 3 times lower than the PMTDI using the NOEL for nephrotoxicity.

At present it is not known whether a PMTDI, based purely on the nephrotoxicity whilst ignoring the carcinogenic properties, will safeguard humans from the adverse effects of the fumonisins. Therefore, at present two safety limits exist i.e. a higher safe limit 0.7/0.8 µg kg bw/day (lower risk) that focuses on the carcinogenic properties of the fumonisins and a lower safety limit of 2 µg/kg/day (higher risk) that mainly focussed on the nephrotoxicity of the fumonisins and represents a level below the threshold for cancer induction. However, the lower TDI based on carcinogenicity of the fumonisins could have a far more adverse economic impact on international trade and industry, affecting the entire maize industry (Viljoen and Marasas, 2003). In developed countries exporting large quantities of maize a low TDI could severely affect the maize industry whereas a high TDI may be a health risk for populations with a high maize intake in developing countries importing maize. Different scenarios regarding risk assessment of the fumonisins exist which could either underestimate the risk

posed to human health or overemphasize the risk that would negatively impact on international trade and the maize industry.

5.5 Risk paradigms of fumonisin B_1

Fumonisin B_1 causes diverse toxicological effects in a variety of animal species both experimentally and under natural conditions (Marasas *et al*., 1996; 2001). *F. verticillioides* and *F. proliferatum* produce the mycotoxin and its structurally diverse analogues, fumonisins B_2 and B_3 on maize worldwide (Shephard *et al*., 1996b). The ubiquitous nature of these mycotoxins in animal feeds and human foodstuff resulted in the establishment of risk assessment parameters (Gelderblom *et al*., 1996; Marasas, 1997; JECFA, 2001) in order to provide governmental organizations and regulatory bodies with the necessary tools to safeguard human and animal health. The economic impact of the regulatory measures if they should be enforced as maximum tolerated levels (MTLs), has also been considered (Viljoen and Marasas, 2003). The risk parameters, however, are general guidelines proposed by international organisations, such as the WHO via its risk evaluation organisations such as JECFA and and the International Agency for Research on Cancer (IARC) which address different aspect of the possible diverse effects of the fumonisins in humans. The fumonsins were classified as group 2B, ie possibly carcinogenic to humans (WHO-IARC, 2002) These risk measurements do not prescribe to any one country how they have to implement regulations of the fumonisins in a specific crop either produced locally or exported/imported. Regulation of the contamination levels of mycotoxins, such as the fumonisins, therefore differs from country to country (Table 3) and levels in processed food for local consumption may be controlled while food and food products intended for the export markets are not. Such an approach shifted the responsibility to safeguard the health of a specific population to governments importing the food or foodstuff. This resulted in a shift in the risk paradigm from one country to another determined by economic factors and international trade agreements, specifically between developed and developing countries (Marasas *et al.,* 2008; Gelderblom

et al., 2008). Regulation, therefore, of a specific mycotoxin will vary from the strict implementation of risk assessment parameters to the total lack thereof. This will depend on the economic climate and different factors determining the trade of a specific food commodity, such as the protection of certain agricultural commodities by government subsidies. These factors will play a major role in the eventual regulation of a mycotoxin in a specific country which may become a "pandora's box" between health authorities considering health risk parameters, political and economic powers, and international trade agreements.

5.6 Perspectives

Several studies exist regarding the possible role of the fumonisins in the development of human diseases of which oesophageal cancer, liver cancer and the development of neural tube defects received the bulk of attention. At present none of the epidemiological studies conducted thusfar could either prove or disproved a causative relationship of the fumonisins with any of the diseases. The numerous determinants hampering studies to evaluate the role in human health include; the use of validated population specific questionnaires; problems that exist in using food frequency and 24 hr recall questionnaires in populations that are in transition regarding their location, lifestyle and types of food consumed; and the lack of specific methods or biomarkers to monitor exposure at an individual level. Controversies regarding the toxic- and carcinogenic properties of the fumonisins also complicate the establishment of risk assessment parameters. These include; the target organ for the toxic- and carcinogenic characteristics of the fumonisins; site-specific mechanisms for cancer induction; genotoxic vs non-genotoxic effects; and the existence of a threshold. The approach for fumonisin risk assessment applied by JECFA was that the mycotoxins are of the non-genotoxic type and therefore utilised a

Table 3: Recommended maximum residue limits for fumonisins in food.

Maize & maize products	Total FB	References
USA: Whole or partly degermed Maize products	 4mg/kg 2 mg/kg	 FDA, 2001
Switzerland: Maize products	 1mg/kg	 Zoller et al., 1994
France: Whole maize	 3mg/kg	 Soriano and Dragacci, 2004
South Africa: Whole maize Whole maize Maize products	 0.1-0.2 mg/kg 4 mg/kg 2 mg/kg	 Marasas, 1997 Viljoen and Marasas, 2003

theoretical toxic regenerative cell proliferative model for cancer induction supporting the non-linear threshold model for chemical carcinogenesis. A PMTDI of 2ug FB/kg bw/day has been derived for the fumonisins using a NOEL for nephrotoxicity and a safety factor of 100. As this is well below the NOEL for nephrocarcinogenicity, other parameters related to carcinogenicity should rather been considered as will be debated in the next Chapter. Further to this debate is the possible interactive role of nephropathy that prevailed in about 90% in the rats due to the high protein diet used in the chronic study in male Fischer rats (NTP, 2001).

Several aspects regarding the regulation of FB in maize are of importance. Setting tolerance levels of FB in maize could either negatively impact on human health in certain population groups or severely disrupt the maize food chain. The setting of realistic risk assessment parameters in the South African context

where neither the health nor the socio-economic aspects regarding maize production will be compromised requires urgent attention. Aspects regarding international trade and economic realities have also to be taken into account in order to provide realistic control measures. When considering the maximum tolerable levels (MTLs) of fumonisins in maize the following are of importance:

(i) Two types, an “economic” and a “scientific or health” MTLs exist. If the “scientific” MTL is too low, it will destroy the maize industry and people will starve because of the absence of maize rather than become ill because of the consumption of contaminated maize if the “economic” MTL is too high. However, appraisal of the “scientific” MTL makes it abundantly clear that the majority of the South African population, especially children, would be at risk when utilising maize as a sole dietary staple.

(ii) Two different scenarios exist with respect to risk of fumonisin exposure in developed vs developing countries. It becomes evident that MTLs that safeguard human health in the one does not necessarily do so in the other.

(iii) A population that is at the greatest risk with respect to fumonisins in maize are subsistence farmers and children in rural Africa who consume home-grown maize. They have the highest maize intakes and also consume the most highly contaminated maize. Yet they are unaffected by MTLs set globally to regulate international trade, eg a MTL of 1 mg/kg as set by Switzerland will allow a PDI over the PMTDI set by JECFA if the intake profiles exceed only 100 g/person/day (Table 1).

(iv) Protection against fumonisin intake and the induction of an adverse effect such as cancer and possibly NTD can be achieved more effectively by other means than MTLs eg, (a) Effective control of fumonisin contamination of maize and/or food diversification programmes would be important ways to reduce FB exposure and the associated risk and (b) fortification of maize products with folic acid and/or abstinence from maize during the first 6 weeks of pregnancy (Viljoen & Marasas, 2003).

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HEPATOTOXICITY AND -CARCINOGENICITY OF THE FUMONISINS IN RATS

A Review Regarding Mechanistic Implications for Establishing Risk In Humans

W.C.A. Gelderblom[1], S.D. Snyman[1], S. Abel[1], S. Lebepe-Mazur[1], C.M. Smuts[1*], L. Van der Westhuizen[1], W.F.O. Marasas[1], T.C. Victor[2], S. Knasmüller[3], and W. Huber[3]

[1] Programme on Mycotoxins and Experimental Carcinogenesis and
[*] National Research Programme for Nutritional Intervention
P.O. Box 19070, Tygerberg, 7505, South Africa
[2] Department of Medical Physiology and Biochemistry
MRC Centre for Molecular and Cellular Biology
University of Stellenbosch Medical School
P.O. Box 19063, Tygerberg, 7505, South Africa
[3] Institut für Tumorbiologie-Krebsforschung
Universität Wien, Borschkegasse 8a
A-1090 Vienna, Austria

ABSTRACT

Cancer induction by the non-genotoxic mycotoxin, fumonisin B_1, has been investigated by studying the mechanisms involved during cancer initiation and promotion in rat liver. Cancer initiation is effected through a toxic-proliferative response while the inhibitory effect on hepatocyte cell proliferation appears to be a key aspect determining cancer promotion. Dose-response effects of the fumonisins on the induction of early neoplastic lesions in both long- and short-term animal experiments have been established. The biphasic response of FB_1 on hepatocyte proliferation will be discussed in relation to the known mechanisms of cancer induction by the genotoxic hepatocarcinogens. Recent investigations regarding the effect of the fumonisins on lipid biosynthesis and its inhibitory effect on hepatocyte growth stimulatory responses *in vitro* will be highlighted. Integration of our current knowledge regarding the carcinogenic potential of the fumonisins in setting a realistic and applicable risk assessment model for this non-genotoxic carcinogen will finally be addressed.

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INTRODUCTION

The mechanisms involved in cancer induction by chemicals have been investigated extensively during the past 20 years. The dominant view that emerges, is that the major driving force in the development of neoplasia is the interaction of carcinogenic genotoxic chemicals with the cellular genome of the target cells (Bishop, 1987). The importance of these DNA lesions has been recognized and is currently widely utilized in molecular epidemiological studies to assess the extent of human exposure to specific natural and industrial genotoxic carcinogens (Perera, 1987). Although the results accurately reflect carcinogen exposure at the individual level, the impact and actual risk of such an exposure is not yet known, as exposure to genotoxic carcinogens at a specific time point does not reflect the outcome of cancer in a specific population and *vice versa* (Butterworth and Goldsworthy, 1991). However, these data are often used as part of the exposure assessment in establishing risk assessment models, although the correlation of associated changes, some of which might occur in cellular oncogenes and/or tumor suppressor genes, and the ultimate cancer has not been established unequivocally (Farber, 1989; Pitot, 1993).

When considering the steps and mechanisms involved, it becomes clear that genetic changes, cellular adaptive reactions and responses and/or dynamic interaction between cells and tissues are important factors that underline the long and multistep nature of cancer development (Farber and Rubin, 1991; Farber, 1993). The latter considerations form part of the epigenetic way of cancer development, an aspect that is largely ignored when the mechanism of cancer induction by chemicals is considered (Weinstein, 1991). Furthermore, recent investigations indicated that an increasing number of non-genotoxic chemicals that apparently lack the ability to interact with cellular DNA are carcinogenic (Rao and Reddy, 1991; Gelderblom *et al.*, 1992). Therefore, the position of non-genotoxic chemicals in the spectrum of chemical carcinogenesis remains an open one. In this context it has to be realized that the chemical and/or biological basis of cancer induction, whether via genotoxic or non-genotoxic systems still remains unknown.

When considering the above arguments concerning the mechanisms of cancer development, the present paper will focus on fumonisin-related biological effects that could provide information on the possible mechanism involved in the toxic and carcinogenic effects of these apparently non-genotoxic compounds in rat liver.

FUMONISINS AS CANCER INITIATORS

(i) Non-Genotoxicity

The fumonisins are non-mutagenic when tested in the *Salmonella* mutagenicity test (Gelderblom *et al.*, 1991) and lack genotoxicity in *in vitro* DNA repair assays in primary rat hepatocytes (Gelderblom *et al.*, 1992; Norred *et al.*, 1992). Genotoxicity of fumonisins has also been the subject of another very recent series of studies involving different *in vitro* systems (Knasmuller *et al.*, unpublished data). The lack of mutagenesis by the fumonisins was confirmed when different concentrations, ranging from 0.7 to 500 μg per plate, were tested against strains TA 100 and TA 98 in the presence and absence of Aroclor 1254 induced S9 ensyme fraction. The fumonisins also failed to induce any genotoxic effects in the DNA-repair assay with *Eschericia coli* (strains 3431753. uvrB/vecA and 3431765.uvr+/vec+) and the umu-microtest with *Salmonella* TA 1535/pKS 1002) in the absence and presence of rat liver S9 mix. Fumonisin B_1 (FB_1) also failed to induce micronuclei and did not alter the mitotic activity of primary rat hepatocytes. The effect of

FB_1 on the induction of chromosomal aberrations in primary hepatocytes is presently under investigation.

(ii) Short-Term Assays in Rat Liver

Despite the fact that the fumonisins were negative in various genotoxicity and mutagenicity assays, short-term *in vivo* studies have shown that FB_1 mimics genotoxic carcinogens with respect to the induction of resistant hepatocytes in rat liver (Gelderblom *et al.*, 1992; 1994). This was substantiated by the observation that FB_1 induces two important enzymes, gamma glutamyltranspeptidase (GGT) and the placental form of glutathione-S-transferase (GSTP), which are accepted histological markers for putative preneoplastic lesions initiated by genotoxic carcinogens. At present it is not known whether the characteristic enzyme phenotype that is associated with the resistant phenotype (Farber, 1990) is also induced by the fumonisins. Feeding experiments with the fumonisins in rats indicated that an increase in cell proliferation is also likely to play a critical role in the induction of the "resistant" phenotype as hepatoxicity, and the resultant regenerative cell proliferation is a prerequisite for initiation (Gelderblom *et al.*, 1994). In this regard regenerative cell proliferation, known to be a prerequisite for cancer initiation by the genotoxic carcinogens (Columbano *et al.*, 1991), is also a critical event during FB_1-induced carcinogenesis. The only difference noticed thus far in the induction of the "resistant" phenotype between the fumonisins and other genotoxic carcinogens lies in the kinetics of the cancer initiation step. It is known that, with genotoxic carcinogens, cancer initiation is normally completed within a matter of hours or a few days (Farber *et al.*, 1989). However, single and/or multiple dosages of the fumonisins in the presence of a stimulus for regenerative cell proliferation fail to effect initiation (Gelderblom *et al.*, 1992). Prolonged exposure to a dietary level (250 mg FB_1/kg of the diet) that induced hepatotoxicity over a period of 14 to 21 days does effect initiation. A recent investigation indicated that another non-genotoxic carcinogen, the peroxisome proliferator clofibrate, induced resistant hepatocytes after prolonged exposure of up to six months (Nagai *et al.*, 1993). As in the case of the fumonisins, the induced hepatocyte nodules stained positively for GSTP and GGT.

(iii) Long-Term Experiments

Male BD IX rats treated with a diet containing 25 mg FB_1/kg over a period of 2 years showed a significant increase ($P<0.01$) in the amount of GSTP positive foci in the liver (unpublished data). The number of foci in the liver of the rats treated with a 10 mg FB_1/kg diet was also noticeably (however not significantly) increased as compared to the control and 1 mg FB_1/kg diet treated groups. When the number of cells per focus were considered, there was a significant ($P<0.01$) increase in the size of the foci between the rats treated with the 10 mg FB_1/kg diet and the control and 1 mg FB_1/kg groups, implying that cancer promotion was effected. However, the presence of these foci indicated that, as for the 25 mg FB_1/kg treated group, promotion (see next section) and most probably cancer initiation did occur after prolonged feeding at this low dietary level. At the lowest dietary level where "initiation" and promotion (10 mg/FB_1/kg diet) were effected, only a few necrotic cells were observed as a result of the fumonisin treatment. Whether this modest induction of cytotoxicity could result in an increased regenerative cell proliferation to an extent to support cancer initiation (see previous section) is not known at present.

In this regard, the presence of "spontaneous" initiated cells and their subsequent selection (promotion) by the fumonisins need to be considered. Especially since cancer promotion by the fumonisins occurs at dietary levels well below the level that is required for cancer initiation (see next section). There were no significant differences in the number

of GSTP positive ($GSTP^+$) single cells in the liver of rats fed the control diet and at three different dietary levels of FB_1 (1, 10 and 25 mg FB_1/kg diet) over a period of two years (unpublished data). The presence of the $GSTP^+$ cells has been regarded as early evidence that cancer initiation was effected following carcinogen treatment (Satoh *et al.*, 1989). A recent study suggested that a certain number of these cells could give rise to hepatocyte nodules when animals are treated with the cancer promoting regimen consisting of 2-acetylaminofluorene (AAF) and partial hepatectomy (Kato *et al.*, 1993). Studies concerning the peroxisome proliferator, nafenopin, showed that another subclass of hepatocyte foci, which stained negatively for GGT and specific sub-units of GST, are induced after chronic exposure (Grasl-Kraupp, *et al.*, 1993). As these altered hepatocyte foci normally occur in older rats, it was suggested that cancer induction by these compounds originated from initiated cells that occur "spontaneously" in the liver (Kraupp-Grasl *et al.*, 1991). This was deduced from the fact that the ultimate cancer also exhibited a similar characteristic as these foci.

With this as a background, the following arguments concerning the role of the fumonisins in promoting initiated cells that occur "spontaneously" within the liver need to be considered:

- (i)Cell proliferation is required for cancer initiation by the fumonisins (Gelderblom *et al.*, 1994), which is characteristic of cancer initiation by genotoxic agents (Columbano *et al.*, 1991).
- (ii)In a short-term carcinogenesis assay in male Fischer rats there were no hepatocyte nodules in the control animals fed only the initiating dosage of FB_1 (up to 500 mg FB_1/kg diet over a period of 21 days) and/or selected with the strong selecting 2-acetylamino-fluorene/partial hepatectomy (AAF/PH) promoting regimen (Gelderblom *et al.*, 1994).
- (iii)Long-term studies in rats treated with a relatively high dietary level (25 mg FB_1/kg) of FB_1 revealed that the number of $GSTP^+$ single cells was not increased above that of the controls while the number of foci were enhanced significantly. This is in contrast with certain genotoxic carcinogens (Satoh *et al.*, 1989) which significantly increased the number of single $GSTP^+$ cells.
- (iv)None of the control male BD IX rats developed hepatocellular carcinomas during the long-term studies (Gelderblom *et al.* 1991; unpublished data) where the carcinogenicity of FB_1 was evaluated.
- (v)Male Fischer rats subjected to the different cancer promoting regimens such as AAF/PH and/or chronically treated with phenobarbital (PB) did not develop hepatocyte nodules and/or cancer (Ghoshal *et al.*, 1987).
- (vi)It is well accepted that initiated hepatocytes exist within the liver of older rats but there is no evidence that they are the precursors for hepatocyte nodules and/or hepatocellular carcinoma (Ghoshal, *et al.*, 1987).
- (vii)Studies concerning clofibrate and the fumonisins indicated that non-genotoxic carcinogens have the ability to induce "resistant" hepatocytes similar to genotoxic carcinogens. An investigation on the carcinogenicity of ciprofibrate in 24 and 52 week old rats indicated that there was no difference in the tumor incidence and numbers of tumors per liver as would be expected since the number of "spontaneously" initiated cells increased with age (Ward *et al.*, 1987). It was suggested that the latter clearly established the role of ciprofibrate as an initiator (Rao *et al.*, 1991).

Based on these arguments it would appear that the presence of hepatocyte foci in the liver of the rats fed the 10 mg FB_1/kg diet can be ascribed to cancer initiation and not due to the promotion of "spontaneous" initiated cells.

FUMONISINS AS CANCER PROMOTERS

The fumonisins promote the induction of hyperplastic foci in rat liver treated with diethylnitrosamine (Gelderblom *et al.*, 1988), a known genotoxic carcinogen. Subsequent studies were directed to the investigation of the mechanisms possibly involved that could effect the selective outgrowth of initiated cells. Prolonged exposure of diethylnitrosamine-initiated rats over a period of 21 days showed that dietary levels of 50 mg FB_1/kg and above induced the formation of hyperplastic foci and/or nodules. No cancer promoting activity was observed at a dietary level of 10 mg FB_1/kg indicating that, similar to cancer initiation, cancer promotion is also dose-dependent, which in turn is determined by the length of exposure.

Recent investigations showed that the fumonisins, like many genotoxic carcinogens, inhibit cell proliferation (Gelderblom *et al.*, 1994). Dietary levels up to 50 mg FB_1/kg that promoted cancer in DEN-initiated rats, also significantly inhibited and/or delayed hepatocyte cell proliferative stimulus induced by partial hepatectomy (unpublished data). Even a single dose of FB_1 (by gavage) following partial hepatectomy effectively inhibited regenerative cell proliferation (Gelderblom., 1992). The exact mechanism for this inhibitory effect is not known but *in vitro* studies in primary hepatocytes indicated that the interruption of growth stimulatory responses is likely to play a role (Gelderblom *et al.*, 1995). The mitoinhibitory effect on the epidermal growth factor (EGF)-induced DNA synthesis occurs at a level well below (10-15X) concentrations that induced a cytotoxic effect. Binding studies using ^{125}I EGF indicated that the interaction of the growth factor with its receptor was not effected and that inhibition occurs beyond the growth factor mediated events. The effect of FB_1-induced inhibition was shown to be reversible, because hepatocytes retained very little "memory" of FB_1 exposure. Subsequent studies concerning the accumulation of sphinganine also failed to indicate any effect that could be associated with this inhibition of the EGF mitogenic response (Gelderblom *et al.*, 1995). Inhibition of cell proliferation by the fumonisins has also been reported in renal epithelial (LLC-PK_1) cells (Yoo, *et al.*, 1992) and provides a reasonable hypothesis for the mechanism of cancer promotion by these compounds. This is further supported by the fact that many other cancer promoters, such as orotic acid (Pichiri-Coni *et al.*, 1990) and PB (Manjeshwar *et al.*, 1992) also exhibit a mitoinhibitory effect in primary hepatocytes.

In general it became apparent that, based on the short-term studies, the fumonisins are strong cancer promoting agents. This needs to be seen against the background that cancer initiation is effected in rat liver at a dietary level of 250 mg/kg over a period of 21 days (Gelderblom *et al.*, 1994). Initiation by FB_1 was also associated with distinct hepatotoxic effects. Cancer promotion, on the other hand, is effected in the absence of excessive hepatoxicity at a dietary level 5-fold less (50 mg FB_1/kg) as compared to cancer initiation (unpublished data).

However, when considering the long-term experiments on FB_1-induced carcinogenesis it would appear, as discussed above, that at low dietary levels (10 mg FB_1/kg) cancer initiation is likely to occur in the absence of excessive hepatotoxic effects and seems to occur at the same dietary level where promotion is effected. It is clear that short-term effects induced by relatively high dosage levels cannot be directly related to those noticed during long-term experiments using low dietary levels.

HEPATOTOXICITY AND METABOLISM OF THE FUMONISINS

Toxicological experiments with culture material of *F. moniliforme* and the fumonisins in rats showed that apart from the kidney, the liver is the main target organ of the fumonisins

(Gelderblom *et al.*, 1991; Voss *et al.*, 1993). Chronic feeding studies in BD IX rats showed that hepatocarcinogenesis developed against a background of a chronic toxic hepatitis that culminates in cirrhosis (Gelderblom *et al.*, 1991). The role of hepatotoxic effects has been established recently as non-toxic dosages failed to initiate cancer in short-term experiments in Fischer 344 male rats (Gelderblom *et al.*, 1994). This was confirmed in a long-term study in rats where dietary levels up to 25 mg FB_1/kg, which did not induce excessive toxic effects, also failed to induce liver cancer (unpublished data). The mild hepatotoxic effects in the rats that received 25 mg FB_1/kg diet, included single cell necrosis, bile duct proliferation and fibrosis that slightly distorted the liver in some cases, while in one rat a large area of cholangiofibrosis was present. Lipid accumulation and mild to prominent anisonucleosis occurred frequently in the high dosage group. In the rats fed the diet which contained 10 mg FB_1/kg, these changes were far less prominent. The only "pre-neoplastic" effect noticed was the induction of hepatocyte nodules and basophilic foci in some of the rats. There was a marked increase in the size and number of $GSTP^+$ foci in the liver of the rats treated with the 10 and 25 mg FB_1/kg diets compared to the control. Large confluent areas of some liver sections of the high dose group also stained positively for GGT. Therefore, a mild hepatotoxic effect induced by the fumonisins only resulted in the induction of early lesions that can be related to cancer development in the liver. As suggested previously, it would appear that a chronic hepatotoxic effect is a prerequisite for cancer development in the rat (Gelderblom *et al.*, 1994), implying the existence of a threshold value. The importance of a certain threshold value concerning fumonisins in cancer induction and the implications regarding the assessment of the risk to humans will be discussed further on.

(i) *In Vitro* Cytotoxicity and Metabolism

The fumonisins are not very toxic to primary hepatocytes in culture with a CD_{50} dosage of 1000μM and 500μM for FB_1 and FB_2 respectively (Gelderblom *et al.*, 1993). The water solubility of the fumonisins appears to be the reason for the low cytotoxicity as the more polar FB_1 is less cytotoxic than the less polar FB_2 and the hydrolyzed aminopolyol products of FB_1 and FB_2. Binding studies using radiolabelled FB_1 indicated that the compound is associated with both the soluble and insoluble or membraneous compartments in the cells (Cawood *et al.*, 1994). Although very little of the labelled FB_1 interacts with the hepatocytes (<0.01%) the compound is associated tightly with the membraneous compartment. As the fumonisin molecule exhibits both hydrophobic and hydrophilic properties it may readily be associated with cellular membranes.

Subsequent studies regarding the possible metabolism of the fumonisins by various enzyme preparations, including the microsomal cytochrome P450 and esterases and the hepatic triglyceride lipases, showed that the fumonisins are not substrates for these enzymes (Cawood *et al.*, 1994). Fractionation of the incubation medium of primary hepatocytes treated with fumonisins also failed to indicate the presence of any metabolites. Investigations regarding the structure-activity relationships of the fumonisins (Gelderblom *et al.*, 1993) indicated that the intact molecule is responsible for the biological activity of the fumonisins.

(ii) *In Vivo* Toxicity and Metabolism

Short-term studies with fumonisins indicated that they are not very hepatotoxic (Gelderblom *et al.*, 1988; 1992; 1994). Dietary levels as high as 50mg FB_1/kg over a period of 21 days induced only scattered single cell necrosis. However, chronic feeding of the same dietary level over a period of 24 to 26 months caused cirrhosis in the liver. These data seem to imply that the onset of fumonisin toxicity is very slow and that events that precede cell

death occur at a far slower rate during chronic feeding at low dietary levels. The irreversible nature of the interaction of fumonisins with cellular membranes (Cawood *et al.*, 1994) suggests that there could be, as a function of time, a slow accumulation of fumonisins in the cell which eventually precipitates hepatotoxicity. Toxicokinetic studies using radiolabelled FB_1 indicated that after a single gavage dosage, all the radiolabel was recovered in the feces as unmetabolized FB_1 (Shephard *et al.*, 1992). Only trace amounts of the radiolabelled parent molecule were found in the liver, kidneys and red blood cells. This again supports the *in vitro* studies that fumonisins are not readily metabolized by the liver.

(iii) Dose-Response Relationships Between FB_1-Induced Toxicity and Carcinogenesis

With respect to dose response effects in cancer initiation, promotion and the induction of the ultimate cancer, the following need to be considered:

- 1)In short-term cancer initiation/promotion experiments cancer initiation occurs at relatively high dietary (250 mg FB_1/kg) levels which also induce toxic effects in the liver (see above). Contrary to this, a dietary level of 50 mg FB_1/kg that lacks cancer initiation activity, effects cancer promotion.
- 2)In long-term feeding studies liver cancer is induced in rats at a dietary level of 50 mg FB_1/kg after 18 to 24 months. As discussed above a dietary level of 25 mg FB_1/kg failed to induce liver cancer when fed to rats over a period of 24 months. However, cancer promotion and initiation were effected in rats fed dietary levels of 10 and 25 mg FB_1/kg for 2 years, resulting in the formation of hepatocyte nodules in the absence of any liver cancer. Of these dosage levels only the 50 mg FB_1/kg diet exhibited any carcinogenic activity (promotion) during short-term studies (see above).

Therefore, the kinetics for the induction of the early events differ depending on the dosage used and the length of exposure. It became apparent that high-dose/short-term effects cannot be extrapolated to those effects obtained with low dose exposure for longer periods of time. It is known that the incidence and latency period of the development of cancer with genotoxic and non-genotoxic carcinogens differ and depend on the type of carcinogen and the dosage used (Rao *et al.*, 1991). Another perspective is that the stage of carcinogenesis beyond the transformation phase of a fraction of cells is controlled by the dose, as well as the length of exposure (Jones, 1978). This time-dose relationship is a general phenomenon of most carcinogens. With respect to the relationship between toxicity and carcinogenicity in rat liver, no consistent data are available (Melnick and Huff, 1993). It has been shown that the majority of chemicals tested in laboratory animals, even mutagens, showed no relationship between carcinogenesis and chronic toxicity (Hoel *et al.*, 1988). Cancer induction with the trihalomethanes in female mice revealed a striking dose response curve in the absence of hepatocellular necrosis (Melnick and Huff, 1993). In this case overt toxicity and hyperplasia were not the major driving force for cancer induction. It has been argued that if toxic effects such as irritation and inflammation, cellular degeneration and/or regeneration, cytoplasmic alterations, hyperplasia, metaplasia and dysplasia were associated with carcinogenesis, then all chemicals that induce these lesions have to cause cancer. Despite the above arguments a chronic hepatotoxic effect seems to be a prerequisite for FB_1-induced cancer initiation (Gelderblom *et al.*, 1992) and the development of the ultimate cancer (Gelderblom *et al.*, 1991). In the study of Hoel *et al.*, (1988) it was noticed that a few chemicals exhibited toxic effects that may be related to the increase in tumor incidence. They have classified

these compounds as "secondary carcinogens" as they produce carcinogenic effects via a mechanism that includes cytotoxicity and compensatory cell proliferation. This type of classification of carcinogens as "primary" (mutagens) and "secondary" carcinogens is in agreement with that of Cohen and Ellwein (1990) who used similar criteria to classify carcinogens as genotoxins and non-genotoxins. At present it would appear that the fumonisins should be classified as secondary or non-genotoxic carcinogens.

ADVANCES IN THE ELUCIDATION OF MECHANISMS OF FUMONISIN-INDUCED HEPATOTOXIC AND -CARCINOGENIC EFFECTS

(i) Interruption of Sphingolipid Biosynthesis

The interruption of sphingolipid biosynthesis by fumonisins and the toxicological and carcinogenic effects thereof have been discussed in detail elsewhere (Riley *et al.*, 1994; Merrill *et al.*, 1993; Schroeder *et al.*, 1994). Experiments have been described that strongly suggest that the disruption of sphingolipid metabolism leads to cell death in a pig kidney renal epithelial cell line (Yoo *et al.*, 1992). A recent study has implicated the mitogenic effect of FB_1 in Swiss 3T3 cells in the hepatocarcinogenicity of the mycotoxins (Schroeder *et al.*, 1994). The FB_1-induced accumulation of sphinganine was associated with the mitogenic effect as both sphingosine and sphinganine induced mitogenesis when added exogenously to Swiss 3T3 cells.

However, an *in vitro* study in primary hepatocyte cultures indicated that disruption of sphingolipid biosynthesis is apparently not involved in the hepatotoxicity and cancer promoting activity of the fumonisins (Gelderblom *et al.*, 1995). In this regard the following need to be considered:

- 1) The sphinganine/sphingosine (Sa/So) ratio is maximally altered in primary hepatocytes at a concentration of 1 μM FB_1 while cytotoxicity is effected at 250 μM.
- 2) Neither FB_1, Sa or So exhibited a mitogenic effect in primary hepatocytes.
- 3) Sa and/or So does not stimulate the EGF mitogenic response in primary hepatocytes.
- 4) The disruption of sphingolipid biosynthesis is not involved in the mitoinhibitory response of FB_1 on the EGF mitogenic response.
- 5) The mitoinhibitory effect of FB_1 in primary hepatocyte cultures is reversible while the accumulation of Sa is not (unpublished data).

In vivo studies indicated that the disruption of the Sa/So ratio occurs below the level that effects cancer initiation, while a slight increase (not significant) was observed at the lowest dietary level of FB_1 that induces cancer promotion and inhibition of cell proliferation (unpublished data). As the *in vitro* data also did not implicate the sphingolipids in the mitoinhibitory effect of the fumonisins, it is unlikely that their accumulation plays a role during cancer promotion.

(ii) Effect on *in Vitro* and *in Vivo* Lipid Biosynthesis

Studies in primary hepatocyte cultures treated with toxic (500 μM) and non-toxic (150 μM) concentrations revealed that FB_1 alters the incorporation of ^{14}C palmitic acid into

cellular lipids indicating that, apart from the effect on sphingolipid biosynthesis, the synthesis of cellular lipids is also affected (Gelderblom *et al.*, 1996). As expected the radiolabelling of sphingomyelin (SM) was decreased as a result of the reduced synthesis of the phospholipid. In contrast, the radiolabelling of both phosphatidylcholine (PC) and phosphatidylethanolamine (PEA) was enhanced as a result of the increase in their respective concentrations. Fatty acid (FA) analysis of the major phospholipids (PC and PEA) and the neutrål lipid triacylglyceride (TAG) showed marked alterations with respect to the n-6 fatty acid profiles. At the highest and cytotoxic dosage (500 μM/dish) C18:2 was markedly increased in PC, PEA and TAG while C20:4 was increased in TAG and PC. At the lower and non-cytotoxic dosage (150 μM) both C18:2 and C20:4 were increased in TAG while C18:2 was increased in PC only. In addition to these changes the free cholesterol (membrane associated) was also markedly decreased in hepatocytes treated with the highest dosage of FB_1. These data seem to imply that, apart from the effect on sphingolipid biosynthesis, FB_1 has important effects on the structure of the major membrane components, the FA storage pool (TAG-FA's) and the accumulation of long chain FA's within the cell. In this regard fat accumulation, noticed histochemically in rat liver treated with toxic dietary levels (Gelderblom *et al.*, 1994) of the fumonisins, is of relevance and could have important implications regarding the toxicity of these compounds. Changes in the membrane structure and function could also eventually lead to the disintegration of membrane continuity and eventually result in cell death.

The lipid profiles of rat liver, subjected to different dietary levels of FB_1, were also monitored *in vivo* in short-term and long-term experiments (unpublished data). In the short-term experiments three different dietary levels (50, 100 and 250 mg/kg) of FB_1 were fed to rats over a period of 21 days. Only mild to moderate toxic effects were noticed at the low dietary levels while more prominent toxic lesions such as single cell necrosis, bile ductule proliferation and early signs of fibrosis were observed at the high dosage level. Of the fumonisin containing diets only the 250 mg FB_1/kg diet initiated cancer while all three dietary levels exhibited cancer promoting activity (Gelderblom *et a*l., unpublished data). In the long-term studies three different diets, containing 1, 10 and 25 mg FB_1/kg, were fed over a period of 24 months. The major toxicological effects obtained in the liver were similar to those described above. The major changes observed in the short-term studies were noticed in the PEA phospholipid fraction in which the level of C18:2 was significantly increased. As a result of the negative feedback of C18:2 on the delta-4-desaturase the level of C22:5 was decreased. The levels of C18:2 and C22:5 were also markedly effected in a similar manner in PC but the differences were not significant. Analyses of the FA profiles in the livers (total FA and individually of PC and PEA) of the rats fed low dietary levels of FB_1 over a period of 24 months (see above) showed that C18:2 was again markedly increased.

In contrast to the *in vitro* studies, no effect was noticed on the arachidonic acid (AA) content in either the PEA and PC phospholipid fractions, presumably due to the fact that no excessive toxic effects were noticed as compared to the *in vitro* studies where 30 to 40 % cell death was recorded. Another difference was that *in vitro* exposure of hepatocytes to FB_1 significantly decreased the total cholesterol by reducing the concentration of free cholesterol. In the *in vivo* study, the highest dietary level (250 mg FB_1/kg) significantly increased the serum and the total cholesterol in the liver.

(iii) Fatty Acid Accumulation and Cell Proliferation

It has been suggested that cell proliferation plays a determining role during cancer initiation and promotion (Gelderblom *et al.*, 1994; 1995). In this regard, the inhibitory effect of FB_1 on cell proliferation is likely to be the prominent determinant during cancer promotion (see above)). *In vitro* studies in primary hepatocytes indicated that FB_1 inhibited the EGF

growth response, thereby mimicking many cancer promoters such as AAF and PB (Gelderblom *et al.*, 1995). The exact mechanism of this inhibition is not known at present. As discussed above the increase in polyunsaturated fatty acids (linoleic acid [C18:2] and AA) in phospholipid and neutral lipid fractions in primary hepatocytes after treatment with FB_1 indicated that the FA biosynthesis is altered. Short-term feeding studies in rats also showed that the concentration of PEA was increased while the level of C18:2 was increased markedly in PEA and PC and no effect was observed on AA (see above). As mentioned above, in long-term feeding studies, low dietary levels of FB_1 also markedly alter the C18:2 FA profiles of PEA. The accumulation of C18:2 suggests that FB_1 alters the n-6 FA metabolic pathway, presumably by affecting the activity of the delta-6-desaturase.

Regarding the disruption of FA metabolism it is known that long chain fatty acids control cell proliferation in different cell culture types via their modulation of prostaglandin levels (Cornwell and Norisaki, 1984). It was shown that, depending on the cell type, prostaglandins of the E series can either inhibit and/or stimulate cell proliferation. *In vitro* studies using Balb/c 3T3 cells, have shown that AA metabolism is required for the mitogenic response of the EGF (Nolan *et al.*, 1988; Handler *et al.*, 1990). Indomethacin inhibits the response while this inhibitory effect is overcome by the addition of prostaglandins, specifically prostaglandin $F2_{\alpha}$. Therefore, the disruption of the prostaglandin levels within the cell regulates the EGF response. Our studies (unpublished data) have shown that ibuprofen, a non-steroidal anti-inflammatory drug, also inhibits the EGF mitogenic response; again implicating the role of AA metabolism. The addition of PGE_2 counteracts the inhibitory effect of FB_1 indicating that the fumonisins also interfere with AA metabolism. The increased levels of AA within primary hepatocytes (see above) suggest that FB_1 inhibits the release and/or the metabolism of AA in primary hepatocytes, an effect that is greatly enhanced at a cytotoxic dosage of FB_1.

(iv) Fumonisin-Induced Toxicity and Oxidative Stress

Chronic inflammation increased the risk of liver cancer in humans as indicated by the fact that both alcohol and hepatitis B infection are important risk factors as they chronically damaged the liver. In the absence of these agents the incidence of liver cancer in humans is low (Ames and Gold, 1990). It has been suggested that the phagocytes liberate oxidants (superoxide) while destroying dead cells which resulted from the chronic infection and/or exposure to a toxic agent (Halliwell, 1994). Another physiological free radical that could be potentially harmful during chronic hepatic injury is nitric oxide which could be involved in the formation of carcinogenic nitrosamines (Bartsch *et al.*, 1992). These oxidants are similar to the process of ionizing radiation and thus could affect the cell by damaging cellular membranes and/or DNA (Ward *et al.*, 1987). It is known that tissue injury leads to oxidative stress when the antioxidant defenses become depleted. Exposure of cells to hepatotoxicants leads to oxidative stress due to various causes (Halliwell, 1994) and increased the probability to further cell injury. Cancer initiation and the induction of the ultimate cancer by FB_1 occur during the development of a chronic active hepatitis that eventually culminates in cirrhosis (Gelderblom *et al.*, 1991; 1994). It has been indicated that primary hepatocytes exposed to FB_1 showed an accumulation of polyunsaturated fatty acids (PUFA) (Gelderblom *et al.*, 1996). The cytotoxic effects of PUFA when added to both normal and cancer cells have been studied extensively and has been associated with an increase in the extent of lipid peroxidation (Gavino *et al.*, 1981; Begin, *et al.*, 1988). These studies imply that FB_1-treated cells could be more susceptible to lipid peroxidation than normal cells. Investigations regarding the effect of the fumonisins on the oxidative mechanisms within the cell are in progress.

(v) The Fumonisins as Peroxisome Proliferators

As the fumonisins appear to be non-genotoxic the possibility that they belong to another class of non-genotoxic carcinogens, the peroxisome proliferators, was investigated (Huber *et al*, unpublished data). The activities of two marker enzymes for peroxisomes, i.e. peroxisomal β-oxidation and acetyl-carnitin-transferase were monitored (Huber *et al*., 1992) in the liver of rats exposed to 3 dietary levels (1, 10, 25 mg FB_1/kg) of FB_1 over a period of 24 months. As discussed above the high dosage levels (10 and 25 mg FB_1/kg) did not cause cancer but induced both GGT^+ and $GSTP^+$ foci and/or nodules in the liver. However, with respect to the parameters measured, no changes were observed above those of the groups receiving the control and low FB_1-containing (1 mg/kg) diets. With respect to the induction of early hepatic lesions associated with liver cancer development, the carcinogenicity of FB_1 is not associated with peroxisome proliferation.

(vi) The Role of p53-Associated Changes in FB_1-Induced Hepatocarcinogenesis

Epidemiological and molecular studies on patients have shown a close association between aflatoxin B (AFB), hepatitus B virus (HBV) infection and hepatocellular carcinoma as well as an association with a mutation at codon 249 in the p53 gene (Bressac, 1991). The synergistic role of FB_1 that has been shown to co-occur with aflatoxin B_1 under natural conditions (Chamberlain *et al*., 1993; Chu and Li, 1994) needs to be elucidated. Studies, currently in progress, are investigating the role of p53-associated mutations in early hepatocyte lesions and the ultimate cancer induced by FB_1 in rats fed a FB_1-containing diet (50 mg FB_1/kg) chronically for two years. DNA from rat liver nodules was amplified by PCR with gene specific primers, corresponding to codon 249 of the human gene. Aflatoxin B_1 (AFB_1), used as a positive control, induced mutations at a very low frequency at codon 243, the equivalent gene to codon 249 in humans, whereas no mutations were detected after cancer initiation with FB_1. In the long-term study with FB_1, only one rat fed 50 mg/kg for 12 months, showed a mutation at codon 243 while none of the rats with hepatic carcinomas after 18 to 26 months showed any mutations. Single strand conformation polymorphism (SSCP) analysis (Sheffield *et al*., 1993) also failed to indicate any additional mutations in the vicinity (110 base pairs) of this codon. The single mutation detected with FB_1 and the low frequency of mutations induced by AFB_1 may not represent primary oncogenic effects. Subsequent studies using immunohistochemical staining procedures with monoclonal antibodies against the wild type and mutant protein will be used to assess whether the p53 protein is altered during FB_1-induced hepatocarcinogenesis.

IMPLICATIONS FOR ESTIMATION OF RISK FOR HUMAN EXPOSURE

(i) Exposure Assessment

Very little information is currently available regarding human exposure to fumonisins at a population level, i.e. estimates obtained from the levels that occur in food combined with the consumption pattern based on dietary recall. In a recent report, a fumonisin (total) intake profile was calculated based on the estimated consumption of "healthy" (14 μg/kg body weight/day) and "moldy" (440 μg/kg body weight/day) home-grown corn in high and low areas for esophageal cancer in southern Africa (Thiel *et al*., 1992). More accurate data

that include the actual daily corn consumption patterns of the populations at risk need to be obtained, since moldy corn does not normally form part of the daily food rations, but rather as an important component of the brewing of traditional beer or used in animal feeds. Therefore, studies need to be performed to monitor fumonisin-intake profiles (daily meals and traditionally brewed beer) at an individual level, over an extended period of time.

Numerous studies have been performed to investigate the natural contamination of corn and corn-based foods and feeds with the fumonisin B (FB) mycotoxins worldwide. High levels of fumonisin contamination of home-grown "moldy" and "healthy" corn have been recorded in the high esophageal cancer risk regions in Transkei, southern Africa (Rheeder *et al.*, 1992; Sydenham *et al.*, 1991) and in Linxian, China (Chu and Li, 1994). In Transkei, mean values of 53.7 (ranging between 3.3-117.5) and 13.7 (ranging between 0.8-23.0) mg/kg for FB_1 and FB_2, respectively, were recorded in homegrown "moldy" corn during the 1989 crop year, giving a total fumonisin content (mean) of 67.4 mg/kg. The corresponding level (total content) in home-grown corn harvested during the 1985 crop year was 31.5 mg FB/kg. The mean total values of the FB contamination of "healthy" corn during the 1985 and 1989 crop years were 9.0 and 5.1 mg FB/kg, respectively (Rheeder *et al.*, 1992). In Linxian and Cinxian, high esophageal cancer areas in China, FB_1 levels up to 155 mg/kg were recorded in "moldy" corn The mean FB_1 levels in the "moldy" and healthy corn were 74 and 35.3 mg/kg, respectively (Chu and Li, 1994).

The mean levels of total FB recorded in commercial corn-based foodstuffs (cornmeal) from the USA was in the order of 1.3 mg FB/kg (Sydenham *et al.*, 1991). The corresponding mean total levels of the fumonisins (FB_1 + FB_2) in South African commercial products were in the order of 0.2 mg/kg. A survey done in South African corn harvested in different locations during the 1989 to 1991 crop years indicated that the bulk of the samples (75%) contained FB (total) levels of 0 to 0.5 mg/kg, while less than 15% have levels of 0.5 to 1 mg/kg and the remaining samples contained levels below 2 mg/kg (Viljoen *et al.*, 1993).

When the total FB level, found in commercial South African corn (0.3 mg/kg), is used and extrapolated to a rural black population that uses corn as a staple diet (70 kg person consuming 460 g corn/day, Thiel *et al.*, 1992) a probable daily intake (PDI) of 2 μg FB/kg body weight/day can be estimated (Table 1).

In exported South African and American corn, containing FB levels of 0.4 and 1.1 mg/kg, respectively, corresponding PDI values are 2.6 and 7.3 μg FB/kg body weight/day respectively. When considering the levels of the fumonisins (total) that occur in commercial corn meal products in SA and the USA (Sydenham *et al.*, 1991), with a mean level of 0.2 and 1.5 mg FB/kg, respectively, the corresponding PDI values are 1.3 and 9.9 μg FB/kg body weight/day (Table 1). The corn consumption pattern of urban black people (Bourne *et al.*, unpublished data) is almost 50 % (276g/70kg body weight/day) when compared to the rural area, (Table 1). In developed countries, such as the European Community, consumption (Smith *et al.*, 1994) of corn products is much lower (7.2g/70kg body weight/day) with PDI values that fall well into the ng FB/kg body weight/day range (Table 1). The corresponding PDI values obtained when using home-grown corn are between 20- to 200-fold higher when compared to the values mentioned above.

(ii) Hazard Assessment

Long-term studies in rats have indicated that the hepatotoxicity of FB_1 is a pre-requisite for the hepatocarcinogenic effect of the compound (Gelderblom *et al.*, 1991). Additional studies on the cancer initiating/promoting potential of the fumonisins indicated that the control of cell proliferation appears to be the key aspect with regard to cancer induction of the fumonisins (Gelderblom *et al.*, 1994). The stimulation of cell proliferation (regenerative) presumably occurs via its hepatotoxicity while the compound inhibits cell

Table 1. Fumonisin (FB) contamination (mean values of FB_1 and FB_2) of corn and corn products in different localities, including high incidence areas for esophageal cancer

Origin	Product	No. of samples	Mean FB level (µg/kg)	PDI (µg/kg body weight/day)		
South Africa (SA)	Exported corn	68[#]	400	2.6	1.6	0.04
	Commmercial corn	209[***]	300	2.0	1.2	0.03
	Corn meal	52[##]	200	1.3	0.8	0.02
SA (Transkei)	Healthy corn	18[*]	7100	46.6	28.0	0.73
	Moldy corn	18[*]	54000	354.9	212.9	5.54
China (Linxian & Cixian)	Healthy corn	15[**]	35300	231.9	139.2	3.60
	Moldy corn	19[**]	74000	486.2	291.8	7.60
United States of America	Exported corn	1682[***]	1100	7.2	4.3	0.11
	Corn meal	16[##]	1500	9.9	5.4	0.15
Corn consumption profiles/70 kg body weight				460g	276	7.18

TDI (µg/kg/day): NOEL/5000 = 0.16; NOEL/1000 = 0.8; NOEL/100 = 8.0.
[*]1985 and 1989 crop years (Rheeder et al., 1992). [**]Based only on FB_1 content (Chu and Li, 1994). [***]1991 and 1992 crop years (Viljoen et al., 1993). [#]1989 crop year (Rheeder et al., 1994). [##]Sydenham et al., 1991). PDI values calculated on the basis that a person (70 kg) in a rural area consumes 460 g (Thiel et al., 1992) , 267 g in an urban area (Bourne et al., 1990, BRISK Study, MRC, Tygerberg) and 7.18g maize/70 kg body weight/day in the European community(Smith et al., 1994).

proliferation via the disruption of growth effects related to normal cell growth processes (Gelderblom *et al.*, 1995). The inhibitory effect on cell proliferation, an important determinant for cancer promotion, is effected at non-toxic dosage levels both *in vitro* and *in vivo* (see above). Short-term studies (21 days) in Fischer rats showed that cancer initiation is effected at a dosage level of $0.7<$effective dosage level(EDL)<1.5 mg FB_1/100 g body weight/day while cancer promotion and inhibition of cell proliferation are effected at a dosage level of $0.17<EDL<0.3$ mg FB_1/100 g body weight/day (Gelderblom *et al.*, unpublished data). Long-term studies using BD IX rats indicated that cancer develops at a daily dietary dosage of $0.09<EDL<0.16$ mg FB_1/100 g body weight/day while cancer initiation and promotion occur at a dietary dosage of 0.03 mg FB_1/100 g body weight/day and above. Comparison of the data obtained from the short-term and the long-term feeding studies with respect to the dosages required for cancer initiation and promotion, indicated differences of 50- and 10-fold, respectively.

In the long-term feeding studies only the rats that received the 50 mg FB_1/kg diet for a period of 26 months developed hepatocellular carcinoma. Apart from the induction of hepatocyte nodules, no cancers were observed in the long-term feeding experiments in which FB_1-containing diets (1, 10 and 25 mg FB_1/kg) were fed for 24 months. The dietary level of 25 mg FB_1/kg diet was considered as the no observed effect level (NOEL) with respect to cancer induction. Since only the highest dietary level induces cancer, the level at which 50 % of the rats would be expected to develop cancer, the TD_{50} value (44 mg FB_1/kg) was determined by linear extrapolation which reflects a numerical estimate of carcinogenic potency (Kuiper-Goodman, 1990). Although 5 animals died during the final stages of the experiment, the 50 mg FB_1/kg diet is below the maximum tolerated dose (MTD) of FB_1 in BD IX male rats. These data represent an intake profile of 800 µg FB_1/kg body weight/day

for NOEL and 1548 μg FB_1/kg body weight/day for the TD_{50}. To determine the potential for human disease two different approaches can be followed, i.e. NOEL/safety factor (SF) using a scale factor of 1000-5000, and the TD_{50}-SF using a scale factor of 50000 (Kuiper-Goodman, 1990).

Using this approach, the estimated tolerable daily intake (TDI) based on the NOEL in carcinogenicity studies (divided by 5000), is 160 ng/kg body weight/day while based on the TD_{50}/50 000 is 31 ng/kg body weight/day. The corresponding TDI values for aflatoxin B_1, as determined in male Fischer rats are 0.15 ng/kg body weight/day for NOEL/SF and 0.023 ng/kg body weight/day for TD_{50}/SF. For ochratoxin A, values of 4.2 (NOEL/SF) and 1.5 (TD_{50}/SF) ng/kg body weight/day were reported in male Fischer rats (Kuiper-Goodman, 1990). Based on these data the estimated TDI values for FB_1 are more than 1100 X (NOEL/SF) and 1350 X (TD_{50}/SF) higher than those of aflatoxin B_1 and 40 X (NOEL/SF) and 20 X (TD_{50}/SF) as compared to ochratoxin A. However, since cancer induction by the fumonisins is dependent on FB_1-induced toxicity, safety factors used for genotoxic carcinogens, such as aflatoxins, cannot be applied directly to the fumonisins. It is suggested that safety factors used for toxic compounds may be more realistic. When a value of 1000, which is the borderline value for differentiating between toxic and carcinogenic effects (Kuiper-Goodman, 1990), is used (safety factors for NOEL are 100-1000), the calculated TDI for NOEL/SF is 800 ng/kg/day. Similarly, with a safety factor of 100, the TDI is 8000 ng/kg/day.

In the case of TDI based on the NOEL/1000, the corresponding acceptable level of total fumonisins in corn is 0.122 mg/kg which is slightly lower than the actual level (0.2 mg/kg) in South African corn meal (Table 1). When NOEL/100 is used, the acceptable value is 1.22 mg/kg, which is well within or higher than the actual mean FB levels in commercial South African corn and corn products (0.2 - 0.4 mg/kg). In contrast, the NOEL/5000 results in a tolerable level of 0.024 mg FB/kg, which is well below FB levels that are known to occur in commercial corn and corn products.

(iii) Thresholds in FB_1 Induced Carcinogenesis and Implications for Assessing Risk to Humans

Most mathematical models treat all carcinogens as mutagens (genotoxins). They assume that even at low doses, DNA reactive molecules could escape the cell's detoxifying mechanisms and induce a mutation in a critical site on the DNA. As a result, many regulatory policies of various countries rely upon the outcome of these models. However, oversimplified speculations on mechanisms of carcinogenesis induced by non-genotoxic carcinogens, such as the fumonisins, should therefore not serve as the basis for risk assessment procedures. Compounds, specifically cancer promoters that act through specific receptors, tend to be active at low doses and it is unclear whether a no-effect threshold exists. On the other hand, compounds that act through a cytotoxic mechanism would be expected to have a no-effect threshold (Cohen and Ellwein, 1990). Below the threshold, cytotoxicity and increased cell proliferation would not occur and thus not increase the tumor risk. Recent studies concerning two compounds, uracil and melamine, that are carcinogenic in the urinary bladder, indicated that urothelial proliferation is a prerequisite for the formation of calculi and tumors (Cohen and Ellwein, 1991). Although these two compounds are carcinogenic in animals, dose-related considerations suggest that they are obviously not carcinogenic since humans are only exposed to doses that are unable to induce urothelial proliferation. Therefore, apart from the DNA-interactive role of genotoxins, other parameters including pharmacokinetics and mode of action as well as the induction of cell proliferation need to be incorporated into the risk assessment (Butterworth and Goldsworth, 1991). FB_1 is carcinogenic at a dosage level that causes adverse toxic lesions culminating in cirrhosis, therefore suggesting the requirement

of a cytotoxic-proliferative response for cancer induction. This was confirmed in a study where relatively low dietary levels of FB_1, that failed to induce excessive toxic effects, also failed to induce any cancers in the liver of the rats. A recent study on the cancer initiating potential of FB_1, indicated that cancer initiation is also effected by dietary levels of FB_1 that induce hepatotoxic effects over a period of 21 days. A threshold for the induction of liver cancer by the fumonisins becomes apparent, and therefore the level of concern regarding the fumonisins as potential human carcinogens, may become irrelevant below doses that fail to induce a cytotoxic-proliferative response.

Thus it is reasonable to accept that with respect to the induction of liver cancer, it is unlikely that the fumonisins can be regarded as human carcinogens at dietary levels below the threshold level that could effect a cytotoxic-proliferative response. This can be deduced when comparing the PDI's (μg/kg body weight/day) of the fumonisins in a population using corn as a dietary staple and the TDI for humans calculated from the NOEL/SF value using a safety factor of 1000 (Table 1). Based on these calculations the consumption of South African commercial corn and corn products yielded PDI values of 1.3 to 2.0 (rural area) and 0.8 to 1.8 (urban area) μg/kg/day are in the same order or slighty higher than the TDI value of 0.8 μg/kg/day (Table 1). The PDI values are based on the assumption that a person (70 kg) in a rural and urban area consumes 460 and 276 g corn per day, respectively. The corresponding PDI values for corn and corn products from the USA are about 3 to 6 fold higher, respectively and more closely resembled the NOEL/100 value. However, the PDI values of populations in the high risk areas for esophageal cancer in Transkei and China are well above the TDI value obtained from NOEL/100 (Table 1). As indicated previously the PDI values in developed countries are likely to be overestimated as the corn consumption in these countries is well below 460 g/day/ person of 70 kg (Thiel at al., 1992) as was indicated for the European Community countries (Smith *et al.*, 1994). This again emphasises the need to perform more accurate studies that will closely monitor the corn intake profiles and consumption patterns of different population groups.

Alternative risk assessment models need to be considered for the fumonisins and other non-genotoxic carcinogens, using NOEL for induced cytotoxic-proliferation responses with a safety factor different from that used for the genotoxic carcinogens where there is no apparent threshold (Butterworth and Goldsworthy, 1991). The safety factors used in the case of the fumonisins, need to consider the fact that cancer induction is likely to occur solely via a cytotoxic mechanism and that a no-effect threshold is likely to exist. On the other hand a possible synergistic interaction with other important carcinogenic stimuli for humans, such as hepatitis B virus (HBV) and AFB, could also eventually affect the potential risk of low dietary levels of the fumonisins. Recent reports indicated that the fumonisins co-occur with aflatoxin in corn, suggesting possible synergism (Chamberlain *et al.*, 1993; Chu and Li, 1994). A recent prospective nested case control study by Ross et al (1990), involving analyses of 18 000 urine samples, indicated a relative risk of 2, 5 and 60, respectively, for AFB, HBV and when the combined effect was considered as the causative principle(s) in the development of liver cancer. Comparable data for the fumonisins in combination with other risk factors, including HBV and AFB, are urgently required.

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Chapter 4

Carcinogenesis by the Fumonisins: Mechanisms, Risk Analyses, and Implications

W. C. A. Gelderblom, S. Riedel, H. M. Burger, S. Abel, and W. F. O. Marasas

PROMEC Unit/MRC, P.O. Box 19070, Tygerberg, South Africa 7505

The fumonisin B (FB) mycotoxins are natural contaminants of corn and cause a variety of diseases in animals. Long- and short-term studies on the carcinogenic potency of FB_1 in rats provided information that a cytotoxic/proliferative response is required and a no-effect threshold exists for cancer induction. Oxidative damage and the disruption of lipid biosynthesis with the subsequent alteration of arachidonic acid and ceramide levels are key determinants for the altered growth properties of initiated cells induced by the fumonisins in the liver. Dietary modulation of the FB_1-induced altered growth responses could play an important role in developing chemopreventive strategies. An integrated approach whereby various aspects regarding the diverse toxicological effects, level of exposure in humans and the relevant mechanisms involved need to be considered in establishing realistic risk assessment parameters for the fumonisins in humans. The socioeconomic and health status of population groups in developing countries, utilizing corn as a major dietary staple, should be weighed against economic realities and international trade.

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Introduction

The fumonisin B mycotoxins were characterized as cancer promoters utilizing a short-term cancer initiating promoting model in rat liver (*1*). Studies in various animal species showed that fumonisin B_1 (FB_1), the major fumonisin produced by *Fusarium verticillioides* (previously known as *F. moniliforme*) is responsible for most of the toxicological effects of corn culture material. These include equine leuko-encephalomalacia in horses (*2*), porcine pulmonary edema (*3*), hepato- and nephrotoxic effects in rats and mice (*4, 5*) and neural tube defects in mice (*6*).

The fumonisins have been implicated in several human diseases due to their natural contamination of human foodstuffs. Associations with esophageal cancer (*7*), liver cancer (*8*), and neural tube defects (*9*) have been reported. However, a causative role of the fumonisins in any of these human diseases still needs to be elucidated. These investigations are hampered by the lack of a sensitive and specific biomarker of exposure in humans. A typical example is changes in sphingolipid metabolism that is disrupted by the fumonisins resulting in an alteration in the sphingosine to sphinganine ratio (So/Sa), which has been utilized as a sensitive marker of exposure in different animal species (*10*). Studies in humans, however, yield equivocal findings (*11-13*), although the general consensus is that changes in the So/Sa ratio lack sensitivity to monitor human exposure. In order to develop relevant risk assessment parameters, studies into the mechanisms of the toxicological effects are still in progress and could provide unique opportunities to develop specific and sensitive biomarkers of exposure in humans.

Carcinogenicity of the Fumonisins

First reports regarding the carcinogenicity of the fumonisins stem from studies utilizing naturally contaminated corn with *Fusarium verticillioides* as well as corn culture material of the fungus in rats (*14, 15*). Studies on fusarin C, the major mutagenic compound produced by the fungus (*16*), failed to explain the carcinogenicity of the fungus (*17*), leading to the purification and characterization of the fumonisins as potent cancer promoters in rat liver (1). Despite the fact that a recent study implied the presence of genotoxic constituents in corn cultures of *F verticillioides* (*18*), FB_1 satisfies all the criteria for being the major carcinogenic principle produced by the fungus which can effect both cancer initiating and promoting phases of liver carcinogenesis (*19, 20*). Although the cancer initiating properties of the apparently non-genotoxic fumonisins were not considered as an inherent property during the risk evaluation, recent studies showed that FB_1 induces a clastogenic effect in different cell culture and subcellular nuclear fractions (*21-24*). Studies in rat liver showed that a threshold for cancer initiation exists depending on the time and dose of exposure (*25*). It would appear that FB_1 mimics the cancer initiating properties of genotoxins such as aflatoxin B_1 (AFB_1), as the initiated cell populations can be promoted into focal proliferative lesions by different cancer promoting regimen such as 2-acetyl-aminofluorene/partial hepatectomy (*25*) as well as phenobarbital (unpublished data). The weak cancer initiating potency of FB_1 could be related to the induction of apoptosis known to occur in the liver and kidneys of rats exposed to the mycotoxin

(*26*). A specific role for an increased oxidative damage that could adversely affect the cellular genome and hence have an effect on cancer induction has also been proposed, as initiation by FB_1 seems to be associated with hepatotoxicity (*24*, *27*). Evidence of oxidative damage as a possible mechanism for FB_1-induced DNA damage is emerging fast as recent studies showed that high levels of dietary iron (*28*) and South African herbal teas (*29*) significantly enhanced and reduced, respectively, the carcinogenic potency of FB_1 in rat liver. Dietary constituents, therefore, could modulate the carcinogenic potency of FB_1 by affecting critical cellular events that either increase or decrease the susceptibility of a cell to undergo genetic transformation. The different diets utilized in the carcinogenicity studies of FB_1 showed that a high protein diet could predispose the kidney to the carcinogenic effects of FB_1 in male Fischer rats whilst a low protein diet exhibited a protective effect (*30*). Diets low in protein, vitamins and minerals, on the other hand, could have played an enhancing role in the induction of hepatotoxic and -carcinogenic effects. Synergistic interactions with other mycotoxins such as AFB_1 have been reported in rats (*31*) and rainbow trout (*32*). This implies that dietary approaches to reduce the toxic and economic impact of the fumonisins and other mycotoxins could be a viable option in the future as was suggested recently (*33*).

The role of apoptotic cell death and the resultant regeneration in the kidney has been proposed as a driving force for the development of kidney tumors by FB_1 (*26*, *34*). In the liver, however, both apoptotic and necrotic cell death are induced which complicates a similar type of mechanism. When fed to rats, FB_1 reduced the relative liver and kidney weights suggesting that the net rate of cell proliferation is reduced, creating a negative pressure on the organ homeostasis. The latter was proposed to be the main mechanism for the selection of initiated cells, resistant to the toxic and/or apoptotic effects of FB_1. A similar hypothesis was developed for the kidneys of rats where genetically altered initiated cells are resistant to the apoptotic effects of FB_1 caused by the disruption of sphingolipid metabolism (*35*). In general it would appear that the genesis of a FB_1-associated resistant phenotype in an altered cell population in the liver or kidneys forms the basis for explaining their selective progression into neoplasia.

Proposed Mechanisms of Cancer Promotion

The fumonisins were characterized as active cancer promoting principles utilizing a liver cancer initiating-promoting model (*1*). Two different mechanisms have been proposed for the selective stimulation or enhancement of the growth of altered lesions in the liver and/or kidneys by FB_1. The first hypothesis is centered on the disruption of sphingolipid metabolism where the accumulation of sphingoid bases or the depletion of complex sphingolipids results in the induction of apoptosis leading to the stimulation of compensatory regenerative cell proliferation to maintain cellular homeostasis (*26*, *34*, *35*). This continued level of increased compensatory cell proliferation is suggested to be a key driving force in the genesis of early altered proliferative lesions due to an increased susceptibility of proliferating cells to undergo genetic alterations that eventually result in the development of neoplasia. The second hypothesis is based on

the growth selection of altered cells, induced during cancer initiation, on the basis of selective inhibition of normal cell growth by FB_1 whilst the altered cells escape these growth inhibitory signals (*36*). Both these hypotheses focus on an increase in cell proliferation, through either compensatory or differential growth mechanisms that eventually result in the abnormal growth of an altered cell population leading to the development of cancer. These different approaches to cancer promotion in the liver were originally developed by Farber (*37*), Dragan and Pitot (*38*) and Schulte-Hermann *et al.* (*39*). The current models for cancer promotion by FB_1 integrate some of the basic biochemical mechanisms induced by FB_1 in the liver and kidneys to provide alternative views regarding the selective outgrowth of resistant initiated cells (*40*).

Alterations in Lipid Metabolism

The disruption of lipid metabolism has been implicated in the toxicological and carcinogenic effects of the fumonisins (*41-43*). In the liver of rats the main emphasis was on the disruption of cholesterol, phospholipid and fatty acid metabolism, while the disruption of sphingolipid metabolism has been proposed to be a major mechanism in the development of liver and kidney cancer (*26, 35*). In both cases a balance between apoptosis and cell proliferation in selected cell populations forms the basis of cancer promotion and the interaction between the different pathways has been proposed (*36*). Changes in the sphingolipid pathway in the liver and kidneys include (*35, 44*):

- Inhibition of ceramide synthase.
- Accumulation of sphinganine and sphingosine.
- Depletion of ceramide and other complex sphingolipids.
- Accumulation of sphingolipid -1-phosphates.

Alterations in lipid metabolism in rat liver include (*41 – 43, 45*):

- Increase in the level of phosphatidylethanolamine (PE).
- Increase in cholesterol.
- Inhibition of the delta -6 desaturase.
- Depletion of polyunsaturated fatty acids (PUFA) in phosphatidylcholine (PC).
- Increase in the PE/PC C20:4n-6 ratio.

The changes in the phospholipids and fatty acid parameters closely mimic alterations known to prevail in hepatocyte nodules that are early proliferative lesions induced by the fumonisins and many other hepatocarcinogens (*46*). These lesions showed a reduced level of oxidative damage (*47*) mainly due to an increased level of reduced glutathione and low levels of PUFA. The reduced oxidative status has been suggested to be one of the major growth determinants in neoplastic lesions as oxidative damage is an important signal for apoptotic cell death. As fumonisins are known to

induce oxidative damage in the liver, resistance against excessive oxidative damage in hepatic lesions could be a major mechanism for their selective outgrowth, whilst the proliferation of normal cells is inhibited. In this regard primary hepatocytes were more susceptible to the cytotoxic effects of FB_1 than Chang liver cells (*48*). The low level of long chained PUFA in Chang liver cells was suggested to be the main determinant for the differential cytotoxicity as it renders the cell more resistant to FB_1-induced oxidative damage. Another important alteration effected by FB_1 is the increased level of C20:4n-6 in the PE phospholipid, resulting in an increase in the C20:4n-6 PC/PC ratio in different subcellular liver fractions (*45*). Changes to the balance of C20:4n-6 in cellular membranes could be important in the regulation of cell proliferation and/or apoptosis. An interactive role between C20:4ω6 and ceramide in the mitochondria was suggested to regulate the balance between proliferation and apoptosis in altered initiated hepatocytes, resulting in their selective outgrowth during cancer promotion. Although FB_1 disrupts ceramide syntase to the same extent in the nodules and surrounding liver tissue, the accumulation of sphingosine in nodules was proposed to also regulate nodular growth via the formation of sphingosine-1-phosphate (*49*).

The disruption of these upstream events, including sphingolipid, phospholipids and fatty acids in the liver, has been associated with the stabilization of cyclin D_1 via the activation of Akt and the inhibition of GSK-3b activity in preneoplastic and neoplastic lesions (*50*). The subsequent elevated CDk4 activity and increased phosphorylation of retinoblastoma (Rb) protein in early as well as neoplastic lesions may be important role players in the hepatocarcinogenic effects of the fumonisins. With respect to cancer promotion, the induction of TGF-β1 and *c-myc* in the liver was suggested to be involved in the proliferation in TGF-β1 resistant cells (*51*). In addition to the increased expression of TGF-α, *c-myc* could contribute to the modification of cyclin D_1 and the inactivation of Rb. The depletion of growth factors and/or disruption of growth factor signaling pathways and the over-expression of *c-myc* could lead to the induction of apoptosis, which could further assist in the selective outgrowth of resistant initiated hepatocytes. The interaction between the different parameters discussed above seems to be the main differential that will allow altered cells to proliferate and eventually result in the development of liver cancer (Figure 1).

This interactive model provides a challenging approach that could impact on our current understanding of the health risks of cancer promoting substances that naturally occur in major staple diets. Information about their existence in humans exposed to the fumonisins could set new paradigms to determine risk. The question arises whether the aspects regarding the carcinogenic potency of the fumonisins discussed above should be included when establishing risk parameters, such as the Provisional Maximum Tolerable Daily Intake (PMTDI), to safeguard humans from their possible adverse effects. As the mechanism of cancer induction is not known and seems to be associated with a chronic toxicity, the fumonisins are regarded as indirect or non-genotoxic carcinogens. However, it has been suggested that an attempt to develop a regulatory policy for indirect carcinogens solely based on a 2-yr rodent study is ineffective and/or inaccurate (*52*). The use of supplementary biological information should be considered with respect to the fumonisins, as the use of nephrotoxicity as the only measure for determining risk seems inappropriate.

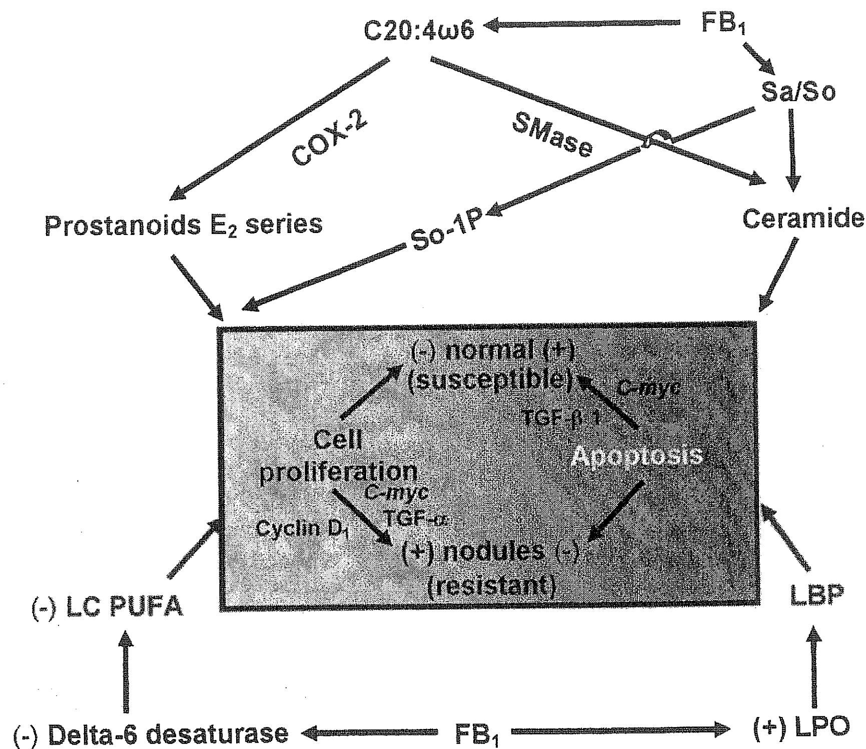

Figure 1. Biphasic effect of FB_1 on the normal vs hepatocyte nodules in the liver of rats. FB_1 increases the level of C20:4ω6 in the liver that selectively stimulates the proliferation of hepatocyte nodules via the induction of prostaglandin E_2 series and regulating apoptosis through ceramide via the sphingomyelinase. FB_1 also inhibits the delta-6 desaturase which results in a low level of LC PUFA and low level of oxidative damage known to stimulate cell proliferation in the hepatocyte nodules. In the normal cells, apoptosis is increased either by the low levels of ceramide or the accumulation of sphingosine or sphinganine, low levels of LC PUFA and the lipid breakdown products as a result of the FB_1-induced oxidative damage. The differential effects of growth regulatory signals such as sphingosine-1-phosphate, C20:4ω6 and ceramide on downstream events such as cyclin D_1, TGFα and β-1, c-myc could selectively stimulate the proliferation of hepatocyte nodules resulting in neoplastic development. LBP=lipid breakdown products; LPO= lipid peroxidation.

Risk Assessment Parameters of the Fumonisins

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) used the No Observed Effect Level (NOEL) for nephrotoxicity and a safety factor of 100 to provide a PMTDI of 2 μg/FB/day/kg bodyweight (*53*). The numerical size of the safety factor used may differ depending whether a compound is classified as having toxic effects or upon long term exposure could result in the induction of a carcinogenic response. In general larger safety factors are used for carcinogenic compounds depending on the mechanism of cancer induction related to epigenetic (non-genotoxic compounds) or genotoxic mechanisms. When using a safety factor of 1000 for carcinogenicity, the NOEL for hepatocarcinogenicity in male BD IX rats resulted in a Tolerable Daily Intake (TDI) of 0.8 mg FB_1/day/kg body weight (*40*). The NOEL for nephrotoxicity was obtained from a long-term chronic feeding study (2 yr) in male Fischer rats and was equivalent to a dietary level of 5 mg FB_1/kg diet, resulting in a FB_1 intake of 200 μg/kg body weight/day (*34*). A 90 day study in male Fischer rats also provided a similar NOEL for nephrotoxicity (*54*). However, when considering the carcinogenicity of FB_1 the NOEL for nephrotoxicity of 200 μg/kg bw/day is below the no-effect threshold for carcinogenicity (700 μg/kg bw/day) for the induction of adenoma and carcinoma in the kidneys. The NOEL for nephrocarcinogenicity and the hepatocarcinogenicity are very similar and vary between an FB_1 intake of 700 μg to 800 μg/kg bw/day, respectively. A TDI of 0.8 μg FB/kg bw/day, using the NOEL for hepatocarcinogenicity and a safety factor of 1000 has been proposed (*40*) which is very similar to the 0.7 μg/kg bw/day using the NOEL for nephrotoxicity with a similar safety factor. The latter approach resulted in a PMTDI that is in the order of 3 times lower than the PMTDI using the NOEL for nephrotoxicity. At present it is not known whether a PMTDI, based purely on the nephrotoxicity whilst ignoring the carcinogenic properties, will safeguard humans from the adverse effects of the fumonisins.

When comparing the risk parameters between different mycotoxins, the PMTDI for deoxynivalenol (DON) is 1 μg/day/kg bw, using a safety factor approach of 100 based on toxicity (*55*). The level is lower than that calculated for fumonisins which exhibit carcinogenic properties and could have a more adverse impact during chronic low levels of exposure. This becomes apparent when considering the Provisional Tolerable Weekly Intake (PTWI) of ochratoxin A (OA) which is 0.1 μg/kg bw/week which was obtained using a safety factor of 1500 and the NOEL for nephrocarcinogenicity in male rats (*56*). When considering the carcinogenic risk to humans, the International Agency for Research on Cancer (IARC) has evaluated DON as not classifiable as to its carcinogenicity and OA as a possible carcinogenic (group 2B) to humans (*57, 58*). Some discrepancy therefore exists when considering the PMTDI of fumonisins exhibiting both hepato- and nephrocarcinogenic effects but, like OA, classified a group 2B carcinogen (*58*). At present two tolerable limits exist i.e., a lower limit 0.7/0.8 μg/kg bw/day (lower risk) that focuses on the carcinogenic properties of the fumonisins and a lower limit of 2 μg/kg/day (higher risk) that was based on the nephrotoxic effects. In developed countries exporting large quantities of corn, a lower tolerable limit (0.8 μg/kg/day) could severely affect the corn industry whereas in developing countries with a high corn intake profile and high level of contamination, even the lower tolerable limit will pose a health risk. In this regard it was suggested that a maximum tolerance level (MTL) of 100 to 200 μg fumonisins/kg

corn should safeguard human health in South Africa when considering the high corn intake profiles in certain rural and urban environments (*59*). In developed countries higher MTL's would be acceptable, e.g., 1 mg fumonisins/kg corn in Switzerland (*60*) due to the low intake profiles in the European Community. Similar situations prevail in France and the USA where levels of 3 and 2 to 4 mg fumonisins/kg whole corn, respectively, have been proposed (*61*, *62*). However, certain population groups in developed countries consuming higher quantities of corn or corn products could be at risk, such as people with celiac disease that consume 162 g/person/day (*63*). A lower PMTDI based on carcinogenicity of the fumonisins could safeguard certain high intake corn consumers worldwide but could also have negative economic impact on international trade affecting the entire corn industry (*64*). As suggested above, different scenarios regarding risk assessment of the fumonisins therefore exist which could either underestimate the risk posed to human health or overemphasize the risk that would negatively impact on international trade and the corn industry in large exporting countries. The current PMTDI of the fumonisins has to be considered against the background of different determinants including health, economy, technology, and politics (Figure 2). The question exists whether the PMTDI for fumonisins addresses the health risk to human populations in developing countries or is in fact driven by economic considerations, protecting the corn industry and international trade agreements of developed countries.

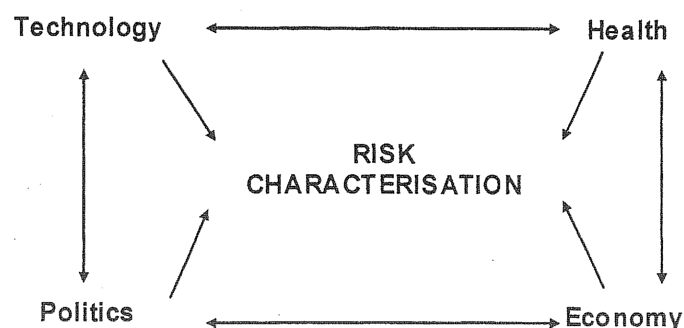

Figure 2. Interactive role of different parameters determining the risk and regulation of food contaminants such as the fumonisins. A balanced approach is required to ensure maximum protection (health PMTDI) of human populations worldwide while conserving the economic viability of corn production and international trade (economic PMTDI).

Fumonisins as Food Contaminants in Developing Countries

Differences exist in establishing and implementing risk assessment parameters from a global perspective versus remote microenvironments in developing countries where corn is used as a sole dietary staple. The health issues associated with these micro-environments are largely ignored by global trade between industrialized countries despite the potential negative impact on human health due to the exposure to

foodborne toxins and carcinogens. In order to evaluate the risk of fumonisin contamination of corn, the production and consumption profiles of corn in different countries need to be considered. According to the FAO corn production figures, Africa (41.6 million metric tons) is the third largest producer of corn globally following the United States of America (298.2 million metric tons) and China (131.1 million metric tons) (*65*). However, according to the food balance sheets corn consumption profiles, Africa is the largest consumer exceeding 36 million metric tons in 2002 followed by China (20.1 million metric tons) and the USA (3.9 million metric tons). In a recent paper it was argued that corn is replacing sorghum, a staple diet of black people in Africa due to the higher yield (*66*). It is clear from the FAO database on corn production that since 1961 there was a steady increase in corn production in West African countries with a decrease of sorghum and millet production while in southern Africa corn remained the dominant crop compared to millet and sorghum (Figure 3) (*65*).

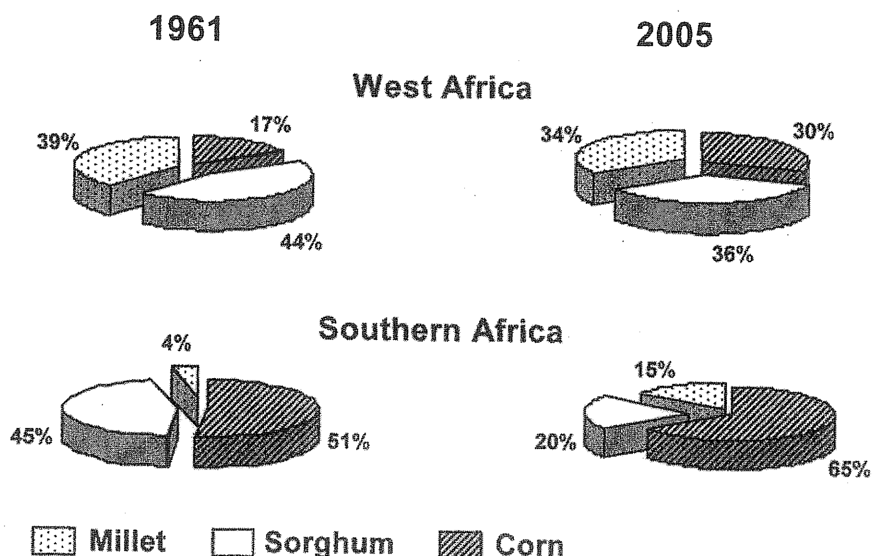

Figure 3. Changes in the millet, sorghum and corn production profiles between 1961 and 2005. Each segment represents the relative % of each crop relative to one another as projected by the FAO.

The dependence on corn as a basic dietary staple in Africa resulted in major imports during droughts typical of the continent. Countries such as the USA, Brazil, and Argentina are large exporters of corn, but apart from the usage for animal feed only 10 % of the harvest is consumed by humans (*65*), mainly as processed foods that significantly reduced the level of FB contamination (*67*). In the African context corn directly enters the human food chain and in many areas it is used as a mono cereal diet for the larger part of the year when the deficiency of micronutrients may exacerbate the susceptibility of consumers to adverse effects of mycotoxins such as the fumonisins.

Persons who are at the greatest risk with respect to fumonisins in corn are subsistence farmers and children in rural Africa who consume large quantities of mostly contaminated home-grown corn or imported corn contaminated with high levels of fumonisins. Different scenarios therefore exist with respect to the corn production and consumption patterns in different regions and the level of fumonisin contamination. In order to further evaluate the possible risk that fumonisins could pose to humans these parameters should be considered against the current PMTDI when utilizing an interactive approach (Table I).

Fumonisin exposure in rural settings in Africa reaches levels that are far above the PMTDI levels set by JECFA especially when considering the maize consumption patterns and fumonisin contamination levels in different so-called "hotspots" of exposure. In certain of these reports corn intake values approach the 500g/day/person with fumonisin contamination levels exceeding 1 mg FB/kg corn. A recent study in Burkina Faso (*68*) indicated that the mean exposure of people consuming market purchased corn would be 12 to 60 times the PMTDI level of fumonisins. It was suggested that children and infants represents a more vulnerable group as they consumed larger amounts per unit body weight. A study in the Western Highlands of Cameroon showed mean FB levels between 1.74 to 5.8 mg/kg in homegrown corn earmarked for human consumption (*69*). In the Transkei region of South Africa, FB intake profiles showed approximately 3 to 7 and 2 to 4 times the PMTDI levels in children and adults respectively, when consuming home-grown corn (*70*). Studies in other developing countries such as Argentina showed FB intake profiles of up to 1.5 and 5 times the PMTDI in adults and children, respectively (*71*), while in China intake levels in adults varying from 1 to 4 times above the PMTDI have been reported (*72*).

Mean FB contamination levels in white corn cultivated in the commercial maize production areas in South Africa recorded over a period of 6 crop years (1989 to 1994) were 0.28 to 0.78 mg/kg corn (*64*). With an approximate intake of commercial corn of 200 g/day in children in the Eastern Cape Province of South Africa (*73*) at the upper contamination level of 0.78 mg FB/kg, a level of approximately 4 times the PMTDI is calculated. When considering the urban South African consumption patterns amongst adults of 267 g/day (*40*) the intake profile is 1.5 times the PMTDI. Imported corn from the USA during 1992 contained considerably higher FB levels that varied between 2.23 to 2.65 mg FB/kg (*74*). If the imported corn enters the human food chain in South Africa a 10- and 4-fold intake above the PMTDI is calculated for children and adults, respectively, in an urban setting. When considering the higher corn intake of adults at levels up to 500 g per person per day for a rural population, a further 2 times increase above the PMTDI is obtained, a situation that is worsened if home-grown corn is utilized as discussed above. This is of particular importance during dry seasons when large quantities of corn need to be imported, some of which is channeled into the human food chain. As commercial South African corn contains low levels of FB compared to corn supplied on the international markets, the latter could increase the risk due to FB intake in the local population. In addition, impoverished rural communities in Africa depending on corn as a sole dietary staple and with a reduced intake of micronutrients, could be exposed to the adverse health effects by the consumption of corn containing high FB levels. Children appear to be the most vulnerable group of the population and PDI values of 2-3 fold above those

Table I. The probable daily intake of fumonisins, as a function of corn intake profiles and FB contamination level, compared to the PMTDI level related to toxicity and carcinogenicity. The light shaded areas reflect the PMTDI for carcinogenicity (*40*) and the dark shaded areas that for nephrotoxicity (*53*).

MAIZE INTAKE [g/person (60kg)/day]

FB (ppm)	*10*	*50*	*100*	*150*	*200*	*400*	*500*	
0.2	0	0.2	0.3	0.5	**0.7**	**1.4**	**1.7**	
0.5	0.1	0.4	**0.8**	**1.3**	**1.7**	***3.4***	***4.2***	***PDI***
1	0.2	**0.8**	**1.7**	***2.5***	***3.3***	***6.6***	***8.3***	***(µg/kg bw/day)***
2	0.3	**1.7**	***3.3***	***5.0***	***6.7***	***13.4***	***16.7***	
3	0.5	***2.5***	***5.0***	***7.5***	***10.0***	***20.0***	***25.0***	
4	**0.7**	***3.3***	***6.7***	***10.0***	***13.3***	***26.6***	***33.3***	

PMTDI = 2 µg/kg bw/day (nephrotoxicity)
PMTDI = 0.7/0.8 µg/kg bw/day (carcinogenicity)

calculated for adults are obtained. Children are considered to be more susceptible to the adverse effects of mycotoxins as they general have a higher intake on a per kg body weight basis (*75*). Aspects of under-nutrition and the HIV AIDS epidemic in sub-Saharan Africa can only further exacerbate the risk to fumonisin exposure.

The established risk assessment parameters for the fumonisins seem to be limited and failed to safeguard humans residing in developing countries such as Africa with high corn intake profiles and more susceptible to the adverse effects of these mycotoxins. The MTL of 2 to 4 mg FB/kg milled corn as proposed by the USA (*62*) and recently suggested for South Africa (*64*) could further worsen the situation with respect to the proposed PMTDI value. Major obstacles that prevail and lack of regulation in developing countries are ignored when setting risk assessment parameters whereas FB intake levels of 10 to 20 times above the PMTDI are common. It is not known at present what effects the long-term exposure to fumonisins at these levels would have on the health status of specific populations at risk. The need to perform studies to quantify the exposure patterns as well as determining the nutritional status of the people at risk is self-evident. Proper intervention measures to reduce exposure to match the established risk assessment parameters is therefore of critical importance in developing countries and/or the setting of a new PMTDI level to safeguard human population groups in developing countries is urgently required. A MTL of 0.2 mg FB/kg maize as suggested by Marasas (*59*) appears to be remarkably close to a safe contamination level of maize in a South African setting where adults may consume up to 500 g and more maize per day. In children, however, the situation is even worse with a corn intake of up to 200 g/child/day according to a recent survey in the Eastern Cape Province of South Africa (*73*) resulting in PDI levels that far exceed the PMTDI level proposed by JECFA (*53*).

Several aspects regarding the regulation of FB in corn are of importance. Setting a MTL for FB could either negatively impact on human health in certain population groups or severely disrupt the corn industry and international trade. The setting of realistic risk assessment parameters in an African context where the health of certain population groups and corn production are not compromised requires urgent attention. Aspects regarding international trade and economic realities have also to be taken into account. The complexities and implications of setting control measures of toxic food contaminants have been debated recently (*76*, *77*). When taking AFB_1 as an example, losses in nut export from Africa will be in the order of \$40 million if the EU standard of 4 μg/kg is adopted (*77*). If the guidelines of 2 mg/kg with respect to fumonisin set by the FDA isadopted the total export losses to the USA, China and Argentina would be about \$100 million, but if this is reduced to 0.5 or 0.2 mg/kg as suggested by Marasas (59), losses will amount to an excess of \$300 million (77).

Since African countries rarely export corn the economic loss due to international trade will be minimal while control measures to safeguard its inhabitants should be the focus. However, if mycotoxin monitoring programmes are not in place in Africa, the implementation of a MTL for exposure at the proposed safety limit for fumonisins will not be possible.

The following aspects regarding the regulation of fumonisin mycotoxins need to be considered in the future:

- Introducing risk assessment parameters should consider that people who are at the greatest risk are subsistence farmers and children in certain developing countries, as they have the highest intakes and also consume the most highly contaminated corn. Food security is a constant problem for populations in developing countries leading to under-nutrition that could increase their susceptibility to the adverse effects of mycotoxins. The synergistic interaction of the fumonisins with other dietary constituents such as aflatoxin contamination, increased levels of dietary iron and diets low in antioxidants could predispose these populations to the adverse effects of fumonisins.
- The carcinogenic potential of the fumonisins needs to be included in determining the risk assessment parameters which will result in a 3x reduction in the PMTDI value (*36*). Such a PMTDI should include aspects regarding its cancer inducting potency and the disruption of growth regulatory signals within a cell that could, upon chronic exposure at low doses, lead to the induction of specific cell populations with the potential to develop into cancer. Such an approach will be in accordance with the carcinogenic evaluation of FB_1 by the International Agency for Research on Cancer as a Group 2B carcinogen (*58*).
- The establishment of a MTL in corn that is either too high due to economic considerations or too low to reduce a possible health risk to humans needs to be debated. The technological feasibility to reduce mycotoxin contamination should be developed to comprise both aspects and to sustain a viable corn industry worldwide. At present the proposed MTL level of 2 to 4 mg FB/kg in dried milled corn products (*62*) could adversely affect human health if entered into the human food chain in developing countries.

- Effective control of fumonisin contamination of corn or development of chemopreventive measures to reduce its impact would be important ways to reduce FB exposure and the associated risk to human health.

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4

Mycotoxins: A Global Problem

Walter F. O. Marasas*, Wentzel C. A. Gelderblom,
Gordon S. Shephard and Hester F. Vismer

Abstract

The five most important naturally occurring mycotoxins in human foods and animal feeds are aflatoxin, ochratoxin, deoxynivalenol, zearalenone and fumonisin. Risk assessment is used to manage the risk from mycotoxins to protect human and animal health. Conventional risk assessment has two major components, *i.e.*, exposure assessment and hazard assessment, which data are used to establish Maximum Tolerated Levels (MTLs). Most countries have established MTLs for total aflatoxins ranging from 4-20 ng/g. The US Food and Drug Administration (FDA) has proposed MTLs for total fumonisins of 4 μg/g in whole maize and 2 ng/g in maize products for human consumption. The MTLs proposed by developed countries apply to commodities that they import and to foodstuffs consumed within their borders, but not to agricultural products that they export. Thus conventional risk assessment has helped manage the risk from mycotoxins in developed countries but has not helped in developing countries that import foodstuffs (or receive food aid). The situation with fumonisins in maize is complicated further by large differences in maize consumption by different populations, *e.g.*, from ~5 g/person/day in Europe to ~500 g/person/day in rural Africa. The differences in maize intake have a marked effect on the Probable Daily Intake (PDI) of fumonisins by different populations. Subsistence farmers in Africa who consume home-grown maize have the highest maize intakes and also consume maize with the highest levels of fumonisin contamination. Conventional risk assessment has not been of value to them and leaves the people who are at the highest risk for mycotoxin exposure the least protected.

Introduction

Mycotoxins have undoubtedly presented a global problem to human and animal health since the earliest times, and this threat will only increase as the demand on the available food supply increases in response to the growth of the world population (Marasas and Nelson, 1987). If the food supply is limited, the mycotoxin hazard is exacerbated in at least two ways. First, more fungus-damaged, potentially mycotoxin-containing foodstuffs are consumed rather than discarded, and second, malnutrition enhances the susceptibility to lower levels of foodborne mycotoxins.

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Natural outbreaks of mycotoxicoses occur world-wide, from the humid tropics to Siberia. Although the climate in a particular country may not favor the elaboration of a specific mycotoxin, such as aflatoxin, the problem may be imported from another country in the form of agricultural products, such as peanuts or maize.

Mycotoxins affect both animals and humans acutely as well as chronically. Acute outbreaks of mycotoxicoses are the tip of the iceberg, whereas chronic effects, such as growth stunting, immune suppression and cancer, are much more important although they may not be as evident. A problem cannot be controlled before it is recognized, and acceptance by governments of developed and developing countries that mycotoxins represent a serious health hazard in addition to serving as a trade barrier with significant economic impacts, is a matter of urgency. Mycotoxins are a global problem that requires a global solution to prevent or reduce the development of mycotoxigenic fungi, their insect vectors and the resulting mycotoxin contamination of agricultural crops in the field and in storage. The extent of the mycotoxin problem, particularly with respect to the foodborne carcinogenic mycotoxins, aflatoxin and fumonisin, risk assessment and possible solutions are discussed in this chapter.

Mycotoxigenic Fungi, Mycotoxins and Mycotoxicoses

Globally, the five most important mycotoxin-producing fungi are (Miller, 2002): *Aspergillus flavus, Aspergillus ochraceus, Penicillium verrucosum, Fusarium graminearum* and *Fusarium verticillioides*. The five most important mycotoxins that occur naturally in agricultural products are (Miller, 1995): aflatoxin produced by *A. flavus*; ochratoxin produced by *A. ochraceus* and *P. verrucosum*; deoxynivalenol and zearalenone produced by *F. graminearum*; and fumonisin produced by *F. verticillioides*. Human diseases that have been associated with two of these mycotoxins in foods are: acute toxic hepatitis and liver cancer with aflatoxin; and esophageal cancer and neural tube defects with fumonisin.

Aflatoxins

Aflatoxins are carcinogenic mycotoxins produced by some *Aspergillus* species in a wide range of agricultural commodities, primarily by *A. flavus* in maize and peanuts. Aflatoxin B_1 was first identified in the United Kingdom in 1960 in a shipment of peanuts from Brazil. Subsequently, aflatoxin B_1 was shown to cause outbreaks of acute hepatitis in animals and humans, to cause liver cancer in animals, and to be associated with liver cancer in humans, particularly in combination with hepatitis B virus infection in sub-Saharan Africa and Southeast Asia (Turner *et al.*, 2002). The International Agency for Research on Cancer (IARC) evaluated aflatoxin B_1 as a Group 1 carcinogen, *i.e.*, carcinogenic to humans (IARC, 1993). Maximum tolerated levels of aflatoxins in foods and feeds are regulated in most countries world-wide and commonly range from 4-20 ng/g (FAO, 2004). The danger of consuming foodstuffs contaminated with aflatoxin at levels above the regulatory limit was again demonstrated in 2004 in Kenya where 125 people died following the consumption of home-grown maize containing high levels of aflatoxin (Lewis *et al.*, 2005).

Fumonisins

Fumonisins are carcinogenic mycotoxins produced by some *Fusarium* species, primarily *F. verticillioides* growing in maize. Fumonisins were first isolated and identified in South Africa in 1988 from cultures of *Fusarium verticillioides* (= *F. moniliforme*) strain MRC 826 (Gelderblom *et al.*, 1988). During 1989/1990, broken maize kernels (screenings) from the 1989 maize crop in the United States caused widespread outbreaks of leukoencephalomalacia (LEM) in horses and pulmonary edema syndrome in pigs throughout the country. By 1990 both of these syndromes were proven to be caused by fumonisin B_1 (Marasas, 2001). Analytical methods for the detection of fumonisin B_1 and fumonisin B_2 in maize also were developed in 1990 (Shephard *et al.*, 1990). Reports followed of naturally occurring levels of the toxin in maize screenings associated with field outbreaks of leukoencephalomalacia and pulmonary edema syndrome as well as in home-grown maize in high-incidence areas of human esophageal cancer in the Transkei region of South Africa (Rheeder *et al.*, 1992) and China (Chu and Li, 1994). During 1991, fumonisin B_1 was shown to cause liver cancer in rats (Gelderblom *et al.*, 1991). The carcinogenicity of fumonisin B_1 was confirmed by the National Toxicology Program (NTP) of the United States Food and Drug Administration (FDA) in a two-year feeding study in rats and mice (Howard *et al.*, 2001). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated the fumonisins and allocated a group provisional maximum tolerable daily intake (PMTDI) of 2 µg/kg body weight to fumonisin B_1, fumonisin B_2 and fumonisin B_3, alone or in combination (WHO, 2002). The IARC evaluated fumonisin B_1 as a Group 2B carcinogen, *i.e.*, possibly carcinogenic to humans (IARC, 2002). Although the role of fumonisin B_1 in esophageal cancer has not been proven, fumonisin has to be considered as a risk factor, particularly in rural populations living on a maize-based staple diet.

Fumonisin B_1 is known to inhibit folic acid transport by the folate receptor and because folic acid deficiency causes neural tube defects, some birth defects in humans may be caused by dietary exposure to fumonisin B_1 (Hendricks, 1999). Fumonisin B_1 causes cranial neural tube defects in mouse embryos and folic acid prevents fumonisin B_1-induced neural tube defects in these animals (Sadler *et al.*, 2002; Gelineau-van Waes *et al.*, 2005). The high-incidence areas of esophageal cancer in South Africa and China, where high levels of fumonisin in the maize staple diet have been reported, also are high incidence areas for neural tube defects in humans (Marasas *et al.*, 2004). The possible role of fumonisin as a cause of birth defects in high incidence areas of Africa, Asia and South America requires further investigation.

At least 28 fumonisin analogs are now known (Rheeder *et al.*, 2002) and three of these (fumonisin B_1, fumonisin B_2 and fumonisin B_3) occur naturally in maize world-wide, sometimes at very high levels of up to 330 µg/g (Shephard *et al.*, 1996). Fumonisins and aflatoxins often co-occur in maize and a synergistic interaction between fumonisin B_1 and aflatoxin B_1 is known (Gelderblom *et al.*, 2002).

The FDA has published a "Guidance for Industry" (FDA, 2001) with respect to fumonisin levels in human foods and animal feeds that the FDA considers to be adequate to protect human and animal health. These levels range from 2-4 µg/g in maize intended for human consumption and 1-50 µg/g in animal feeds. However, the risk of fumonisin contamination of maize to the consumer is determined by both maize intake and level of contamination (Table 1). In general, the highest maize consumers in rural areas also consume the most highly contaminated home-grown maize (Gelderblom *et al.*, 1996; Marasas, 1997).

Table 1. Interactive associations between fumonisin contamination (µg/g maize) and maize intake profiles (g/60 kg person/day) shown as Probable Daily Intake (PDI; µg/kg body weight/day). Provisional Maximum Tolerable Daily Intake (PMTDI) calculations are based on nephrotoxic effects as set forward by the JECFA (WHO, 2002) or hepatocarcinogenic effects as proposed by Gelderblom *et al.* (1996).

FB (µg/g)	Maize intake (g/60 kg person/day)						
	10	50	100	150	200	400	500
0.2	0	0.2	0.3	0.5	0.7	1.4	1.7
0.5	0.1	0.4	0.8	1.3	1.7	3.4	4.2
1	0.2	0.8	1.7	2.5	3.3	6.6	8.3
2	0.3	1.7	1.3	5.0	6.7	13	17
3	0.5	2.5	5.0	7.5	10	20	25
4	0.7	3.3	6.7	10	13	27	33
5	0.8	4.2	8.3	13	17	33	43
10	1.7	8.3	17	25	33	67	83
12	2	10	20	30	40	80	100

White areas: PMTDI falls within the tolerable daily intake level; lightly shaded areas: PMTDI = 0.8 µg/kg body weight/day (hepatocarcinogenicity); medium shaded areas: PMTDI between 0.8 and 2 µg/kg body weight/day (nephrotoxicity); dark shaded areas: PMTDI values above the maximum tolerable daily intake levels.

Such maize is not subject to national or international regulations based on the MTLs of aflatoxin or the guidelines proposed for fumonisin by the FDA.

Economic Impacts of Mycotoxins

Few attempts to estimate the economic costs of mycotoxins in monetary terms have been published. Lubulwa and Davies (1995) estimated the social costs of the impacts of fungi and aflatoxins in maize and peanuts in Indonesia, Philippines and Thailand during 1991 to be Aus.$ 477 million. The economic models used to make this estimate included the evaluation of product spoilage effects, human health effects with respect to disability and premature death due to aflatoxin-related primary liver cancer and livestock health effects due to reduced feed efficiency and increased mortality. The estimate, however, did not include the costs associated with immune suppression and growth stunting (see Chapters 5 and 6) or those from the loss of export markets for the contaminated commodities.

The effects of regulating mycotoxin levels on trade in agricultural products have been investigated by the World Bank (Otsuki *et al.*, 2001*a*,*b*). The implementation of a new European Union (EU) aflatoxin standard which is lower (4 ng/g aflatoxin B_1) than the internationally accepted *Codex Alimentarius* standard would reduce health risks by 2.3 deaths per billion people per year, but with a reduction of 64% in the export of cereals and peanuts from Africa to Europe at a cost of US$ 670 million (Otsuki *et al.*, 2001*b*). In a subsequent World Bank study by Jaffee and Henson (2004) these findings were challenged because the estimated "cost" of US$ 670 million had been misinterpreted as actual losses of trade rather than an estimate from an econometric simulation. Jaffee and Henson (2004) concluded that

EU imports from Africa would increase due to the more stringent aflatoxin standards, whereas some competing countries, *e.g.* Turkey, incurred more rejections. Similarly, Wu (2004*a*) stated that the developing countries most likely to experience large losses from the tighter mycotoxin standards are not sub-Saharan African nations, but China and Argentina. Among developed countries the United States would experience the heaviest economic losses. The three largest maize exporting countries are the United States, China and Argentina. Wu (2004*a*) calculated that if the current FDA guideline of 2 µg/g fumonisin were adopted internationally, then the export losses to each of these three countries would range from US$ 20-40 million annually with a total loss amongst the three countries of US$ 100 million. A fumonisin standard of 0.5 µg/g would increase the maize export losses to the United States to US$ 170 million, to China to US$ 60 million, and to Argentina to US$ 70 million, for a total of US$ 300 million.

The potential annual cost of contamination of food and feed crops in the United States with three mycotoxins (aflatoxin, fumonisin and deoxynivalenol) is estimated to range from US$ 418 million to US$ 1.66 billion, with a mean estimated cost of US$ 946 million (CAST, 2003). In addition, the costs of mycotoxin management, including research and monitoring, are estimated at between US$ 500 million and US$ 1.5 billion (Robens and Cardwell, 2003).

Risk Assessment and Regulation of Mycotoxins

Conventional risk assessment of mycotoxins has two major components, *i.e.*, exposure assessment and hazard assessment (Gelderblom *et al.*, 1996; Marasas, 1997). Exposure is calculated from food intake and naturally occurring levels of a mycotoxin and expressed as the Probable Daily Intake (PDI). Hazard is calculated from toxicological studies in experimental animals and is expressed as the Tolerable Daily Intake (TDI). The PDI and TDI data are used to assess the risk of a mycotoxin and establish MTLs.

Risk assessment of fumonisins to human health has been performed (IARC, 2002; WHO, 2002) and MTLs proposed ranging from 100-200 ng/g (Gelderblom *et al.*, 1996, Marasas, 1997), to 2-4 µg/g (FDA, 2001). It remains to be seen if and when the fumonisin levels proposed in the FDA Guidance for Industry (FDA, 2001) will be implemented. Viljoen and Marasas (2003) supported the fumonisin levels proposed by the FDA and pointed out that lower MTLs could seriously limit the food supply and affect the entire grain chain from producer to consumer.

Conventional risk assessment and MTLs do not apply to subsistence farmers in Africa who consume the largest amounts of maize containing the highest levels of fumonisins due to the interaction between maize intake and fumonisin contamination (Table 1). The intake profiles for the best quality maize with the lowest fumonisin contamination levels provide PDI values well below the PMTDI of 2.0 µg/kg bw/day proposed by the JECFA (WHO, 2002). This scenario reflects the typical situation in developed countries where maize consumption is low and mycotoxin contamination of foodstuffs is strictly regulated. In contrast, a completely different situation prevails in developing countries, particularly in rural and subsistence farming communities, where home-grown maize is the major dietary staple. High fumonisin contamination levels together with high maize consumption patterns (400-500 g/person/day) result in PDI values well above (10-50 fold higher) the PMTDI. The risk of developing disease due to fumonisin intake is further increased when consider-

ing the maize consumption patterns in children. Detailed maize intake profiles in different population groups, particularly in Southern and Eastern Africa where maize is the staple diet, are required to accurately assess the risk of fumonisins to human health. The implementation of MTLs based on conventional risk assessment in developed countries to protect the health of the lowest maize consumers may make the situation worse as food security problems will lead the highest consumers in the producing countries to consume the contaminated maize rejected by the importing countries. A similar rationale applies to MTLs for aflatoxin in peanuts.

People in rural areas of developing countries, who are at the highest risk from mycotoxins in staple foods, particularly subsistence farmers, are completely unprotected by mycotoxin regulations. Moreover, in developing countries in Africa and elsewhere, food safety is an issue that frequently must be balanced against issues of food security (Shephard, 2003). Given the choice between starvation and consuming foods containing mycotoxins at levels higher than the prescribed MTLs, most people in developing countries would probably eat the foodstuffs that would be rejected by developed countries. Thus, people who are at the highest risk, also have the most urgent need for solutions other than regulation for the mycotoxin problem.

Possible Solutions

The ultimate solution to the global mycotoxin problem is not regulation, but reduction of fungal infection and mycotoxin levels in crop plants (Marasas and Nelson, 1987; WHO, 2000). Attempts to achieve this goal by conventional plant breeding have not been very successful for various reasons including the lack of major single genes and difficulties in selecting appropriate germplasm due to time-consuming and expensive mycotoxin analyses (Gressel *et al.*, 2004; Munkvold, 2003). Although several sources of resistance to *A. flavus* infection and/or aflatoxin production in maize have been identified, the levels of genetic resistance are not sufficient to prevent the development of unacceptable aflatoxin levels. The same problem also occurs with the polygenic sources of resistance to *F. verticillioides* and fumonisin levels in maize (Munkvold, 2003). Molecular markers are being used increasingly to facilitate selection and to combine resistance genes from different sources in order to develop varieties with high yields and low mycotoxin levels, but potentially commercial lines have yet to be identified.

The most promising approach for innovative solutions is biotechnology. Genetic engineering approaches are the most attractive methods now under development, and the future of fumonisin reduction may lie in the hands of biotechnologists. The potential of transgenic resistance to mycotoxigenic fungi and/or their mycotoxins as biotechnology solutions for the global mycotoxin problem is receiving intensive international attention. The following strategies that might be used to reduce fumonisins in maize were reviewed by Duvick (2001).

Reducing infection by the mycotoxigenic fungus

Several antifungal compounds in plants are potential candidates for genetic engineering to alter maize genotypes for resistance to mycotoxigenic fungi.

Inserting genes capable of degrading the mycotoxin

Progress has been made with this strategy of *in planta* detoxification of fumonisins in maize. Duvick (2001) reported that two species of saprophytic fungi (*Exophiala spinifera* and *Rhinocladiella atrovirens*) from moldy maize ears can utilize FB_1 as their sole carbon source. These fungi produce enzymes capable of hydrolyzing and further metabolizing fumonisins by oxidative deamination. The genes coding the specific enzymes that carry out the detoxification steps have been cloned and the effects of the expression of these genes in transgenic maize on fumonisin levels are currently being evaluated in the United States (Duvick, 2001).

Interfering with mycotoxin biosynthesis

An α-amylase inhibitor has been identified in the legume *Lablab purpureus* that inhibits aflatoxin biosynthesis (Munkvold, 2003). This gene is a candidate for expression in genetically modified crops to reduce aflatoxin contamination. Genes that regulate fumonisin production by *F. verticillioides* have been identified (Proctor *et al.*, 2003; Brown *et al.*, 2005) and this information has the potential to be used in transgenic maize to disrupt fumonisin biosynthesis.

Inserting genes for insect resistance

There is a close association between insects, *e.g.*, the European corn borer (*Ostrinia nubilalis*), and the infection of maize by *F. verticillioides*, so transgenic maize hybrids carrying genes encoding insecticidal proteins from *Bacillus thuringiensis* (*Bt*) are a potential solution to the fumonisin problem. *Bt* hybrids, which are resistant to the European corn borer and have correspondingly less *F. verticillioides* ear rot, had significantly lower fumonisin levels than did conventional hybrids grown in Iowa, USA (Munkvold *et al.*, 1999). Similar results were subsequently reported for *Bt* hybrids elsewhere in the world where fumonisin contamination of maize is associated with insect damage such as France, Italy and Spain (Munkvold, 2003). *Bt* hybrids represented approximately 25% of the field maize planted in the United States and the estimated annual saving to farmers due to reduced mycotoxin (fumonisin and deoxynivalenol) levels alone, is US$ 17 million (Wu *et al.*, 2004). Whether currently available *Bt* hybrids also contain significantly less fumonisin than non-*Bt* hybrids in countries where other species of corn borers predominate, *e.g.*, *Busseola fusca* in West Africa (Cardwell *et al.*, 2000) and South Africa (Flett and van Rensburg, 1992), remains unknown. In South Africa, significant reductions in fumonisin levels have been found in some *Bt* cultivars in some seasons in some locations (Vismer *et al.*, 2005). If additional *Bt* genes are deployed to control a broader range of maize insects, then the reduction in fumonisin levels may be improved further.

Conclusions

The EU continues to make mycotoxin standards more stringent by lowering the MTL in imported agricultural products on the one hand, while prohibiting the importation of GM crops on the other. This impasse is a serious obstacle to the implementation of biotechnolo-

gy solutions to the mycotoxin problems of exporting countries. The reasons why decision makers at both the government and individual consumer levels have not endorsed *Bt* maize and other GM crops are complex (Wu, 2004*b*). The potential benefits of *Bt* maize, *e.g.*, increased yield, decreased use of pesticides and reduced mycotoxin levels, should be emphasized in educational programs to improve public understanding of biotechnology by providing accurate and balanced information.

During the Workshop on Mycotoxins in Food in Africa (Cardwell, 1996; Cardwell and Miller, 1996) held in Cotonou, Benin, 6-10 November 1995 it was resolved by an international, multidisciplinary team of scientists that:

Recognizing the increasing importance of food grains in sub-Saharan Africa;

Realizing that heavy losses are caused by mycotoxins at all levels of production, storage, processing, and utilization; and being

Concerned that mycotoxins are having a direct negative impact on human and animal health, and on trade; and being

Aware that African governments are fully committed to the promotion of food security and safety, and to the improvement of public health and quality of life for their citizenry

Be it Resolved that The Pan African Mycotoxins Initiative Committee:

Does Hereby Reiterate that appropriate measures must be taken to reduce the grain losses caused by mycotoxins to internationally accepted standards, and to increase production of good quality grains; and

Advocates that resources be mobilized by African governments and the international community for support to mycotoxin research and intervention initiatives.

We strongly recommended that not only governments of African countries, but that governments of all developed and developing countries support the search for, as well as the development and implementation of, solutions for the global mycotoxin problem.

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The implications of naturally occurring levels of fumonisins in corn for human and animal health

Pieter G. Thiel, Walter F.O. Marasas, Eric W. Sydenham, Gordon S. Shephard & Wentzel C.A. Gelderblom
Research Institute for Nutritional Diseases, South African Medical Research Council, P.O. Box 19070, Tygerberg 7505, South Africa

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Abstract

Contamination of corn with the fungus *Fusarium moniliforme* and its secondary metabolites, the fumonisins, has been associated with several human and animal diseases. This paper summarizes present knowledge and presents new data on the levels of fumonisins present in foods and feeds associated with these diseases as well as in commercial corn and corn-based products. The doses of fumonisins to which humans and animals consuming these products would be exposed are compared with those doses known to produce LEM in horses and hepatocarcinogenesis in rats. It is concluded that the known naturally occurring levels of fumonisins present a potential threat to human and animal health and realistic tolerance levels need to be set.

Introduction

Contamination of cereals with the fungus *Fusarium moniliforme* Sheldon, a common contaminant of corn throughout the world, has been associated with several human and animal diseases [1]. Culture material of strains of *F. moniliforme* has long been known to produce the neurotoxic disease, leukoencephalomalacia (LEM) in horses under experimental conditions [2–4] and to produce toxic pulmonary effects in swine [2]. Cultures of *F. moniliforme* were furthermore hepatotoxic to horses, swine and rats [2, 5] and produced two types of cancers in the livers of rats after long-term exposure [6, 7].

The recent observation that cultures of *F. moniliforme* exhibit cancer promoting activity in rat liver in a short-term bioassay [8] resulted in the isolation [9] and characterization [10] of the fumonisin B (FB) mycotoxins. Four of the FB toxins have thus far been described; namely, FB_1 and FB_2 [9] as well as FB_3 and FB_4 [11]. Only FB_1 and FB_2 appear to be of any toxicological significance as FB_3 and FB_4 as well as the two fumonisin A toxins, FA_1 and FA_2, occur at extremely low concentrations under natural conditions [10]. FA_1 and FA_2 also exhibited no cancer initiating activity when tested at concentrations where FB_1 and FB_2 were active [11].

Pure FB_1 has since been shown to cause LEM in horses [12, 13], pulmonary edema in swine [14] and to be hepatotoxic and hepatocarcinogenic in

rats [15]. Future risk assessment studies on exposure of animals to the fumonisins should consider FB_2, in addition to FB_1, as FB_2 most probably exhibits toxic and carcinogenic properties similar to FB_1 [11] and occurs at significant levels in cereals under natural conditions [16]. FB_1 and FB_2 also pose a potential risk to humans as they occur in human food and have been shown to be statistically correlated with the prevalence of human esophageal cancer (EC) in Transkei, a high incidence area for EC in southern Africa [16].

This paper reports new data on the natural occurrence of FB_1 and FB_2 in corn, foods and feeds. The doses of fumonisins used in experimental animal studies to precipitate various lesions are also compared with the doses of fumonisins to which humans and animals will be exposed if they consume corn-based products contaminated with fumonisins at the naturally occurring levels reported.

Analytical methodology

In order to assess human and animal exposure to the fumonisins by the ingestion of foods and feeds contaminated with *F. moniliforme*, it is necessary to develop suitable analytical procedures whereby naturally occurring levels can be quantified accurately and reproducibly. The fumonisins do not possess any chromophores and therefore do not absorb UV or visible light, nor do they fluoresce. Published methods make use of derivatization to yield either UV-absorbing or fluorescent derivatives. The first quantitative HPLC determination of the fumonisins involved UV-detection at 230 nm of their maleyl derivatives [14]. Although this technique has been used successfully for the analysis of fungal cultures [17], more sensitive techniques were required for the determination of the fumonisins in naturally contaminated corn samples. The HPLC technique of Shephard et al. [18], which involves fluorescence detection of the o-phthaldialdehyde (OPA) derivatives, fulfilled all the criteria for a suitable technique to quantify naturally occurring levels of fumonisins; i.e., the technique is sensitive, accurate, reproducible and detects and quantifies both FB_1 and FB_2. It is presently being studied collaboratively by 10 laboratories in 7 countries as a project of the Commission on Food Chemistry of the International Union of Pure and Applied Chemistry. The results reported in this paper were obtained using the OPA-derivatization procedure of Shephard et al. [18].

Several other techniques have been proposed for the determination of the fumonisins but each lacked certain basic elements to make it suitable for monitoring natural occurrence. The HPLC procedure of Ware [19] employs fluorescence detection of the naphthalene-2, 3-dicarboxaldehyde (NDA) derivatives. The procedure is sensitive and the derivative is stable, in contrast to the OPA-derivatization procedure, but it only measures FB_1 while there is a necessity to determine FB_2 also. The GC/MS procedure of Plattner et al. [20] has the advantage of spectrometric confirmation of the observations, but lacks the necessary sensitivity and requires sophisticated, expensive equipment which is not generally available. This method should, however, be suitable for quantifying the high levels of fumonisins occurring in cultures of fumonisin-producing fungi and in feed samples from outbreaks of LEM. Other reagents such as fluorescamine have been used for pre-column derivatization before HPLC determination with fluorescence detection. However, fluorescamine derivatization yields two products for each fumonisin [21], a situation which is considered to be undesirable for quantification purposes.

Fumonisin levels in LEM feeds

The levels of fumonisins detected by Shephard et al. [18] in the feed associated with a field outbreak of LEM in South Africa, by Thiel et al. [22] in feeds associated with confirmed cases of LEM in

Table 1. Fumonisin levels in feeds associated with outbreaks of equine leukoencephalomacia[a]

Source	No. of samples	Fumonisins (ng/g)	
		FB_1	FB_2
South Africa [18]	1	(8850)	(3000)
USA [22]	14	1300–27000 (7700)	100–12800 (3100)
USA [23]	3	37000 122000 (72000)	2000–23000 (12000)
Brazil	21	0–38500 (8900)	0–11800 (2850)

[a]Values in parentheses = means

the USA and by Wilson et al. [23] in feeds associated with an epizootic of LEM in Arizona, USA are in good agreement (Table 1). Wilson et al. [23] used information on diet, animal weights and feeding practices to estimate the FB_1 dosage rates involved in the LEM cases. These values will be referred to again later (Table 5). In addition to the feed samples from confirmed cases of LEM, very high levels of both FB_1 and FB_2 were also recorded in 21 feed samples from Brazil associated with cases of LEM and other suspected mycotoxicoses (Table 1). The mean levels of the fumonisins in the Brazilian samples were similar to the levels recorded in the South African case of LEM [18] and the LEM cases from the USA reported by Thiel et al. [22]; namely, approximately 8,000 ng/g FB_1 and 3,000 ng/g FB_2. Wilson et al. [23], however, recorded even higher levels of both toxins ranging from 37,000 to 122,000 ng/g FB_1 and from 2,000 to 11,800 ng/g FB_2 (Table 1).

Fumonisin levels in a high human esophageal cancer area

F. moniliforme infection of corn has been correlated with human esophageal cancer risk in Transkei, where corn is the dietary staple [24, 25] and in China [26, 27]. Sydenham et al. [16] have since detected statistically significantly higher levels of both FB_1 and FB_2 in corn samples of the 1985 crop from the high esophageal cancer rate area than in corresponding samples from the low rate area. This observation has now been confirmed on samples of the 1989 crop and is compared with the 1985 values in Table 2 for 'healthy corn' samples and in Table 3 for 'moldy corn' samples, from the two areas. The levels of fumonisins detected in these samples are higher than those normally found in human foods (See next Section) while the levels of both FB_1 and FB_2 in the 'moldy corn' samples were alarmingly high, especially in the samples from the high esophageal cancer rate area. These samples in fact contained levels of fumonisins which were higher than levels known to have precipitated LEM in horses (Table 1).

Table 2. Fumonisin levels in 'healthy' corn from opposing esophageal cancer (EC) areas in Transkei[a]

Area	Year	No. of samples	Fumonisins (ng/g)	
			FB_1	FB_2
Low EC	1985	12	0–550 (63)	0–150 (<50)
Low EC	1989	8	0–3310 (500)	0–970 (130)
High EC	1985	12	50–7900 (1600)	0–2250 (500)
High EC	1989	6	0–5380 (1530)	0–1320 (420)

[a]Values in parentheses = means

Table 3. Fumonisin levels in 'moldy' corn from opposing esophageal cancer (EC) areas in Transkei[a]

Area	Year	No. of samples	Fumonisins (ng/g)	
			FB_1	FB_2
Low EC	1985	12	460–18900 (6520)	150–6750 (2500)
Low EC	1989	7	110–11340 (4050)	0–3700 (1090)
High EC	1985	12	3450–46900 (23900)	900–16300 (7550)
High EC	1989	6	3020–117520 (53740)	750–22960 (13680)

[a]Values in parentheses = means

Table 4. Fumonisin levels in commercial corn products for human consumption[a]

Source	No. of samples	Fumonisins (ng/g)	
		FB_1	FB_2
South Africa	68	0–475 (105)	0–120 (21)
Egypt	2	1780–2980 (2380)	410–780 (595)
USA	29	0–2790 (711)	0–1070 (190)
Peru	4	0–660 (165)	0–135 (34)

[a]Values in parentheses = means.

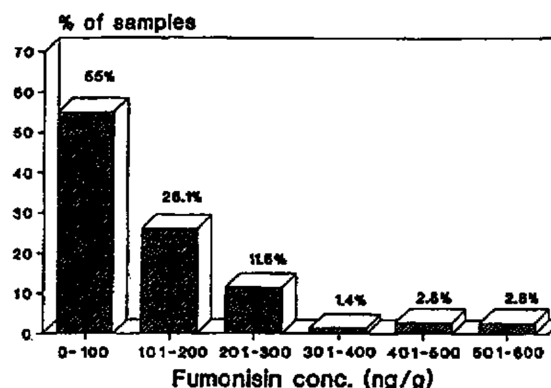

Fig. 1. The distribution of fumonisin concentrations ($FB_1 + FB_2$) in commercial corn products for human consumption in South Africa (68 samples).

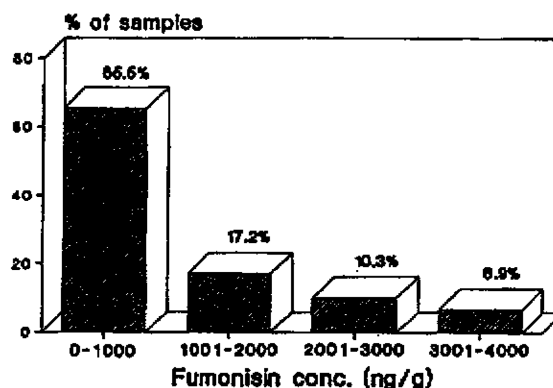

Fig. 2. The distribution of fumonisin concentrations ($FB_1 + FB_2$) in commercial corn products for human consumption in the USA (29 samples).

The potential implications of the consumption of commodities containing these levels of fumonisins to human health risks are discussed in a later section. It must be stressed that the corn samples from the high and low cancer rate areas in the Transkei are not commercial corn samples but home-grown corn produced, harvested, stored and consumed by the people living in this rural area.

Fumonisin levels in commercially available foods

Samples of commercially available corn or corn-based products were obtained from retail stores in the USA, South Africa, Egypt and Peru and analyzed for their fumonisin content (Table 4). The levels detected in samples from South Africa and Peru were relatively low (total fumonisins <1,000 ng/g) while a large portion of the samples from the USA and Egypt contained more than 1,000 ng/g total fumonisins ($FB_1 + FB_2$). The distribution of the total fumonisin content of the South African samples is graphically illustrated in Fig. 1, and of the samples from the USA in Fig. 2. None of the South African samples contained more than 600 ng/g total fumonisins while 55% of these contained less than 100 ng/g total fumonisins (Fig. 1). In contrast to this, 35.5% of the samples from the USA contained more than 1,000 ng/g total fumonisins and 17.2% contained in excess of 2,000 ng/g (Fig. 2). It must be stressed that it is not the intention to imply that the figures quoted reflect the general situation with respect to the fumonisin concentration of commercially available products in the different countries, especially since the number of samples analyzed is very low. The figures do, however, serve as an indication of potential exposure of humans to fumonisins in the respective countries and as such should be treated as important. The toxicological significance of exposure to the levels of fumonisins present in commercial products is discussed later.

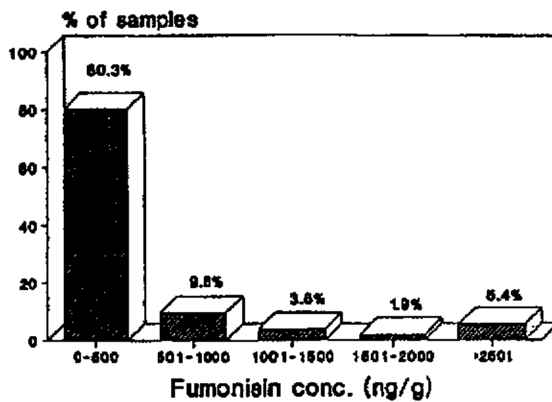

Fig. 3. The distribution of fumonisin concentrations (FB_1 +FB_2) in samples of the 1989 corn crop, representative of all the corn production areas in South Africa.

Table 5. Fumonisin doses in relation to pathological effects

Exposure	References	Fumonisin concentration (ng/g)	Fumonisin intake (mg/kg/day)
Natural outbreak of LEM (USA)	[23]	72,000	0.6–2.1
Experimental LEM in horses	[12]	–	1.25–4.0
Experimental carcino-genesis[a] in rats	[15]	50,000	3.75
Person eating 'healthy'[b] Transkeian corn	–	2.100	0.014
Person eating 'moldy'[b] Transkeian corn	–	67,420	0.44

[a]Based on a 200 g rat eating 15 g feed per day.
[b]Based on 70-kg person eating 460 g corn per day.

Fumonisin levels in South African corn

Representative samples of the 1989 South African corn crop were drawn from the major corn producing areas and from the three different grades into which commercial corn is graded in South Africa. The distribution of the fumonisin content of these samples is graphically illustrated in Fig. 3. A significant proportion of these samples (89.9%) contained less than 1,000 ng/g total fumonisins. Data such as those summarized in Fig. 3 will in the future be invaluable in establishing which fraction of a particular harvest presents a health hazard and in setting realistic standards with respect to both economic and health criteria.

Fumonisin dosage rates in experimental animal studies

Marasas et al. [13] succeeded in reproducing LEM in a horse by the intravenous (i.v.) injection of 7 daily doses of 0.125 mg of FB_1 per g live mass spread over 10 days. Harrison et al. [14] similarly induced pulmonary edema in a pig by i.v. injection of 0.4 mg FB_1 per g body weight daily for 4 days. Apart from demonstrating the ability of FB_1 to induce LEM in a horse and pulmonary edema in a pig, these dosage rates could give no indication of the dietary levels of fumonisins which would potentially induce the disease when the toxin is ingested *per os* (p.o.). Kellerman et al. [12] succeeded in inducing LEM in two horses by oral dosing of FB_1. The daily doses varied between 1.25 and 4 mg FB_1 per g body weight (Table 5). Both animals developed typical clinical symptoms of LEM in approximately 25 days.

Gelderblom et al. [15] demonstrated the hepatocarcinogenic activity of FB_1 to rats by incorporating 50,000 ng/g FB_1 in their diet over a period of 26 months. They concluded that FB_1 can be responsible for the hepatocarcinogenic and the hepatotoxic effects of culture material of *F. moniliforme* MRC 826 in rats. Assuming a daily feed intake of 15 g by a 200 g rat, the daily dose of FB_1 was approximately 3.75 mg/g body weight (Table 5).

Health risks of exposure to naturally occurring fumonisins

The estimated daily intakes of fumonisins which led to natural and experimental LEM in horses

and the daily dose of FB_1 which caused hepatocarcinogenesis in rats are compared in Table 5 with the potential intakes of fumonisins by persons consuming corn containing the levels occurring in 'healthy' or 'moldy' corn in the Transkei (Tables 2 and 3). The estimated daily intakes of 0.014 and 0.44 mg/g/day for 'healthy' and 'moldy' corn, respectively, are based on an intake of 460 g corn by a 70 g person daily [28]. It is evident that the daily intakes of fumonisins by individuals consuming especially the 'moldy' Transkeian corn are alarmingly high when compared to those daily intakes which gave rise to LEM in horses (0.6 to 4.0 mg/g/day) or hepatocarcinogenesis in rats (3.75 mg/g/day). Exact risk assessment can, however, not yet be done as the lowest levels of fumonisins which can precipitate lesions in animals have not yet been determined. It is entirely possible that much lower doses than those reported in Table 5 may induce LEM in horses or liver cancer in rats. In the cancer experiment in rats, approximately 66% of the animals developed hepatocellular carcinomas while a cancer rate as low as 0.02% (20 per 100,000) can be considered to be a significant risk to a human population.

Conclusion

All these facts point towards a real health risk to both animals and humans exposed to the known levels of naturally occurring fumonisins. In order to set realistic tolerance levels, from both a health and economic perspective, more carefully planned experimental animal studies as well as epidemiological data from areas where animals and humans are exposed to fumonisins, will be needed.

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CHAPTER VI

SUMMARY AND CONCLUSIONS

The involvement of *Fusaruim verticillioides* in several animal diseases and its association with maize, a major dietary staple in certain population groups, especially in developing countries, suggests that the fungus could adversely affect human health. The discovery that maize cultures of the fungus are hepatotoxic and hepatocarcinogenic in male BD IX and Fischer 344 rats stimulated research efforts in characterising the causative principles. It was further strengthened by the fact that maize, either artificially cultured or naturally infected by the fungus, induces leukoencephalomalacia in horses. This led to the notion that a single compound could be responsible for the diverse toxic syndromes. The characterization of the highly mutagenic compound, fusarin C, from maize culture material, however, failed to explain the carcinogenic properties of the fungus, presumably due to its heat liability and the rapid rate of inactivation by liver drug metabolizing enzymes. An alternative approach, utilising the cancer promoting properties of the fungal culture material in a short-term cancer initiation/promotion rat liver model, resulted in the characterization of the fumonisin B mycotoxins. Subsequent studies using purified fumonisin B_1, the major compound produced in maize, provided evidence that it is the causative principle responsible for the major mycotoxicological effects in experimental and farm animals. Once chemically characterised and sensitive analytical methodology developed, it was shown to occur naturally in maize worldwide. It is known to be produced mainly by *F. verticillioides* and *F. proliferatum* on maize, although other fungal spp including, *F. nygami* and recently also *Aspergillus niger* produce the mycotoxin in culture.

With respect to human health the main focus was on the toxicological effects in rats and mice, conducted by the US National Toxicology Program (NTP, 2001), the outcome of which played an important role in setting risk assessment parameters for exposure of the fumonisins to humans (WHO, 2001). Based on an IARC evaluation the fumonisins were characterized as a Group 2B carcinogen, i.e. a possible carcinogen to humans (IARC, 2001). However, several controversial findings regarding the toxicological effects of the culture material of the fungus and pure FB_1 in rats have been reported that should be clarified prior to assessing the risk in humans. Differences have been reported in the extent of liver damage induced by culture material of *F. verticillioides* strain MRC 826 and FB_1 in male BD IX rats over a period of two years which depends on the FB content of the diet as well as the type of diet used (Gelderblom *et al*., 2004). In all of these studies, the kidney was not a

prominent target organ in this rat species, although it was consistently affected, especially the epithelial cells in the proximal convoluted tubules. However, these lesions never progressed into renal adenoma and/or carcinoma as reported by the long-term study in male Fischer rats when using pure FB_1 up to a level of 50 mg/kg diet and higher (Howard *et al*., 2001a). The NTP study provides some novel findings in that, for the first time, kidney tumours were induced in male Fischer rats without adversely affecting the liver, while opposite findings were reported in BD IX rats. Whether species differences would explain the differences in the outcome of these two carcinogenicity studies still needs to be explored as, irrespective of the rat species and the method of purification used, FB_1 induced very similar toxic lesions in the kidney at similar dosage levels. However, with respect to the liver major differences seem to exist in the dietary levels and rat species used. The main point for debate is the lack of hepatotoxicity observed in male Fisher rats at a dietary level of 5.7 mg FB_1/kg bw/day for 90 days (Voss *et al*., 1995) and even at a mean intake of 6.6 mg FB_1/kg bw/day over a period of 2 years (Howard *et al*., 2001a). These findings are in contrast with the studies of Gelderblom *et al*. (1996; 2001) where mild toxic effects were induced in the liver of male Fisher rats at an intake as low as 3.5 mg FB_1/kg bw/day over a period of 21 days.

Different aspects that could alter FB_1-induced toxicity and carcinogenicity may impact on setting risk assessment parameters of the fumonisins:

(i) One modifying factor that could possibly explain the differences in the outcome of the various experiments is the diets used implying specific FB_1 dietary interactions (Gelderblom *et al*, 2004). In male BD IX rats a marginally deficient diet was used in the long-term studies with the culture of MRC 826 as well as pure fumonisins (Jaskiewicz *et al*., 1987; Gelderblom *et al*., 1991). The open formula NIH-31 cereal based diet, developed according to a similar formulation as the NIH-07 diet, and was used in both the 90 day and long-term study of FB_1 in male Fischer rats (Voss *et al*., 1995; Howard *et al*., 2001a). The AIN 76 diet was used in the short-term rat liver cancer initiating/promoting studies of FB_1 in male Fischer rats (Gelderblom *et al*, 1994, 1996). The underlying differences between the diets have been summarized (Gelderblom et al., 2004) with the high protein levels in the NIH derived diets likely to sensitise the kidneys to FB_1-induced toxic and carcinogenic effects. In this regard the induction of specific atypical tubule proliferative lesions in the kidneys due to the development of chronic progressive nephropathy in older rats is likely to explain the FB_1-induced nephrocarcinogenc effects. Several other dietary factors, such as plant extracts (antioxidants) and

dietary Fe could either stimulate or inhibit cancer induction of FB_1, which complicates the comparison of toxicological effects of FB_1 between different studies and animal species. Cognisance should be taken of the modulating role of dietary constituents as it will determine the outcome of toxicological assays and therefore determine the threshold of an adverse effect in a specific target organ to be used in determining risk assessment parameters.

(ii) Another determining aspect of fumonisin toxicology that will impact on risk assessment is the underlying mechanism for cancer induction in the liver and kidneys of rats. It is generally accepted that the disruption of lipid metabolism could explain the toxicological effects in animals. This includes the disruption of sphingolipid, phospholipids and fatty acid (FA) metabolism, which play a major role in the modulation of apoptotic and cell proliferative pathways related to cancer development. Depending on the cell type the disruption of these pathways could, via interactive mechanisms, either stimulate or inhibit cell proliferation, which eventually will determine the induction of apoptosis and hence affect cell survival. In general it would appear that the disruption of lipid metabolism results in the induction of apoptosis in normal cells whilst pre-neoplastic cells are resistant to these effects and therefore will survive and proliferate resulting in cancer development. However, two different types of mechanistic approaches have been suggested to explain the genesis of cancer in the liver or kidney, which are based on the role of cell proliferation and the differential effects of the fumonisins. The one approach followed the probabilistic model developed by Cohen and Ellwein (1990) and Butterworth et al., (1992) amongst others whereby an increased rate of cell proliferation resulted in an increased risk of cells to undergo mutations (DNA damage), culminating in the formation of genetically altered cells that develop into cancer. This theoretical cell proliferative model was debated in the literature and questioned the exclusive role of cell proliferation as a major risk factor for cancer (Perera et al., 1991; Farber, 1995; 1996). The theoretical regenerative cell proliferative model was applied to describe cancer induction of the apparent non-genotoxic fumonisin B_1 in the kidneys (Howard et al., 2001b; Dragan et al., 2001). This model, which is in agreement to the hypothesis of Cohen and Ellwein (1990), accepts that the fumonisins are nongenotoxic carcinogens and stimulate cell proliferation in response to a chronic toxic effect in the kidney. The second model for cancer induction was developed in the liver and based on the resistant hepatocyte model of Solt et al. (1977) classifying a carcinogen as cancer initiators and promoters. During initiation, which is regarded as a mutation-like event, a rare cell is induced with altered

proliferative properties that develop into cancer. Based on this model, fumonisin B_1 exhibits both initiating and promoting properties although the kinetics of initiation differs when compared to the classical genotoxic carcinogens. Cancer promotion by FB_1 in the liver also follows the differential inhibition hypothesis whereby the proliferation of normal cells is inhibited while the altered cells proliferate due to the acquired resistance. The underlying mechanisms of the altered proliferative capacity of preneoplastic cells involve an interactive response between C20:4n6 and ceramide affecting downstream cell survival growth regulators.

(iii) As a NOEL for toxicity and carcinogenicity for the fumonisins exist in the kidneys of rats, the aspect of thresholds for genotoxic and apparently non-genotoxic carcinogens has to be debated. Depending on the level and duration of exposure in rat liver hyperplastic foci are induced at low dietary levels over a period of two years even as low as 0.03 mg/FB_1/kg bw/day. The induction of these lesions is not related to the promotion of spontaneous initiated cells known to occur in older animals. It would appear that FB_1 initiated cancer in the liver despite the fact that it is generally regarded as a non-genotoxin. *In vitro* studies showed that FB_1 induced clastogenic effects which suggest that it exhibits some genotoxic effects which could raise some doubts about the existence of a perfect threshold effect as proposed for non-genotoxic carcinogens. However, recent developments suggest that thresholds should also apply for genotoxic carcinogens when considering the induction of an adverse biological effect. In this regard interaction with the DNA is just one event in the multi-step process of cancer development and therefore could not be taken as the basis for applying a no-effect threshold for genotoxins. It would appear that a carcinogen such as fumonisin, whether it is labeled genotoxic or non-genotoxic *per se*, exhibits some degree of risk at any level due to additive or synergistic interactions with other xenobiotics and/or dietary constituents.

(iii) Although the kidney is the most sensitive target in male Fischer rats the NOEL for cancer development in the liver (0.8 mg/kg bw/day) and kidneys (0.7 mg/kg bw/day) is similar and is effected at a dietary level of 50 mg FB_1/kg diet over a period of two years. The rat kidney carcinogenesis model was utilized in assessing the risk for fumonisins in humans, yielding a Provisional Maximum Tolerable Daily intake (PMTDI) of 2 ug FB/kg bw/day using a safety factor of 100. The selection of the NOEL was based on kidney toxicity (0.2 mg/kg bw/day) rather than carcinogenicity with the notion that it is a prerequisite for

nephrocarcinogenicity based on the theoretical compensatory proliferative model (Dragan et al., 2001; Howard, 2001b). The validity of such a decision is debatable as the NOEL for nephrotoxicity was far below the NOEL for carcinogenicity. The uncertainty regarding the nephro-carcinogenicity due to a possible FB_1/nephropathy interaction, raise some doubts about using the outcome of the NTP study in establishing risk assessment parameters in humans. This becomes apparent as a variety of hyperplastic lesions occur in the kidneys as a result of nephropathy, which, in the presence of FB_1, could play a role in cancer development, especially the occurrence of a subtype referred to as atypical tubule hyperplasia (Hard et al 2004). It is therefore of interest, when comparing the situation in the liver, neoplasia also developed from hyperplastic liver foci and nodules, however, with the difference that these lesions are induced by FB_1 and not by any dietary related response. The hepatocarcinogenicity of the fumonisins therefore, provides a far better established model to be used in risk assessment for the fumonisins as was indicated by the majority of long-term and short-term studies using either fungal cultures or pure fumonisins in different animals species conducted in different locations as discussed previously (Chapter III).

When using the NOEL for hepatocarcinogenicity (0.8 mg/kg bw/day) and a higher safety factor of 1000 for non-genotoxins (Kuiper Goodman, 1990; Gelderblom *et al.*, 1996b) a TDI of 0.8 μg/kg/day was obtained which is approximately 3 times lower than the PMTDI proposed by JECFA. This is also in agreement with the upper tolerable intake of 1 μg/kg bw/day recommended by the Nordic Working group (Peterson and Thorup, 2001). When considering the maize intake vs FB contamination levels (Chapter V) the PDI levels could be easily obtained in developed countries where maize consumption levels above 50 to 100 gm/person/day are rare while FB contamination of maize is unlikely to reach levels of 0.2 to 0.5 ppm (Soriano and Dragacci, 2004a,b). This in agreement with fumonisin tolerance levels on maize proposed by Marasas (1997) of between 0.1 to 0.2 mg/kg. However, as fumonisin exposure is directly related to the level of contamination and the amount of maize consumed, population groups in both developed and developing countries utilising maize as a main dietary staple are the most vulnerable. When considering specific population groups, exposure of children is of additional concern as it can adversely affect their growth and development. Regulation of the fumonisins in food and the associated risk has to be considered from many perspectives. In developed countries maize is not a major dietary staple and is mainly used to generate income for a country by exporting the bulk to other countries including developing countries. In many of these developing countries there is a lack of quality

control implying that maize highly contaminated with mycotoxins may directly enter the food chain of adults and children. Many of these countries are politically unstable and due to poor socioeconomic status and underdeveloped agricultural practices control of mycotoxins is difficult or in some cases totally absent. The interaction of politics, economy and technology will eventually determine the impact on health which will differ between countries. Specific and simple measures should therefore be devised and introduced to reduce the levels of the fumonisin in maize by targeting populations at risk.

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ADDENDUM

Abel S, Smuts CM, and Gelderblom WCA (2001) Changes in essential fatty acid patterns associated with normal liver regeneration and the progression of hepatocyte nodules in rat hepatocarcinogenesis. Carcinogenesis **22**: 795-804.

Abel S, De Kock M, Smuts CM, de Villiers C, Swanevelder S, and W.C.A. Gelderblom (2004) Dietary Modulation of Fatty Acid Profiles and Oxidative Status of Rat Hepatocyte Nodules: Effect of Different ω6/ω3 Fatty Acid Ratios. Lipids **39**: 963-976

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Changes in essential fatty acid patterns associated with normal liver regeneration and the progression of hepatocyte nodules in rat hepatocarcinogenesis

S.Abel[3], C.M.Smuts[1], C.de Villiers[2] and W.C.A.Gelderblom

Programme on Mycotoxins and Experimental Carcinogenesis, [1]National Research Programme for Nutritional Intervention, [2]Experimental Biology Programme–Primate Unit, Medical Research Council (MRC), PO Box 19070, Tygerberg 7505, South Africa

[3]To whom correspondence should be addressed
Email: sabel@mrc.ac.za

Changes in lipid metabolism were monitored in rat hepatocyte nodules at certain time points over 9 months. Tissue obtained from partially hepatectomized rats, collected over a period of 7 days, were included as a control for normal hepatocyte cell proliferation. Two important features regarding the lipid profiles of hepatocyte nodules and normal regenerating liver were the increased concentrations of phosphatidylethanolamine (PE), resulting in a decreased phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratio, and cholesterol. These changes coincided with increased membrane fluidity in the nodules and regenerating liver. With respect to the fatty acid (FA) profiles of the nodules, C18:1ω9 and C18:2ω6 increased in PE and PC whereas C20:4ω6 decreased in PC and increased in PE. C22:5ω6 and C22:6ω3, the end products of the ω6 and ω3 metabolic pathways, respectively, decreased in PC and remained unchanged in PE. The FA levels in PC reflected an impaired δ-6 desaturase enzyme, whereas this effect was masked in PE due to the increased concentration of this phospholipid fraction. In regenerating liver, the FA profiles of PC and PE showed the same pattern as described for the hepatocyte nodules, except for C18:1ω9 which decreased in PC and increased non-significantly in PE. The increased C18:1ω9 level, a FA with anti-oxidative properties, as well as the decreased levels of the long-chain polyunsaturated fatty acids (C20 and C22 carbon chains), have been associated with the decreased lipid peroxidation level in hepatocyte nodules. The resultant decrease in peroxidative metabolites, known to affect apoptosis, could be important in the progression of the nodules into neoplasia. The present results indicate that the altered lipid parameters associated with hepatocyte nodules closely mimics cellular proliferation in regenerating liver and could be responsible for the enhanced proliferation and/or altered growth pattern in these lesions. The altered FA profiles suggest various pathways in which FA could play a role in transmembrane signalling related to the altered cell proliferative and apoptotic pathways. The persistent changes in the hepatocyte nodules suggest that the lipid metabolism escapes the regulatory mechanisms required for normal cellular homeostasis at different levels.

Abbreviations: Chol, cholesterol; CM, chloroform/methanol; DPH, 1,6-diphenyl-1,3,5-hexatrine; FA, fatty acid(s); FAME, fatty acid methyl esters; LC-PUFA, long chain polyunsaturated fatty acids; P/S, polyunsaturated to saturated fatty acid ratio; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT2, *N*-methyltransferase-2; PL, phospholipid(s); PUFA, polyunsaturated fatty acid(s).

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Introduction

The process of carcinogenesis is complex, resulting from alterations in the normal patterns of cellular growth (1). In the resistant hepatocyte model for liver carcinogenesis in the rat, a key event is the appearance of numerous altered or 'resistant' cells during initiation which, upon promotion, results in the formation of hepatocyte nodules with a characteristic altered phenotype (2). Although the majority of these hepatocyte nodules disappear or re-differentiate to normal appearing liver, a few 'persistent nodules' develop into malignant tumours (3). In the hepatocyte nodules, the balance between cell death and proliferation is disrupted resulting in a net increase in cell proliferation. This phenomenon changes, though, in the persistent nodules where cell death increases to counteract the increased cell proliferation, resulting in the retardation in growth of the persistent nodules. With the onset of cancer, this balance is again disturbed by an increased growth rate observed in the neoplastic tissue (4).

Studies have shown that the occurrence of potentially 'neoplastic' hepatocyte lesions is associated with changes in the polyunsaturated fatty acid (PUFA) profile, especially the long-chain PUFA (C20 and C22 carbon chains, LC-PUFA) and the lipid peroxidative status (5,6). The differences in LC-PUFA levels and extent of lipid peroxidation in pre-neoplastic lesions are possibly due to an abnormal essential FA metabolism involving Δ-6 desaturase (7–9). Changes in the FA profiles have a wide range of effects regarding the integrity of cellular membranes. These changes are known to affect the membrane structure and fluidity, the activity of membranal enzymes and the affinity of growth factor receptors. Furthermore fatty acids act as signalling molecules involved in cell proliferation and/or apoptosis (5,10).

Both LC-PUFA, which form the main substrates for lipid peroxidation, and membrane lipid peroxidation have been found to be lower in hepatocyte nodules than in surrounding 'normal' tissue (11,12). The level of lipid peroxidation has also been found to influence tumour growth (11) and, together with changes in the membrane lipid status, is likely to play an important role in the abnormal cellular growth which prevails in pre-neoplastic lesions. The integrity of the cellular membrane is therefore important in the normal functioning of the cell and its responses to external growth stimulatory and/ or inhibitory factors.

Studies in experimental animals have indicated that focal hepatocyte proliferations or hepatocyte nodules are the critical, relatively early lesions in the development of liver cancer in rats (2). The current study investigated the lipid profiles associated with the progression of hepatocyte nodules as well as in normal regenerating liver in order to delineate alterations

Table I. Comparative lipid parameters in the Resistant Hepatocyte model in control, nodule and surrounding tissue and in regenerating liver

Month		Resistant Hepatocyte Model			Regenerating liver	
		Control	Nodule	Surrounding		
Cholesterol (µg/100mg liver)						
1		15.80 ± 3.26[a]	21.77 ± 3.98[b]	17.60 ± 0.96	Control	15.43 ± 3.18[A]
3		15.00 ± 1.39	17.85 ± 1.34	16.88 ± 3.09	1 Day	15.08 ± 3.17[A]
6		22.15 ± 0.38	25.30 ± 2.36[a]	19.68 ± 3.74[b]	2 Days	24.52 ± 2.59[B]
9		22.42 ± 1.34[A]	37.06 ± 6.72[B]	26.11 ± 3.66[A]	7 Days	29.88 ± 4.96[B]
Phospholipids (µg Pi/100mg liver)						
1	PC	133.84 ± 23.29[A]	183.65 ± 23.28[B]	129.71 ± 11.68[A]	Control	167.45 ± 29.14[a]
	PE	29.63 ± 5.38[A]	58.57 ± 6.83[bB]	40.72 ± 1.97[cA]		37.07 ± 6.73[A]
3	PC	160.66 ± 16.73	161.26 ± 28.75	132.29 ± 20.22	1 Day	148.46 ± 14.45
	PE	38.21 ± 2.96[a]	50.07 ± 2.96[bB]	36.35 ± 5.92[aA]		81.09 ± 7.79[B]
6	PC	153.82 ± 45.71	162.13 ± 18.64	129.29 ± 12.15	2 Days	119.59 ± 31.91[b]
	PE	29.96 ± 2.03[aA]	72.82 ± 5.66[bB]	41.89 ± 10.77[cA]		65.93 ± 18.78[B]
9	PC	131.25 ± 14.02	138.38 ± 32.05	135.56 ± 19.17	7 Days	146.42 ± 18.90
	PE	39.30 ± 3.08[A]	62.63 ± 7.99[B]	45.59 ± 4.94[A]		63.93 ± 7.53[B]

Values are means ± SD. Significant differences are represented by uppercase ($P < 0.01$) and lowercase ($P < 0.05$) superscript letters. PC, phosphatidylcholine; PE, phosphatidylethanolamine.

in lipid metabolism with respect to cancer development in the liver of rats.

Materials and methods

Experimental animals

Male Fischer rats ($n = 144$) were fed the AIN 76A diet (13) *ad libitum* when weaned, and housed under controlled lighting (12 h cycles) and temperature (23–25°C) with free access to water. Upon reaching a body weight of 150 g, they were housed separately in wire-bottomed cages and weighed three times a week.

Resistant Hepatocyte Model

Hepatocyte nodules were induced according to the method described by Solt and Farber (14). Briefly, the rats (body weight approximately 150 g) were injected intraperitoneally (i.p.) with diethylnitrosamine (DEN, 200 mg/kg body weight) to effect cancer initiation. Promotion was effected 3 weeks later by a daily intragastric dose of 2-acetylaminofluorene (2-AAF, 20 mg/kg body weight) on three consecutive days followed by partial hepatectomy on the fourth day. The rats ($n = 60$) were terminated at intervals of 1 ($n = 15$), 3 ($n = 15$), 6 ($n = 15$) and 9 ($n = 15$) months after cancer promotion and the hepatocyte nodules and surrounding tissue were collected. Control tissue was collected at similar time intervals from rats ($n = 60$) that did not receive the initiating and promoting treatments. The tissue samples were immediately frozen on dry ice and stored at –80°C prior to analyses.

Regenerating liver

Rat liver samples ($n = 18$) were collected at intervals of 1, 2 and 7 days following partial hepatectomy in order to obtain tissue sections representing different stages of the regenerative response (15). Livers of untreated rats ($n = 6$) were also collected at the time of partial hepatectomy. All the samples were stored at –80°C until analysed.

Lipid analyses

Lipids were extracted from the control, nodule, surrounding and regenerating liver tissue with chloroform/methanol (CM; 2:1; v/v) (16) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant according to the method of Smuts *et al.* (17). In short, approximately 100–150 mg liver was ground to a fine powder in liquid nitrogen and weighed in glass-stoppered tubes. The tissue was suspended in 0.5 ml saline and the lipids were extracted with 24 ml CM. The CM mixture was filtered (sinterglass filters using Whatman Glass Microfibre filters; Whatman International, Maidstone, UK) and the filtrate was evaporated to dryness *in vacuo* at 40°C, transferred to glass-stoppered tubes, washed with saline saturated with CM, and stored at 4°C under nitrogen for 2 weeks until analysed. The lipid extracts were fractionated by thin layer chromatography (TLC) and the major phospholipid fractions, PC and PE, were collected for phospholipid and FA analyses (18). Phospholipid levels were determined colorimetrically using a malachite green dye after digestion with perchloric acid (16 N) at 170°C for approximately 1 h (19). For the FA analyses, the phospholipid fractions, PC and PE, were transmethylated with 2 ml methanol/18 M sulphuric acid (95:5; v/v) at 70°C for 2 h. The FA methyl esters (FAME) were extracted in hexane and analysed by gas chromatography on a Varian 3400 Gas Chromatograph equipped with 30 m fused silica Megabore DB-225 columns of 0.53 mm internal diameter (J&W Scientific). The individual FAME were identified by comparison of the retention times to those of a standard mixture of free FA, C14:0 to C24:1, and quantified using an internal standard (C17:0) and expressed as µg FA/100 mg liver weight.

Total cholesterol of the lipid extracts was determined by an enzymatic iodide method using cholesterinoxidase and -esterase (20). The cholesterol/phospholipid molar ratio was calculated using the molar weights of 386.7, 787 and 744 for cholesterol, PC and PE, respectively.

Membrane fluidity/fluorescence polarization

Fluorescence polarization studies were performed on homogenized control, surrounding, nodule and regenerating liver tissue with a fluorescence spectrofluorimeter (MPF 44A, Perkin-Elmer). Samples were diluted to a concentration of 0.2–0.3 mg protein/ml with 10 mM Tris–HCl, pH 7.4, sonicated for 10 s and 5 µl 1,6-diphenyl-1,3,5-hexatrine (DPH; 2 mM in tetrahydrofuran) added (21,22). The suspension (2.5 ml) was incubated in a water bath (37°C) for 30–60 min in the dark. Measurements were done manually with an emission polarizer at 0° (V component) and 90° (L component) with the excitation polarizer first at 0° (vertical component v) and then at 90° (horizontal component h). The excitation and emission slit widths were 14 nm and the excitation and emission wavelengths were 357 and 425 nm, respectively. The temperatures selected for screening ranged from 25 to 41°C.

Protein determination

Powdered liver preparations (10–15 mg) were solubilized in 5% SDS at 37°C and the protein content determined using a modified method of Lowry (23).

Statistical analyses

Statistical analyses were performed using the analyses of variance (ANOVA). The Tukey Studentized Range Method was used to determine differences between the means. Lipid changes as a function of time were analysed with the Parametric Paired Difference t-test. Values were considered significant if $P < 0.05$.

Results

Cholesterol content (Table I)

The cholesterol content was significantly increased within the control, nodule and surrounding tissue, as a function of time. This increase was significant between months 3 and 6 ($P < 0.01$) in the control group, between months 6 and 9 ($P < 0.05$) in the nodules and between months 6 and 9 ($P < 0.05$) in the surrounding tissue. In the hepatocyte nodules, the cholesterol was significantly increased at months 1 ($P < 0.05$) and 9 ($P < 0.01$) compared with the respective controls. Compared with the surrounding tissue, the cholesterol in the nodules was significantly increased at months 6

Table II. Comparative membrane fluidity parameters of regenerating liver versus control, hepatocyte nodules and surrounding tissue generated in the Resistant Hepatocyte Model

Month	Resistant Hepatocyte Model			Regenerating liver	
	Control	Nodule	Surrounding		
Fluorescence polarization					
1	0.249 ± 0.002[A]	0.261 ± 0.004[B]	0.245 ± 0.004[A]	Control	0.238 ± 0.006[A]
3	0.249 ± 0.003[A]	0.267 ± 0.006[B]	0.247 ± 0.002[A]	1 Day	0.171 ± 0.007[B]
6	0.243 ± 0.015[A]	0.276 ± 0.013[B]	0.240 ± 0.005[A]	2 Days	0.130 ± 0.030[B]
9	0.226 ± 0.004[A]	0.175 ± 0.008[B]	0.210 ± 0.002[A]	7 Days	0.152 ± 0.030[B]
Cholesterol/phospholipid molar ratio*					
1	0.19 ± 0.01	0.18 ± 0.02[a]	0.21 ± 0.01[b]	Control	0.15 ± 0.01[A]
3	0.15 ± 0.01[a]	0.17 ± 0.02	0.20 ± 0.04[b]	1 Day	0.13 ± 0.02[A]
6	0.25 ± 0.05	0.22 ± 0.04	0.24 ± 0.07	2 Days	0.28 ± 0.05[B]
9	0.27 ± 0.02[a]	0.38 ± 0.02[b]	0.29 ± 0.02[a]	7 Days	0.28 ± 0.03[B]
PC/PE ratio					
1	4.54 ± 0.43[A]	3.14 ± 0.16[B]	3.20 ± 0.39[B]	Control	4.54 ± 0.43[A]
3	4.21 ± 0.43[a]	3.21 ± 0.47[b]	3.69 ± 0.73	1 Day	1.84 ± 0.17[B]
6	5.14 ± 1.51[aA]	2.22 ± 0.11[B]	3.20 ± 0.54[b]	2 Days	1.83 ± 0.26[B]
9	3.34 ± 0.21[A]	2.19 ± 0.24[B]	2.97 ± 0.22[A]	7 Days	2.31 ± 0.36[B]

Values are means ± SD. Significant differences are represented by uppercase ($P < 0.01$) and lowercase ($P < 0.05$) superscript letters. PC, phosphatidylcholine; PE, phosphatidylethanolamine.
*The phospholipid molar ratio was calculated from the sum of PC and PE which constitute the major phospholipid fractions in rat liver.

($P < 0.05$) and 9 ($P < 0.01$). No significant changes were observed between the controls and the surrounding tissue. The cholesterol concentration in the regenerating liver was significantly increased ($P < 0.01$) at days 2 and 7 when compared with the control.

Phospholipid content (Table I)

The PE level in the hepatocyte nodules, increased significantly at 1 ($P < 0.01$), 3 ($P < 0.05$), 6 ($P < 0.01$) and 9 ($P < 0.01$) months when compared with the respective controls and surrounding tissue ($P < 0.01$). PE in the surrounding tissue was only significantly increased at months 1 ($P < 0.05$) and 6 ($P < 0.05$) compared with the respective controls. In contrast, PC in the nodules increased significantly only at month 1 compared with the control and surrounding tissue. The level of PE in the regenerating liver was significantly increased ($P < 0.01$) at days 1, 2 and 7 compared with the control, whereas PC was significantly decreased ($P < 0.05$) at day 2.

Membrane fluidity (Table II)

The measurement of DPH-labelled membranes relates to membrane micro-viscosity which is inversely related to membrane fluidity i.e. an increase in micro-viscosity indicates a decrease in membrane fluidity (19). The membrane fluidity in the nodular tissue, decreased at months 1 ($P < 0.01$), 3 ($P < 0.01$) and 6 ($P < 0.05$) as compared with the respective controls. However, at month 9 the membrane fluidity was significantly higher ($P < 0.01$) than the respective control. The same pattern was observed when comparing the nodular tissue with the surrounding tissue. No significant differences were observed between the surrounding tissue and the respective controls. In the regenerating liver, the membrane fluidity was significantly increased at days 1, 2 and 7 ($P < 0.01$) when compared to the control.

A significant increase in the cholesterol/phospholipid molar ratio was observed in the surrounding tissue ($P < 0.05$) at 3 months. At 9 months the ratio was significantly increased ($P < 0.05$) in the nodules. The PC/PE ratio in the nodules was significantly decreased at months 1, 3, 6 and 9 (months 1, 6 and 9, $P < 0.01$; month 3, $P < 0.05$) compared with the respective controls. The surrounding tissue PC/PE ratio was also significantly decreased from the controls at months 1 and 6 ($P < 0.01$ and $P < 0.05$, respectively). In the regenerating liver, the cholesterol/phospholipid molar ratio was significantly ($P < 0.01$) increased at days 2 and 7 compared with the control and day 1. The PC/PE ratio was significantly ($P < 0.01$) decreased from the control level at days 1, 2 and 7.

Comparative FA profiles in PC and PE of hepatocyte nodules compared with surrounding and control tissue (Tables III and IV)

The general trend of the FA profiles in the surrounding tissue tended to mimic that of the control tissue, especially after 3 months. Before 3 months, some values fell in between the control and nodule values.

Saturated FA: (C16:0, C18:0)

In the nodules, the levels of C16:0 in PC increased significantly at months 1, 6 and 9 ($P < 0.01$) compared with the controls, whereas C18:0 was significantly decreased at months 6 and 9 ($P < 0.01$). However, in PE C16:0 was significantly decreased at month 3 ($P < 0.05$), whereas C18:0 was significantly increased at months 1, 6 and 9 ($P < 0.01$). No significant changes were observed in the total saturated FA levels of PC, but in PE the levels increased significantly at months 1, 6 and 9 ($P < 0.01$).

Monounsaturated FA: (C16:1, C18:1)

The values of C16:1 increased significantly in PC and PE in the nodules at months 1, 6 and 9 ($P < 0.01$), while C18:1 increased significantly ($P < 0.01$) in PC and PE at months 1, 3, 6 and 9 ($P < 0.01$). The total monounsaturated FA level was significantly increased in PC at 1, 6 and 9 months ($P < 0.01$) in the nodules and in PE at 1, 3, 6 and 9 months ($P < 0.01$).

Polyunsaturated FA

ω6 PUFA: (C18:2, C20:4, C22:4, C22:5)

The level of C18:2 in the nodules increased ($P < 0.01$) at

Table III. Fatty acid analyses of the phosphatidylcholine (PC) fraction of control liver, nodule and surrounding liver in the Resistant Hepatocyte Model

	1 Month			3 Months			6 Months			9 Months		
	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding
Saturates												
C16:0	11.92 ± 1.68^{A}	18.13 ± 2.18^{B}	13.61 ± 0.98^{A}	16.03 ± 1.39^{a}	13.55 ± 3.60	10.03 ± 3.40^{b}	15.31 ± 0.91^{A}	23.42 ± 2.48^{B}	14.46 ± 1.77^{A}	14.20 ± 1.90^{A}	22.10 ± 2.99^{B}	14.78 ± 1.62^{A}
C18:0	13.13 ± 1.80	11.34 ± 1.96	10.90 ± 0.51	15.50 ± 0.84^{a}	10.64 ± 3.18	9.22 ± 2.38^{b}	16.71 ± 2.08^{A}	10.99 ± 1.85^{B}	15.89 ± 2.24^{A}	21.40 ± 1.05^{A}	12.98 ± 3.76^{B}	19.13 ± 1.87^{A}
Total	25.04 ± 3.42	29.48 ± 4.03	24.51 ± 1.18	31.53 ± 2.07^{A}	24.18 ± 5.97	19.24 ± 5.77^{B}	32.02 ± 2.57	34.42 ± 4.25	30.35 ± 3.33	35.60 ± 2.74	35.08 ± 6.34	33.91 ± 3.45
Monounsaturates												
C16:1	0.56 ± 0.27^{A}	1.75 ± 0.71^{B}	0.82 ± 0.18^{A}	0.91 ± 0.18	1.00 ± 0.32	0.68 ± 0.28	0.71 ± 0.07^{A}	1.79 ± 0.27^{B}	0.65 ± 0.29^{A}	0.57 ± 0.17^{aA}	1.71 ± 0.34^{bB}	0.99 ± 0.14^{cA}
C18:1	4.81 ± 0.70^{A}	10.95 ± 2.19^{B}	6.88 ± 0.46^{A}	6.55 ± 1.03	8.58 ± 1.87^{a}	5.22 ± 1.47^{b}	6.23 ± 0.31^{aA}	12.73 ± 0.91^{bB}	7.73 ± 0.93^{cA}	5.77 ± 0.70^{A}	16.70 ± 4.51^{B}	7.23 ± 0.78^{A}
Total	5.38 ± 0.96^{A}	12.71 ± 2.87^{B}	7.70 ± 0.56^{A}	7.46 ± 1.20	9.59 ± 2.14	5.90 ± 1.75	6.93 ± 0.34^{A}	14.52 ± 1.07^{B}	8.38 ± 1.15^{A}	6.33 ± 0.82^{A}	18.41 ± 4.53^{B}	8.23 ± 0.85^{A}
ω6												
C18:2	6.13 ± 1.33^{A}	11.92 ± 2.09^{B}	7.80 ± 1.10^{A}	7.22 ± 0.70	9.04 ± 2.61^{a}	5.45 ± 1.60^{b}	7.80 ± 0.89^{A}	13.57 ± 2.25^{B}	8.97 ± 1.47^{A}	8.37 ± 1.56^{A}	15.97 ± 5.17^{B}	8.58 ± 1.12^{A}
C20:4	20.19 ± 2.41	18.67 ± 3.35	18.58 ± 0.91	25.85 ± 1.72^{aA}	18.53 ± 5.39^{bAB}	15.40 ± 4.35^{bB}	26.36 ± 2.21	22.77 ± 2.49	25.00 ± 2.73	32.01 ± 3.40	28.03 ± 4.85	28.96 ± 3.11
C22:4	0.56 ± 0.06	0.54 ± 0.11	0.71 ± 0.14	0.74 ± 0.07^{a}	0.50 ± 0.15^{b}	0.51 ± 0.11^{b}	0.69 ± 0.07^{aAB}	0.57 ± 0.06^{bB}	0.71 ± 0.07^{aA}	0.97 ± 0.19	1.26 ± 0.82	0.78 ± 0.09
C22:5	2.45 ± 0.54^{a}	1.48 ± 0.29^{b}	2.27 ± 0.60	3.19 ± 0.42^{A}	1.45 ± 0.66^{B}	1.75 ± 0.40^{B}	2.93 ± 0.15^{aA}	0.92 ± 0.19^{bB}	2.38 ± 0.47^{cA}	4.49 ± 0.85^{A}	1.66 ± 0.78^{B}	4.19 ± 0.55^{A}
Total	29.92 ± 4.16	33.11 ± 5.92	29.88 ± 1.43	37.81 ± 2.33^{A}	30.05 ± 8.42	23.63 ± 6.46^{B}	38.52 ± 2.80	38.43 ± 4.82	37.74 ± 3.91	46.71 ± 5.78	47.53 ± 11.31	43.95 ± 4.89
ω3												
C22:5	0.18 ± 0.03^{a}	0.18 ± 0.04^{a}	0.25 ± 0.03^{b}	0.23 ± 0.01^{aA}	0.13 ± 0.05^{bB}	0.15 ± 0.06^{bAB}	0.23 ± 0.02^{A}	0.16 ± 0.02^{B}	0.24 ± 0.03^{A}	0.11 ± 0.03	0.08 ± 0.03	0.07 ± 0.01
C22:6	1.62 ± 0.19^{A}	0.82 ± 0.13^{B}	1.30 ± 0.08^{C}	1.69 ± 0.12^{A}	0.78 ± 0.33^{B}	0.98 ± 0.30^{B}	1.86 ± 0.11^{A}	0.64 ± 0.11^{B}	1.53 ± 0.17^{C}	0.82 ± 0.15^{A}	0.41 ± 0.18^{B}	0.87 ± 0.13^{A}
Total	1.88 ± 0.21^{A}	1.13 ± 0.18^{B}	1.64 ± 0.08^{A}	2.02 ± 0.12^{A}	1.08 ± 0.42^{B}	1.22 ± 0.35^{A}	2.24 ± 0.09^{A}	0.94 ± 0.14^{B}	1.88 ± 0.19^{C}	1.04 ± 0.20	0.58 ± 0.23	1.01 ± 0.14
ω6/ω3	15.89 ± 0.64^{A}	29.27 ± 2.71^{B}	18.20 ± 0.74^{A}	18.79 ± 1.04^{A}	29.19 ± 4.98^{B}	19.40 ± 0.37^{A}	17.16 ± 0.86^{A}	41.11 ± 3.32^{B}	20.05 ± 0.73^{A}	45.51 ± 2.97^{A}	85.18 ± 12.59^{B}	43.67 ± 4.00^{A}
PUFA	31.80 ± 4.36	34.24 ± 6.08	31.52 ± 1.48	39.83 ± 2.41^{A}	31.13 ± 8.79	24.85 ± 6.81^{B}	40.76 ± 2.08	39.37 ± 4.94	39.63 ± 4.09	47.74 ± 5.97	48.12 ± 11.53	44.97 ± 5.00
P/S	1.27 ± 0.05	1.16 ± 0.09^{a}	1.29 ± 0.08^{b}	1.26 ± 0.03	1.28 ± 0.09	1.30 ± 0.05	1.27 ± 0.04	1.15 ± 0.08^{a}	1.31 ± 0.10^{b}	1.34 ± 0.12	1.37 ± 0.16	1.33 ± 0.06

Values are expressed as means ± SD µg FA/100 mg liver weight. Significant differences are indicated by uppercase ($P < 0.01$) and lowercase ($P < 0.05$) superscript letters. Statistical comparisons (rows) of control, nodule and surrounding tissue were conducted within each time interval. PUFA, polyunsaturated fatty acids; P/S, polyunsaturated to saturated fatty acid ratio.

Table IV. Fatty acid analyses of the phosphatidylethanolamine (PE) fraction of control liver, nodule and surrounding liver in the Resistant Hepatocyte Model

	1 Month			3 Months			6 Months			9 Months		
	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding
Saturates												
C16:0	6.51 ± 1.44^{a}	8.08 ± 0.79	8.31 ± 0.75^{b}	9.01 ± 0.89a	5.57 ± 0.95^{b}	6.01 ± 2.89	8.37 ± 0.74	10.02 ± 1.20	8.39 ± 1.05	4.32 ± 0.66	4.54 ± 0.64	5.34 ± 0.69
C18:0	8.55 ± 1.80^{A}	15.26 ± 1.26^{B}	10.41 ± 1.25^{A}	9.65 ± 0.49	12.81 ± 2.57^{A}	5.91 ± 2.38^{B}	10.24 ± 0.59^{A}	19.75 ± 1.90^{B}	11.70 ± 2.32^{A}	7.51 ± 0.62^{A}	11.15 ± 1.52^{B}	8.18 ± 0.98^{A}
Total	15.06 ± 3.12^{aA}	23.34 ± 1.97^{bB}	18.72 ± 1.48^{a}	18.66 ± 1.30	18.37 ± 3.40	11.92 ± 5.25	18.61 ± 0.47^{A}	29.77 ± 2.89^{B}	20.08 ± 3.19^{A}	11.83 ± 1.01^{A}	15.69 ± 1.55^{B}	13.51 ± 1.55
Monounsaturates												
C16:1	0.20 ± 0.08^{A}	0.61 ± 0.12^{B}	0.27 ± 0.05^{A}	0.33 ± 0.09	0.25 ± 0.08	0.23 ± 0.09	0.29 ± 0.03^{A}	0.65 ± 0.10^{B}	0.25 ± 0.11^{A}	0.12 ± 0.04^{A}	0.25 ± 0.07^{B}	0.26 ± 0.04^{B}
C18:1	2.50 ± 0.37^{A}	8.71 ± 1.39^{B}	4.47 ± 0.46^{C}	3.63 ± 0.45^{a}	5.79 ± 1.32^{bB}	2.46 ± 1.12^{aA}	3.58 ± 0.17^{A}	11.90 ± 1.44^{B}	4.96 ± 0.44^{A}	1.90 ± 0.31^{A}	7.03 ± 1.58^{B}	2.54 ± 0.32^{A}
Total	2.70 ± 0.38^{A}	9.32 ± 1.50^{B}	4.75 ± 0.42^{C}	3.96 ± 0.52^{A}	6.04 ± 1.40^{B}	2.69 ± 1.20^{A}	3.88 ± 0.17^{A}	12.55 ± 1.46^{B}	5.21 ± 0.46^{A}	2.02 ± 0.29^{A}	7.28 ± 1.63^{B}	2.79 ± 0.34^{A}
ω6												
C18:2	2.38 ± 0.48^{aA}	5.70 ± 0.68b^{B}	3.56 ± 0.67^{cA}	3.12 ± 0.35	4.14 ± 1.38^{a}	2.35 ± 0.86^{b}	3.13 ± 0.32^{A}	6.94 ± 0.89^{B}	4.54 ± 1.45^{A}	2.15 ± 0.59^{A}	3.66 ± 0.51^{B}	2.48 ± 0.42^{A}
C20:4	11.27 ± 1.87^{aA}	18.19 ± 2.11^{bB}	14.84 ± 1.76^{cAB}	13.28 ± 1.10	16.27 ± 3.28^{A}	7.65 ± 3.99^{B}	13.69 ± 1.25^{A}	25.06 ± 2.29^{B}	16.20 ± 2.00^{A}	10.40 ± 1.27^{aA}	15.35 ± 2.86^{bB}	11.40 ± 1.36^{a}
C22:4	0.76 ± 0.12^{A}	1.52 ± 0.20^{B}	1.38 ± 0.22^{B}	1.09 ± 0.05	1.36 ± 0.36^{A}	0.64 ± 0.34^{B}	1.12 ± 0.22^{A}	2.46 ± 0.36^{B}	1.38 ± 0.13^{A}	1.05 ± 0.22^{A}	1.87 ± 0.37^{B}	1.01 ± 0.14^{A}
C22:5	3.31 ± 0.98	4.32 ± 0.69	4.21 ± 0.96	3.97 ± 0.26^{A}	3.17 ± 1.03	1.80 ± 0.94^{B}	4.09 ± 0.43	4.16 ± 0.80	4.01 ± 0.51	3.74 ± 0.49	3.04 ± 0.75	3.95 ± 0.58
Total	18.04 ± 3.34^{A}	30.12 ± 3.63^{bB}	24.38 ± 2.60^{c}	21.86 ± 1.36	25.33 ± 5.66^{A}	12.69 ± 6.11^{B}	22.38 ± 1.97^{A}	39.06 ± 3.60^{B}	26.57 ± 3.24^{A}	17.61 ± 2.30^{a}	24.15 ± 4.30^{b}	19.29 ± 2.47
ω3												
C22:5	0.26 ± 0.04^{A}	0.50 ± 0.05^{B}	0.50 ± 0.11^{B}	0.34 ± 0.04	0.33 ± 0.10	0.19 ± 0.12	0.33 ± 0.05^{A}	0.62 ± 0.08^{B}	0.43 ± 0.06^{A}	0.09 ± 0.02^{a}	0.16 ± 0.06^{b}	0.10 ± 0.02
C22:6	2.54 ± 0.56	2.62 ± 0.14	2.72 ± 0.61	2.32 ± 0.24^{a}	2.16 ± 0.62^{a}	1.12 ± 0.68^{b}	2.81 ± 0.27	3.11 ± 0.44	2.82 ± 0.29	0.71 ± 0.10	0.89 ± 0.20	0.82 ± 0.12
Total	2.87 ± 0.58	3.28 ± 0.16	3.33 ± 0.73	2.75 ± 0.26^{a}	2.70 ± 0.76^{a}	1.42 ± 0.80^{b}	3.26 ± 0.33	3.89 ± 0.52	3.36 ± 0.35	0.86 ± 0.14	1.12 ± 0.27	0.96 ± 0.14
ω6/ω3	6.31 ± 0.36^{A}	9.18 ± 0.81^{B}	7.50 ± 1.03^{A}	7.98 ± 0.67	9.56 ± 1.16	8.94 ± 0.50	6.87 ± 0.17^{A}	10.08 ± 0.57^{B}	7.90 ± 0.47^{C}	20.68 ± 1.26	21.90 ± 1.40	20.23 ± 0.71
PUFA	20.91 ± 3.90^{aA}	33.40 ± 3.75^{bB}	27.72 ± 3.29^{b}	24.62 ± 1.51^{a}	28.03 ± 6.33^{aA}	14.12 ± 6.90^{bB}	25.64 ± 2.29^{A}	42.96 ± 4.08^{B}	29.93 ± 3.55^{A}	18.47 ± 2.43^{A}	25.27 ± 4.56^{B}	20.25 ± 2.60
P/S	1.39 ± 0.07	1.43 ± 0.05	1.48 ± 0.14	1.32 ± 0.03	1.52 ± 0.09^{A}	1.21 ± 0.08^{B}	1.38 ± 0.13	1.45 ± 0.12	1.50 ± 0.09	1.56 ± 0.12	1.60 ± 0.18	1.50 ± 0.07

Values are expressed as means ± SD μg FA/100 mg liver weight. Significant differences are indicated by uppercase ($P < 0.01$) and lowercase ($P < 0.05$) superscript letters. Statistical comparisons (rows) of control, nodule and surrounding tissue were conducted within each time interval. PUFA, polyunsaturated fatty acids; P/S, polyunsaturated to saturated fatty acid ratio.

Table V. Fatty acid analyses of the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions of regenerating liver

	Regenerating liver fatty acid PC				Regenerating liver fatty acid PE			
	Control	1 Day	2 Days	7 Days	Control	1 Day	2 Days	7 Days
Saturates								
C16:0	22.17 ± 6.69^{a}	14.49 ± 2.03^{b}	15.14 ± 1.78^{b}	13.93 ± 1.55^{b}	10.94 ± 2.30	12.94 ± 0.81	12.88 ± 2.14	11.01 ± 1.64
C18:0	23.84 ± 5.31^{aA}	16.57 ± 2.89^{b}	17.30 ± 2.48^{b}	14.51 ± 2.24^{bB}	14.87 ± 3.80	20.27 ± 3.12	18.59 ± 2.85	15.03 ± 2.08
Total	46.01 ± 11.75^{aA}	31.05 ± 3.46^{b}	32.44 ± 4.16^{b}	28.44 ± 2.32^{bB}	25.82 ± 5.81	33.21 ± 3.63	31.48 ± 4.73	26.04 ± 2.96
Monounsaturates								
C16:1	1.16 ± 0.61	0.57 ± 0.24	0.53 ± 0.11	0.77 ± 0.27	0.33 ± 0.15	0.30 ± 0.09	0.21 ± 0.05	0.29 ± 0.05
C18:1	9.52 ± 3.56^{a}	5.59 ± 1.42^{b}	6.16 ± 0.94	8.10 ± 1.07	4.29 ± 0.67^{a}	5.24 ± 1.34	4.36 ± 0.74^{a}	6.31 ± 0.57^{b}
Total	10.68 ± 4.02^{a}	6.16 ± 1.62^{b}	6.69 ± 1.02	8.87 ± 1.33	4.62 ± 0.70^{a}	5.54 ± 1.41	4.57 ± 0.75^{a}	6.60 ± 0.57^{b}
ω6								
C18:2	10.90 ± 3.47	12.58 ± 3.36^{a}	8.59 ± 1.39	7.31 ± 0.79^{b}	4.25 ± 1.29^{A}	9.97 ± 2.09^{B}	5.31 ± 1.16^{A}	4.23 ± 0.61^{A}
C20:4	35.66 ± 6.74^{aA}	20.75 ± 2.58^{bB}	24.98 ± 2.83^{bB}	26.64 ± 1.72^{b}	18.93 ± 4.43	24.69 ± 3.25	23.59 ± 3.50	22.98 ± 3.23
C22:4	0.99 ± 0.24^{a}	0.66 ± 0.15^{b}	0.87 ± 0.12	0.85 ± 0.15	1.29 ± 0.32^{a}	1.44 ± 0.34	1.95 ± 0.37^{b}	2.02 ± 0.26^{b}
C22:5	4.32 ± 1.27^{A}	1.85 ± 0.67^{B}	2.35 ± 0.28^{B}	3.21 ± 0.57	5.68 ± 1.99^{a}	3.19 ± 0.75^{bB}	5.36 ± 0.58	7.35 ± 0.95^{aA}
Total	52.78 ± 11.22^{aA}	36.25 ± 4.80^{bB}	37.52 ± 4.39^{bB}	39.10 ± 1.50^{b}	30.64 ± 8.09	39.70 ± 5.73	36.74 ± 5.54	37.51 ± 3.26
ω3								
C22:5	0.31 ± 0.06^{a}	$0.20 \pm 0.04b^{B}$	0.35 ± 0.06^{aA}	0.28 ± 0.03	0.46 ± 0.11^{aA}	0.58 ± 0.08^{ab}	0.83 ± 0.18^{bB}	0.70 ± 0.10^{b}
C22:6	2.89 ± 0.59^{A}	1.59 ± 0.20^{B}	1.84 ± 0.20^{B}	1.83 ± 0.19^{B}	4.49 ± 1.13	4.59 ± 0.56	4.51 ± 0.58	5.00 ± 0.64
Total	3.34 ± 0.65^{A}	1.92 ± 0.23^{B}	2.34 ± 0.25^{B}	2.23 ± 0.17^{B}	5.09 ± 1.30	5.29 ± 0.58	5.48 ± 0.73	5.84 ± 0.71
ω6/ω3	15.82 ± 1.03^{a}	18.92 ± 2.21^{b}	16.07 ± 1.44^{a}	17.56 ± 0.95	6.01 ± 0.26^{A}	7.50 ± 0.66^{aB}	6.69 ± 0.23^{b}	6.46 ± 0.45^{B}
PUFA	56.12 ± 11.84^{aA}	38.17 ± 4.95^{bB}	39.86 ± 4.58^{bB}	41.33 ± 1.64^{b}	35.73 ± 9.37	44.99 ± 6.22	42.22 ± 6.26	43.35 ± 3.89
P/S	1.23 ± 0.09^{A}	1.23 ± 0.08^{A}	1.23 ± 0.06^{A}	1.46 ± 0.07^{B}	1.38 ± 0.10^{A}	1.35 ± 0.07^{A}	1.34 ± 0.07^{A}	1.67 ± 0.08^{B}

Values are expressed as means ± SD μg FA/100 mg liver weight. Significant differences are indicated by uppercase ($P < 0.01$) and lowercase ($P < 0.05$) superscript letters. Statistical analyses (rows) of the phospholipid fractions of 1, 2 and 7 days after partial hepatectomy were compared against the control. PUFA, polyunsaturated fatty acids; P/S, polyunsaturated to saturated fatty acid ratio.

months 1, 3, 6 and 9 in PC and PE. In PC, C20:4 was significantly decreased only at month 3 ($P < 0.05$), but tended to be slightly (not significant) lower at months 1, 6 and 9. However, in PE C20:4 was significantly increased at 1, 6 and 9 months ($P < 0.01$) with a non-significant increase at 3 months. The level of C22:4 in PC was significantly decreased at months 3 ($P < 0.05$) and 6 ($P < 0.01$), but in PE the level was increased ($P < 0.01$) at 1, 3, 6 and 9 months. In PC, C22:5 was significantly decreased at 1 ($P < 0.05$), 3, 6 and 9 months ($P < 0.01$). No significant changes were observed in PE. The total ω6 levels in PE were increased significantly at 1, 3, 6 ($P < 0.01$) and 9 ($P < 0.05$) months, while in PC it was significantly lower only at 3 months.

ω3 PUFA: (C22:5, C22:6)

In PC, C22:5 was significantly decreased in the nodules at 3 and 6 months ($P < 0.01$) but increased significantly in PE at 1, 6 ($P < 0.01$) and 9 months ($P < 0.05$). C22:6 was significantly decreased in PC at 1, 3, 6 and 9 months ($P < 0.01$) compared with the controls, while no changes were observed in PE. The total ω3 level in PC was significantly decreased at 1, 3, 6 ($P < 0.01$) and 9 months ($P < 0.05$). In PE, no significant changes were observed although the levels tended to increase (not significant) at months 1, 6 and 9.

Due to the decrease in ω3 FA, the ω6/ω3 ratio was significantly increased in PC at 1, 3, 6 and 9 months ($P < 0.01$), but only at months 1 and 6 ($P < 0.01$) in PE. The PUFA level increased in PE at 1, 6 and 9 ($P < 0.01$) months, and decreased significantly ($P < 0.01$) at 3 months in PC. No significant changes in the P/S ratio were observed in the nodular tissue compared with the respective controls.

FA profiles of PC and PE in regenerating liver (Table V)

Saturated FA: (C16:0, C18:0)

The levels of both C16:0 (1, 2 and 7 days: $P < 0.05$) and C18:0 (1 and 2 days: $P < 0.05$; 7 days: $P < 0.01$) decreased significantly in PC. No changes were observed with regards to C16:0 and C18:0 in PE, although a non-significant increase was noticed at days 1 and 2. As a result of these changes, the total saturate level in PC was significantly decreased at 1, 2 ($P < 0.05$) and 7 days ($P < 0.01$). In PE, no significant changes were observed, but there was a slight increase (not significant) in the level at days 1 and 2.

Monounsaturated FA: (C16:1, C18:1)

No significant changes occurred with regards to C16:1 in PC and PE, although it decreased initially (not significant) in PC. The level of C18:1 was significantly decreased at 1 day ($P < 0.05$) in PC, but increased thereafter at 2 and 7 days towards the control level. In PE, the level of C18:1 was significantly increased at 7 days after partial hepatectomy ($P < 0.05$). The total monounsaturate level was significantly decreased in PC at day 1 ($P < 0.05$) and thereafter the levels at days 2 and 7 tended to increase towards the control level. In PE, the total monounsaturate level was significantly increased at day 7 ($P < 0.05$).

Polyunsaturated FA

ω6 PUFA: (C18:2, C20:3, C20:4, C22:4, C22:5)

At day 1 C18:2 increased in PC ($P < 0.05$) and PE ($P < 0.01$), after which the level tended to revert back to that in the controls. In PE, C20:3 was significantly increased 7 days ($P < 0.01$) after partial hepatectomy (data not shown). The C20:4 level significantly decreased at 1, 2 ($P < 0.01$) and 7 days ($P < 0.05$) after partial hepatectomy in PC, but increased over time towards control levels. No significant changes were observed in PE, although there was a slight increase at day 1, but then tended to decrease back to the control level. C22:4 was significantly decreased in PC at day 1 only ($P < 0.05$), but tended to increase thereafter. There was a significant increase in the level of C22:4 in PE at days 2 and 7 ($P < 0.05$). The level of C22:5 decreased in PC at days 1 and 2 ($P < 0.01$) and in PE at day 1 only ($P < 0.05$). In both PC and PE, C22:5 was decreased initially at day 1 but tended to increase to the control levels at day 7. The total ω6 FA level was significantly decreased at days 1, 2 ($P < 0.01$) and 7 ($P < 0.05$) in PC. No

significant changes were observed in PE, although the level tended to be higher than control levels.

ω3 PUFA: (C22:5, C22:6)

The level of C22:5 was significantly decreased at day 1 ($P < 0.05$) only in PC, but increased significantly in PE at days 1 ($P < 0.05$), 2 ($P < 0.01$) and 7 ($P < 0.05$). In PC, C22:6 was significantly decreased at days 1, 2 and 7 ($P < 0.01$). No changes were observed in PE. The total ω3 FA level was significantly decreased in PC at 1, 2 and 7 days ($P < 0.01$) compared with the control. Once again, no changes were observed in PE.

The ω6/ω3 ratios were significantly increased in PC ($P < 0.05$) and PE ($P < 0.01$) 1 day after partial hepatectomy compared with the control levels. The PUFA level was significantly decreased at 1, 2 ($P < 0.01$) and 7 days ($P < 0.05$) in PC, but no significant changes were observed in PE, although the level tended to be higher (not significant) over the experimental period. The P/S ratio in PC and PE was significantly increased from the respective controls at 7 days ($P < 0.01$) after partial hepatectomy.

Discussion

The structure of plasma membranes in regenerating liver concerning the patterns of membrane proteins have shown no changes compared with that of control liver (5,10). However, changes in the plasma membrane enzyme activity and receptor expression in regenerating liver have been described (24,25). In hepatoma cells, alterations in membranal protein profiles have been reported resulting in changes in the activity of certain membrane enzymes, such as a decrease in 5-nucleotidase, an increase in γ-glutamyltranspeptidase, as well as changes in the affinity of receptors (10,26,27). Changes in membrane protein turnover have also been shown to occur in HTC cells (Morris hepatoma 7288C) (28). Alterations in membrane fluidity can also influence the activity of certain enzymes and the affinity of receptors to their ligands (5,10). Membrane fluidity has been shown to increase in the nuclear membrane following partial hepatectomy in rats (29). This increase is linked to nuclear membrane neutral-sphingomyelinase activity and the content of sphingomyelin. Alterations in lipid content have been closely linked with changes in membrane fluidity which could play an important role in the control of signal transduction pathways and cellular regeneration in the altered growth pattern and progression of hepatocyte nodules to cancer development (5,10).

Important indicators of membrane fluidity are the cholesterol/phospholipid molar ratio (Chol/PL), the PC/PE ratio and the degree of membrane unsaturation (P/S ratio) (5,10). In the present study, fluorescence polarization indicated that the nodule membranes were more rigid, i.e. less fluid, than the respective controls at 1, 3 and 6 months, in contrast to the increased fluidity in regenerating liver. However, at 9 months the nodule membrane fluidity increased above the control, mimicking regenerating liver. The PC/PE ratio in the nodules and the regenerating liver decreased early on due to the increased PE level. This increase in the PE concentration has also been observed in Morris hepatoma 7777 cells (5,10). The Chol/PL molar ratio in the nodules increased only at 9 months due to a significant increase in the cholesterol level. This mimics regenerating liver where the cholesterol level also increased after 2 and 7 days. Cholesterol appears to play an important structural role in maintaining the fluidity of membranes making the outer part of the membrane less fluid, but causing the inner part of the lipid bilayer to be slightly more fluid by organizing the tails of the FA acyl chains (30). The non-polar membrane components, such as the FA acyl chains and cholesterol, seem to have a greater regulatory effect on the activity of membrane bound proteins than the polar phospholipid head groups (31). In this way cholesterol can organize the movement of membrane bound proteins in regenerating liver and also in hepatocyte nodules. Finally, the unsaturation index (P/S ratio) in the hepatocyte nodules did not change, suggesting that this index is tightly controlled in the nodules and does not appear to have a significant effect on membrane fluidity in the hepatocyte nodule. The increased PUFA level in PE, due to the increased PE concentration, did not correlate with the resultant unchanged membrane fluidity, further indicating that unsaturation did not affect fluidity directly. A recent study showed that the FA unsaturation level in a membrane did not have such a large influence on membrane fluidity when compared with the effect of cholesterol (32). This increase in PE appears to be an early event in the nodular lipid profile compared with changes in the cholesterol and membrane fluidity which appear to be late events. Together, the increases in membrane cholesterol and PE are likely to be the major factors determining fluidity changes in the hepatocyte nodules. In regenerating liver, an increase in these two parameters was also associated with an increase in fluidity.

When comparing the lipid profiles of nodular liver with regenerating liver, some similarity in the pattern of lipid changes exists. In the regenerating liver, cholesterol increased significantly at days 2 and 7 while PE increased from day 1. The latter changes coincided with an increase in the fluidity as well as maximal liver regeneration, which occurs 2–3 days after partial hepatectomy (15). As seen in the hepatocyte nodules, the increased PE concentration in the regenerating liver is an early event with the changes in cholesterol occurring later. With regards to the fatty acids, C18:1ω9 decreased significantly in PC only at day 1, but was increased in PE at day 7, presumably due to the increase in the concentration of this phospholipid. It is not known whether the initial decrease in C18:1ω9, known to have antioxidant properties (6,7), could be related to the decreased rate in lipid peroxidation noticed in regenerating liver prior to DNA synthesis (7). After an initial increase of C18:2ω6 in PC after day 1, the level was significantly reduced after 7 days, presumably due to an increased conversion of this FA to C20 and C22 fatty acids as a result of liver regeneration. In comparison with the nodules, it would therefore appear that the Δ-6 desaturase enzyme is not impaired during normal regeneration following partial hepatectomy. The LC-PUFA, C20:4ω6, C22:5ω6 and C22:6ω3, decreased significantly in the regenerating liver PC presumably due to a higher metabolism of these FA during regeneration (7). It has been observed that proliferating cells have a higher utilization of the Δ-6 desaturated FA (11). The resultant decrease in these LC-PUFA supports the low lipid peroxidative status present in regenerating liver (33). In the nodules, the higher rate of cell proliferation, as well as the impaired Δ-6 desaturase enzyme, resulted in a similar decrease of LC-PUFA in PC which also appears to be an early event in the genesis of the nodules.

The lipid pattern associated with regeneration could be instrumental in the signal for cellular growth in regenerating liver under controlled conditions, while it prevails in hepatocyte nodules resulting in a steady increase in their size. However,

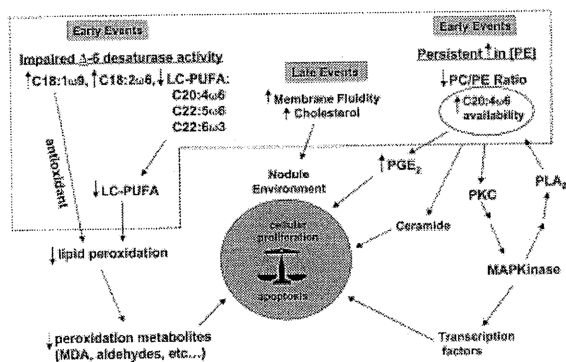

Fig. 1. Critical events associated with the altered growth pattern of hepatocyte nodules. The growth of pre-neoplastic nodules can be influenced by certain critical events with regards to lipid metabolism as summarized in the figure. This involves an impaired Δ-6 desaturase, an increase in PE, cholesterol concentration and membrane fluidity (this study dotted block). The early events involving the Δ-6 desaturase and increased PE level establish an environment critical for the continued proliferation of the nodules. This results in the later events such as the increased cholesterol and membrane fluidity affecting the functionality of the cellular membrane involving membrane enzymes and receptor affinity. The increased PE level is an important event leading to an increased membrane C20:4ω6 availability affecting various systems such as prostaglandin synthesis and PKC, ceramide and MAP kinase activity. These factors in turn play a role in the regulation of cellular proliferation and apoptosis. The impaired Δ-6 desaturase enzyme, as shown by increases in C18:1ω9 and C18:2ω6 and decreases in the LC-PUFA, can result in a decreased lipid peroxidation status leading to an imbalance in the cell proliferation/apoptosis equilibrium in nodules, thereby favouring cell proliferation.

in the majority of these nodules the lipid-associated stimulatory signals revert back to that prevailing in normal tissue, as observed in regenerating liver following partial hepatectomy, contributing to the remodelling process in the majority of the nodules. In the present study this was shown in the FA profiles of PC and PE of the 3 month nodules versus surrounding tissue with differences not being as prominent as that obtained at 6 and 9 months, presumably due to the large amount of nodules that are still remodelling at this stage. In a small subset of nodules, however, these changes 'persist' supporting the increased rate of cell proliferation and facilitating their ultimate development into cancer. It has been reported that after 6 months post-initiation, certain hepatocyte nodules, termed 'persistent' nodules, lose their ability to control cell proliferation (4) i.e. the normal 'regulatory' processes of the cell cycle are impaired. After 9 months, the remaining subset of the original pre-neoplastic nodules develop into cancer (4). Therefore, the normal control processes that regulate cell proliferation, seen in regenerating liver, is not present in a small subset of these nodules. Up to a certain stage, 1–6 months, the hepatocyte nodules are still in a transitional phase i.e. at an early stage most of the nodules revert back/regress to normal hepatocytes and at a later stage, i.e. from 6 to 9 months, the persistent nodules lose their 'pre-neoplastic' features/phase and advance to the neoplastic stage.

Critical events in the nodules are the persistent alterations in lipid parameters involving changes in FA metabolism (Δ-6 desaturase), increased PE and cholesterol levels and changes in membrane fluidity (Figure 1). Except for some changes in FA metabolism related to the impaired Δ-6 desaturase enzyme, the other changes closely mimic that of regenerating liver but with the difference that in the latter, these changes revert back to normal liver. This implies that the normal regulatory mechanisms, related to lipid metabolism to ensure normal liver homeostasis, are disrupted in hepatocyte nodules. It is not known at present whether the increased level of PE is related to an increased synthesis or to a decrease in the conversion to PC involving phosphatidylethanolamine *N*-methyltransferase-2 (PEMT2). Recent studies imply the expression of this enzyme in the regulation of hepatocyte growth (34). PEMT2 expression is transiently inactivated after partial hepatectomy (35) and permanently disappears in hepatocellular carcinoma induced by the resistant hepatocyte model (36), while the transfection of the enzyme to a hepatoma cell line inhibits the cell growth rate (37). The higher PE concentration, a phospholipid normally situated on the inside of cellular membranes (38), together with the resultant increases in C20:4ω6 appears to be an integral part of the growth stimulus in hepatocyte nodules and could play a role in sustaining cellular proliferation in these lesions (Figure 1).

The dual role of C20:4ω6, (i) structural as part of membrane phospholipids and (ii) functional as a precursor to the E_2-series eicosanoids and signal transduction pathways, are of particular interest with respect to its role in maintaining normal cellular homeostasis in the liver (39). The proteins, phospholipase A_2 (PLA_2), phospholipase C (PLC) and protein kinase C (PKC) play an important role in cell proliferation and have shown a tendency to be modulated by FA. Recent studies indicated that membranal FA, specifically C20:4ω6, play an important role as second messengers in signal transduction pathways via the activation of protein kinase C (PKC), mitogen activated protein kinase (MAP kinase) and the generation of ceramide (40). C20:4ω6 can also be involved in apoptosis via the release of ceramide by activating sphingomyelinase, which acts as a second messenger activating the apoptotic process (41). Increased levels of the E_2-series prostaglandins from C20:4ω6 can also be involved in activating the apoptotic process (42). A recent study investigating the role of C20:4ω6 in phenobarbital induced rat liver foci indicated the involvement of this FA in the tumour promoting mechanisms of phenobarbital (43). It would appear that C20:4ω6 plays a key role in controlling events which support the altered growth kinetics in hepatocyte nodules (Figure 1).

Apart from the role of FA in regulating prostaglandin production and signal transduction pathways, they are also key substrates for lipid peroxidation. LC-PUFA can play a role in the control of cell proliferation by inhibiting cell growth and stimulating and/or enhancing apoptosis by the increase of cellular lipid peroxidation and the subsequent breakdown products such as malondialdehyde (44,45). A key event with respect to the changes in FA metabolism in hepatocyte nodules appears to be a change in the Δ-6 desaturase enzyme and the implications this has on the LC-PUFA levels (6,30). The impairment of this enzyme has been observed in BL6 melanoma and Morris hepatoma 9618A cell lines and in various types of liver cancer with different origins (7–9). In the present study, impaired activity of this enzyme was observed in the nodule tissue indicated by the increased levels of the FA substrates C16:1ω7, C18:1ω9 and C18:2ω6 in PC, while the LC-PUFA products, C20:4ω6, C22:5ω6 and C22:6ω3, were decreased. The decreased levels of C22:5ω6 and C22:6ω3 in PC can also be related to the impaired activity of Δ-6 desaturase, as this enzyme has also been shown to be involved in the conversion of C22:4ω6 and C22:5ω3 to C22:5ω6 and C22:6ω3, respectively (46). The increased C18:1ω9 level, a FA with antioxidative properties, as well as the decrease in the LC-PUFA

have been associated with a decreased lipid peroxidation status in malignant lesions (6,7). A loss of lipid peroxidation has also been observed in pre-neoplastic hepatocellular lesions in rats and a decrease in the cytostatic 4-hydroalkenals, other aldehydes and peroxides could be related to the increased growth patterns observed in these lesions (12). The subsequent decrease in peroxidative metabolites, which are known to induce apoptosis (47), is likely to negatively affect the apoptotic process in the nodular environment (Figure 1). In normal regenerating liver, the level of C18:1ω9 decreased, C18:2ω6 increased and the LC-PUFA in PC decreased after day 1, implying a possible controlled involvement of the Δ-6 desaturase enzyme to reduce the lipid peroxidation level. It has been shown that the rate of lipid peroxidation is reduced in regenerating liver following partial hepatectomy which fits into the general hypothesis that increased cell proliferation is associated with a decreased rate of lipid peroxidation (7,48,49). This would suggest that the decrease in lipid peroxidation is another important event in sustaining hepatocyte regeneration both under normal and abnormal conditions (Figure 1).

The present study indicated that the persistent alterations in lipid metabolism in the hepatocyte nodules (dotted block in Figure 1) are likely to play an important role in the development of the malignant phenotype. The dynamic state of the lipid bilayer of cellular membranes would allow the manipulation of the lipid content of the membrane by dietary means, thereby modulating the activity of membrane proteins, the availability of FA for signal transduction pathways and prostaglandin synthesis and thereby determining cell survival (31). The altered cholesterol, phospholipid and FA profiles in the hepatocyte nodules could provide unique targets for developing strategies in chemoprevention with the inclusion of dietary manipulation in order to counteract the increased cellular proliferation and therefore the progression and subsequent development of these lesions into neoplasia.

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Dietary Modulation of Fatty Acid Profiles and Oxidative Status of Rat Hepatocyte Nodules: Effect of Different n-6/n-3 Fatty Acid Ratios

S. Abel[a,]*, M. De Kock[b], C.M. Smuts[c], C. de Villiers[d], S. Swanevelder[e], and W.C.A. Gelderblom[a]

[a]Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC Unit), [c]Nutritional Intervention Research Unit, [d]Diabetes Research Group/Primate Unit, [e]Biostatistics Unit, Medical Research Council, Tygerberg, South Africa, and [b]Department of Physiology, University of the Western Cape, Bellville, South Africa

ABSTRACT: Male Fischer rats were fed the AIN 76A diet containing varying n-6/n-3 FA ratios using sunflower oil (SFO), soybean oil (SOY), and SFO supplemented with EPA-50 and GLA-80 (GLA) as fat sources. Hepatocyte nodules, induced using diethylnitrosamine followed by 2-acetylaminofluorene/partial hepatectomy promotion, were harvested, with surrounding and respective dietary control tissues, 3 mon after partial hepatectomy. The altered growth pattern of hepatocyte nodules in rats fed SFO is associated with a distinct lipid pattern entailing an increased concentration of PE, resulting in increased levels of 20:4n-6. In addition, there is an accumulation of 18:1n-9 and 18:2n-6 and a decrease in the end products of the n-3 metabolic pathway in PC, suggesting a dysfunctional Δ-6-desaturase enzyme. The hepatocyte nodules of the SFO-fed rats exhibited a significantly reduced lipid peroxidation level that was associated with an increase in the glutathione (GSH) concentration. The low n-6/n-3 FA ratio diets significantly decreased 20:4n-6 in PC and PE phospholipid fractions with a concomitant increase in 20:5n-3, 22:5n-3, and 22:6n-3. The resultant changes in the 20:4/20:5 FA ratio and the 20:3n-6 FA level in the case of the GLA diet suggest a reduction of prostaglandin synthesis of the 2-series. The GLA diet also counteracted the increased level of 20:4n-6 in PE by equalizing the nodule/surrounding ratio. The low n-6/n-3 ratio diets significantly increased lipid peroxidation levels in hepatocyte nodules, mimicking the level in the surrounding and control tissue while GSH was decreased. An increase in n-3 FA levels and oxidative status resulted in a reduction in the number of glutathione-S-transferase positive foci in the liver of the GLA-fed rats. Modulation of cancer development with low n-6/n-3 ratio diets containing specific dietary FA could be a promising tool in cancer intervention in the liver.

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Investigations into the lipid content of tumor tissue indicate that the process of carcinogenesis is associated with an altered lipid profile that appears to play an important role in cell survival and the subsequent development into neoplasia. FA, as integral components of cell membranes, may influence neoplastic development by altering cellular integrity, the activation state of pre-carcinogens, and the capacity of the cell to respond to growth regulatory signals (1). Studies by Dyerberg and Bang (2) indicated that dysfunctions in eicosanoid metabolism can lead to certain illnesses and disorders such as cardiovascular and gastrointestinal diseases as well as an increased incidence of cancer (3). These disorders have been linked to an imbalanced PUFA intake, related to diets with a high n-6 and/or low n-3 FA content (3). Diets high in n-6 FA content have been shown to promote colon and breast cancer which is associated with the up-regulation of cyclooxygenase-2 (COX-2) and p21*ras* expression (4,5). In the colon, phospholipase A_2 (PLA_2) and COX-2 (5) are overexpressed in neoplastic cells which may lead to the release of 20:4n-6 from membrane phospholipids with an increased production of prostaglandins such as PGE_2 (6–8). Overproduction of PGE_2 has been implicated in tumor initiation and promotion, cell proliferation, and differentiation and is shown to modulate cellular and humoral immune responses by inhibiting the production of lymphocytes, interleukin, and antibodies. It also has been shown to inhibit the macrophage mediated cytotoxicity to cancer cells (6). However, 20:4n-6 is an important FA in maintaining normal cellular homeostasis because of the multiple roles it plays: (i) structurally as part of membrane phospholipids, (ii) functionally as a precursor to the 2-series eicosanoids, and (iii) as an intermediate involved in signal transduction pathways regulating cell proliferation and apoptosis (9). As 20:4n-6 appears to be one of the major players in the progression of hepatocyte nodules into neoplasia, the modulation of the 20:4n-6 level is therefore of importance (10). The addition of n-3 PUFA in the diet is known to displace and therefore decrease the n-6 FA content of cellular membranes (3,6,11). This displacement and decrease are of particular importance with respect to 20:4n-6. To prevent excessive n-6 FA replacement, it is suggested that a dietary combination of 18:3n-6 and 20:5n-3 should be used to main-

*To whom correspondence should be addressed at PROMEC Unit, Medical Research Council, P.O. Box 19070, Tygerberg, 7505, South Africa. E-mail: stefan.abel@mrc.ac.za

Abbreviations: 2-AAF, 2-acetylaminofluorene; CM, chloroform/methanol; CMS, chloroform/methanol/saline; COX-2, cyclo-oxygenase-2; DEN, diethylnitrosamine; GSH, glutathione (reduced form); GSSG, glutathione (oxidized form); $GSTP^+$, glutathione-S-transferase (placental) positive; LC-PUFA, long-chain PUFA; MDA, malondialdehyde; MUFA, monounsaturated fatty acid(s); SATS, saturated fatty acid(s); P/S, polyunsaturated to saturated fatty acid ratio; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; SFO, sunflower oil; SOY, soybean oil; Δ6 SP, ratio of substrates to products of the Δ6-desaturase enzyme.

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tain a critical level of 20:4n-6 in cell membranes (11,12). It has been shown, *in vitro* and *in vivo*, that supplementation with 18:3n-6 or evening primrose oil can maintain a steady state in the 20:4n-6 level (13,14). Dietary supplementation with 18:3n-6 and 20:5n-3 is also important with regard to supplying FA downstream of the Δ6-desaturase enzyme, which is known to be impaired in cancer tissue (15). In addition, 18:3n-6 is rapidly converted to 20:3n-6, a substrate for the 1-series prostaglandins that counteract the activities of the 2-series prostaglandins (11).

An important property of cancer cells is the low level of lipid peroxidation, partly due to unusually high levels of antioxidants such as vitamin E and 18:1n-9 (15,16). Another key molecule in determining the redox status in cells is the antioxidant glutathione which appears to be altered in cancer tissue (17,18). One of the main reasons, however, appears to be the low PUFA levels, especially n-3 PUFA, due to the impairment of the Δ6-desaturase enzyme (11,15). A previous study utilizing a liver cancer model in rats indicated that a distinct pattern with regard to FA metabolism exists in hepatocyte nodules (10). This entails low levels of the LCPUFA 22:5n-6 and 22:6n-3, and high levels of 18:1n-9 and 18:2n-6 typical of a Δ6-desaturase impairment. The decrease in the LCPUFA is also likely to be associated with a low oxidative status in hepatocyte nodules. Other important properties associated with hepatocyte nodule development include: (i) an increased concentration of the PE phospholipid fraction, resulting in a decrease in the PC/PE ratio and an increase in the level of 20:4n-6, (ii) elevated cholesterol, and (iii) an increase in membrane fluidity (10). Wood *et al.* (19) found similar elevations of PE in the plasma membrane and endoplasmic reticulum from hepatoma (7288CTC) cells grown in the hind legs of rats. With respect to FA levels, 18:1n-9 was increased in the PC and PE phospholipid fractions, while the level of 22:6n-3 was very low.

As tumors are dependent on host circulation for the type and amount of PUFA available, it should be possible to alter the FA composition by dietary means (20). Several studies have shown that the FA composition of cancer cells can be altered *in vitro* and *in vivo* by supplementation with dietary FA (21–23). The resultant changes in the FA content of the tumor may alter prostaglandin synthesis and relevant signaling pathways, thereby modulating the apoptotic/proliferative imbalance (24). The present study was conducted to monitor the effect of low n-6/n-3 FA ratio diets on the PUFA and oxidative status, and the modulating role on hepatocyte nodule development.

MATERIALS AND METHODS

Chemicals. Eicosapentaenoic acid (EPA-50) and GLA-80 were obtained from Callanish Ltd. (Breasclete, Scotland).

Animals and diets. The use of laboratory animals in this study was approved by the Ethics Committee of the Medical Research Council of South Africa. At weaning (body weight 50 g), male Fischer-344 rats were divided into three treatment groups (n = 20 rats per group) and their respective control groups (n = 5 rats per group) and fed the AIN 76A diet (25) containing fat (5% of diet) with varying n-6/n-3 FA ratios for the duration of the experiment. The different fat sources consisted of: (i) sunflower oil with a n-6/n-3 FA ratio of 250:1 (SFO, high n-6/n-3 ratio diet), (ii) SFO supplemented with EPA-50 and GLA-80, yielding a n-6/n-3 ratio of 12:1 (GLA, low n-6/n-3 ratio diet), and (iii) soybean oil yielding a n-6/n-3 ratio of 5:1 (SOY, low n-6/n-3 ratio diet).

To examine the influence of GLA on the nodule FA profile, a separate treatment group (n = 20) was fed the AIN 76A diet containing SFO and EPA-50 (without GLA) with an n-6/n-3 FA ratio of 12:1 (EPA diet). A schematic outline of the experimental design is illustrated in Figure 1.

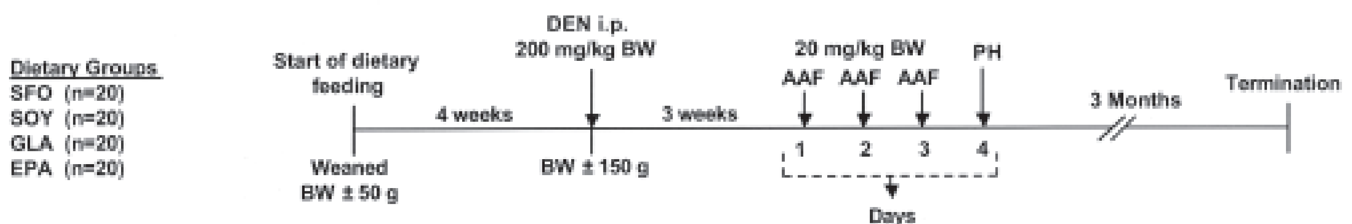

FIG. 1. The diagram depicts a timeline of the experimental procedure. Weaned male Fischer-344 rats (body weight, BW, 50 g) were divided into four experimental treatment groups (n = 20 rats per group) and were fed the AIN 76A diet containing fat (5% of diet) with varying n-6/n-3 FA ratios consisting of sunflower oil (SFO), soybean oil (SOY), sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 (GLA) and sunflower/eicosapentaenoic acid-50 (EPA) for the duration of the experiment. Control groups included rats fed SFO, SOY, and GLA diets without the carcinogen treatment. Hepatocyte nodules were induced in the experimental treatment groups as described in the Materials and Methods section. Rats were terminated 3 mon after the cancer promotion treatment and hepatocyle nodules and surrounding tissue were collected.

TABLE 1
FA Content of the Oils (% of Total) Used in the Experimental Diets[a]

		Low n-6/n-3 FA ratio oils		
	Sunflower (SFO)	Soybean (SOY)	Sunflower EPA-50 GLA-80 (GLA)	Sunflower EPA-50 (EPA)
14:0	0.07	0.10	0.14	0.11
16:0	6.68	11.26	5.77	5.59
17:0	0.04	ND	0.24	0.22
18:0	4.45	4.20	4.23	4.15
20:0	0.29	0.36	0.29	0.32
22:0	0.75	0.43	0.79	0.77
24:0	0.25	ND	ND	ND
Total	12.53	16.35	11.46	11.16
16:1	0.06	0.06	1.17	1.02
18:1	25.17	25.60	24.35	22.08
20:1	0.21	0.24	0.28	0.24
22:1	0.01	0.01	0.04	0.02
24:1	0.05	ND	ND	ND
Total	25.50	25.91	25.84	23.36
n-6				
18:2	62.25	48.53	53.42	60.15
18:3	0.01	ND	6.2	0.12
20:2	0.02	ND	ND	ND
20:3	ND	ND	0.08	0.02
20:4	ND	ND	0.12	0.07
22:4	0.02	0.04	0.03	0.03
22:5	0.01	ND	0.01	0.02
Total	62.31	48.57	59.90	60.41
n-3				
18:3	0.25	9.20	0.31	0.40
18:4	ND	ND	0.35	0.30
20:3	ND	ND	0.05	0.13
20:5	ND	ND	3.40	3.58
22:5	ND	ND	0.27	0.23
22:6	ND	ND	0.56	0.44
Total	0.25	9.20	4.94	5.08
n-6/n-3	249.24	5.28	12.12	11.89
PUFA	62.56	57.77	64.84	65.49
P/S Ratio	4.99	3.53	5.66	5.87

[a]n-6/n-3 = n-6 FA to n-3 FA ratio, ND = not detected, P/S ratio = PUFA to saturated FA ratio.

The n-6/n-3 FA ratios of the different dietary oils used (Table 1) were determined by GC (Varian 3300, Palo, Alto, CA). The diets were prepared and stored at 4°C under nitrogen for the duration of the experiment. The rats were housed separately in wire-bottomed cages under controlled lighting (12-h cycles), humidity, and temperature (23–25°C) with free access to water. They were fed *ad libitum* and weighed three times weekly.

Induction and harvesting of hepatocyte nodules. Hepatocyte nodules were induced in the treatment groups according to the method described by Solt and Farber (26). Briefly, the rats (body weight approximately 150 g) were injected intraperitoneally (i.p.) with a single dose (200 mg/kg body weight) of diethylnitrosamine (DEN) to effect cancer initiation. Promotion was effected 3 wk later by a daily intragastric dose (20 mg/kg body weight) of 2-acetylaminofluorene (2-AAF) on three consecutive days followed by partial hepatectomy on the fourth day. The rats were terminated 3 mon following cancer promotion, and hepatocyte nodules and surrounding tissue were collected. Control tissue was collected from rats only fed the SFO, SOY, and GLA diets without the carcinogen treatment. Tissue sections were immediately frozen on dry ice and stored at –80°C prior to analyses.

Lipid extraction. Lipids were extracted with chloroform/methanol (CM; 2:1, vol/vol) containing 0.01% BHT as antioxidant (27,28). Approximately 100 to 150 mg of the liver tissue was ground to a fine powder in liquid nitrogen and weighed in glass-stoppered tubes. The tissue was suspended

in 0.5 mL saline (0.9% NaCl in distilled water), and the lipids were extracted with 24 mL CM. The CM mixture was filtered (sinterglass filters using Whatman glass microfiber filters, Cat. No. 1820 866; Whatman International, Ltd., Maidstone, England) and evaporated to dryness *in vacuo* at 40°C. The extract was transferred to glass-stoppered tubes, washed with saline saturated with CMS (chloroform/methanol/saline; 86:14:1, by vol) containing 0.01% BHT, and stored at 4°C until analyzed.

FA analyses. The lipid extracts were fractionated by TLC, and the major phospholipid fractions, PC and PE, were collected for phospholipid and FA analyses (29). For FA analyses, the phospholipid fractions were transmethylated with 2 mL methanol/18 M sulfuric acid (95:5, vol/vol) at 70°C for 2 h. The FAME were extracted in hexane and analyzed by GC on a Varian 3300 gas chromatograph equipped with 30-m fused silica Megabore DB-225 columns with a 0.53-mm internal diameter (cat. no. 125-2232; Agilent Technologies, Palo Alto, CA). The individual FAME were identified by comparison of the retention times to those of a standard mixture of free FA, 14:0 to 24:1 (Nu-Chek-Prep Inc., Elysian, MN) and quantified using an internal standard (17:0; Sigma-Aldrich, St. Louis) and expressed as µg FA/mg protein.

Phospholipid and cholesterol analyses. The phospholipid concentrations of PC and PE (µg/mg protein) were determined colorimetrically using malachite green after digestion with perchloric acid (16 N) at 170°C for approximately 1 h (30). Total cholesterol (µg/mg protein) from the lipid extracts was determined by an enzymatic iodide method (31) using cholesterin-oxidase and -esterase (Preciset Cholesterol kit, cat. no.125512; Indianapolis, IN). The cholesterol/phospholipid molar ratio (Chol/PL) was calculated by adding PC and PE together representing the major membrane phospholipids and using the M.W. of 386.7, 787, and 744 for cholesterol, PC, and PE, respectively.

Lipid peroxidation. Liver homogenates were prepared (1:19: m/vol) in a 1.15% KCl/0.01 M phosphate buffer (pH 7.4) on ice and a 0.5 mL aliquot (2 mg protein/mL) incubated with 2.5 mM ferrous sulfate for 1 h at 37°C (32). Malondialdehyde (MDA) was measured by determining the TBARS level according to the method of Hu *et al*. (33), and the results were expressed as TBARS representing the µmol MDA equivalents/mg protein, using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ at 532 nm for MDA (34). Nonspecific lipid peroxidation was prevented by the incorporation of EDTA in the buffers and BHT in the reaction solutions for the TBARS assay.

Glutathione (GSH and GSSG) analysis. The glutathione, reduced (GSH) and oxidized forms (GSSG), was determined according to the method of Tietze (35). Tissue samples were homogenized (1:10 ratio) in a 15% TCA (wt/vol) and 1 mM EDTA solution for GSH and 6% perchloric acid (PCA, vol/vol), 3 mM M2VP (1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate) and 1 mM EDTA solution for GSSG, on ice. The homogenates were centrifuged at $10{,}000 \times g$ for 10 min, and 50 µL of the supernatant was added to glutathione reductase (5 units) and 75 µM DTNB [5,5′ dithiobis-(2-nitrobenzoic acid)], in a microtiter plate. The reaction was initiated with the addition of 0.25 mM NADPH (50 µL) to a final reaction volume of 200 µL, the absorbance monitored at 410 nm for 5 min, and the levels of GSH and GSSG determined from standard GSH and GSSG curves, respectively. The results were expressed as mM GSH or GSSG/g wet liver weight.

Immunohistochemistry. Tissue sections of the major liver lobes were taken from all treatment and control groups at termination of the rats and preserved in buffered formalin for GSTP staining according to the method of Ogawa *et al.* (36). Dewaxed tissue sections (5 µm) were immunostained with a strepavidin-biotin-peroxidase complex and an affinity-purified biotin-labeled goat anti-rabbit IgG serum (Vector Laboratories, Burlingame, CA). Negative controls, without the antibody, were also included to test the specificity of the anti-GSTP antibody binding. The number and size (internal diameter using the largest transverse of longitudinal measurement) of the $GSTP^+$ foci were quantified microscopically (4× objective) and categorized according to the internal diameter of the foci (10 to 20, 21 to 50, 51 to 100, >100 µm, and total, i.e., >20 µm). The results were expressed as number per cm^2.

Protein determination. Powdered liver preparations (10–15 mg) from the liquid nitrogen homogenization step were first solubilized in 5% SDS at 37°C, and the protein content was determined using a modified method of Lowry (37). The protein content in the liver homogenate prepared for the lipid peroxidation determination was determined as described by Kaushal and Barnes (38).

Statistical analyses. Descriptive statistics performed on the data indicated that all groups were normally distributed (Kolmogorov–Smirnof Test) with homogeneity among the variances (Levene's Test). Initial statistical analyses included two-way ANOVA testing for interaction effects between diet and tissue type, which was followed by one-way ANOVA testing for diet effects across all tissue types and also testing for tissue effects across all dietary groups. One-way ANOVA were also used to test for dietary group differences within each tissue type separately, as well as for tissue type differences within each dietary group. When dietary group differences or tissue type differences were present, Tukey's Studentized Range Test was used, testing for multiple pairwise comparisons between the means of the different groups. As the data were unbalanced, the Tukey–Cramér adjustment was made automatically. When only two groups were present, group differences were tested using Student's *t*-test. Statistical significance was considered at $P < 0.05$.

RESULTS

FA content of dietary oils (Table 1). FA analyses of the dietary oils showed that with regard to PUFA, the SFO oil mainly contained 18:2n-6 (62.25%) with an n-6/n-3 FA ratio of approximately 250:1 consisting of total n-6 and n-3 FA levels of 62.31 and 0.25%, respectively. The SOY oil primarily con-

TABLE 2
Comparative Phospholipid and Cholesterol Content and Parameters in the Nodule, Surrounding and Control Liver of Rats Fed a Diet with Varying n-6/n-3 FA Ratios Phospholipid Concentration[a]

		Phospholipid concentration (μg/mg protein)			PC/PE phospholipid ratio		
Diet		Nodule	Surrounding	Control	Nodule	Surrounding	Control
SFO	PC	113.63 ± 31.56^{A}	102.32 ± 11.08^{A}	107.87 ± 14.55^{A}	1.99 ± 0.53^{a}	3.07 ± 0.46^{b}	2.83 ± 0.30^{b}
	PE	57.20 ± 5.08^{A}	35.58 ± 7.05^{B}	39.71 ± 5.68^{B}			
SOY	PC	104.76 ± 11.33^{A}	95.40 ± 13.13^{A}	91.94 ± 6.89^{A}	2.19 ± 0.30^{a}	3.09 ± 0.39^{b}	2.90 ± 0.32^{b}
	PE	51.77 ± 6.71^{A}	30.80 ± 5.46^{B}	32.05 ± 4.64^{B}			
GLA	PC	106.90 ± 20.70^{A}	87.82 ± 10.55^{A}	99.09 ± 9.30^{A}	2.17 ± 0.19^{a}	2.60 ± 0.17^{b}	2.91 ± 0.45^{b}
	PE	49.09 ± 8.45^{A}	35.29 ± 4.72^{B}	31.95 ± 8.60^{B}			
EPA	PC	101.95 ± 21.65^{A}	82.10 ± 12.70^{A}	—	2.19 ± 0.36^{a}	2.83 ± 0.49^{b}	—
	PE	48.73 ± 5.24^{A}	29.71 ± 8.23^{B}	—			
		Cholesterol Concentration (μg/mg protein)			Cholesterol/phospholipid Molar Ratio		
SFO		19.73 ± 3.30^{a}	15.88 ± 3.12a	14.82 ± 2.45	0.24 ± 0.05a	0.22 ± 0.05ab	0.20 ± 0.04
SOY		15.17 ± 2.02^{A}b	13.97 ± 2.14^{AB}ab	12.32 ± 1.98^{B}	0.19 ± 0.02ab	0.22 ± 0.04ab	0.20 ± 0.02
GLA		13.99 ± 2.22b	11.93 ± 1.66b	11.94 ± 2.98	0.18 ± 0.01b	0.19 ± 0.02a	0.19 ± 0.04
EPA		13.84 ± 1.67b	13.05 ± 1.06ab	—	0.18 ± 0.02^{A}b	0.25 ± 0.04^{B}b	—

[a]Values are means ± SD of 5 or 6 replications. Initial statistical analyses included two-way ANOVA testing for interaction effects between diet and tissue type, followed by one-way ANOVA testing for overall diet effects across all tissue types and testing for overall tissue effects across all dietary groups. Separate one-way ANOVA was performed to test for significance between different tissue types separately within the same dietary group, as indicated by superscript uppercase letters within a row ($P < 0.05$). One-way ANOVA was also used to test separately for differences within the same tissue type, but compared between the different dietary groups, as indicated by lowercase letters ($P < 0.05$) and identical color in a column. SFO = sunflower oil diet, SOY = soybean oil diet, GLA = sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 oil diet, EPA = sunflower/eicosapentaenoic acid-50 oil diet. The cholesterol/phospholipid molar ratio was calculated using the sum of PC and PE, which constitute the two major phospholipid fractions in rat liver.

sisted of 18:2n-6 (48.53%) and 18:3n-3 (9.2%). The total n-6 and n-3 FA levels were 48.57 and 9.2%, respectively, with a n-6/n-3 ratio of approximately 5:1. The GLA dietary oil contained 18:2n-6 (53.42%) and 18:3n-6 (6.2%) compared to 60.15 and 0.12% in the EPA dietary oil. Both the GLA and EPA dietary oils contained total n-6 and n-3 FA levels of 60 and 5%, respectively, with an n-6/n-3 ratio of approximately 12:1. Both dietary oils also contained relatively high levels of 20:5n-3 (3.5%).

Due to the complexity and amount of data, all data for the lipid parameters and FA were first analyzed by two-way ANOVA for any diet and tissue interactions. Where a significant ($P < 0.05$) interaction occurred, the data were further analyzed by one-way ANOVA for diet effects across all tissue types and tissue effects across all dietary groups. Where no diet–tissue interactions were observed, the data were described as a diet and/or tissue effect, independent of each other. Because only overall interactions and effects were observed with this type of data analyses, the data were also separately analyzed by one-way ANOVA in which the different diet and tissue types were not grouped together as for the two-way ANOVA. The data in Tables 2, 3, and 4 and Figures 2 and 3 depict the results from these one-way ANOVA analyses.

Lipid parameters: phospholipid and cholesterol content (Table 2). (i) Phospholipids. No diet–tissue interaction was observed. Two-way ANOVA indicated significant ($P < 0.05$) effects due to the diet and tissue for PC and PE. Overall, the PC and PE levels were significantly ($P < 0.05$) higher in the nodule tissue. Overall the tissues, the EPA diet decreased ($P < 0.05$) the PC and PE levels in the nodules when compared to the SFO diet. The separate one-way ANOVA revealed a significantly ($P < 0.05$) higher PE level in the nodule tissue compared with the respective surrounding and control in all the diet groups (Table 2).

(ii) Cholesterol. No diet–tissue interaction was observed. Two-way ANOVA revealed significant ($P < 0.05$) effects due to the diet and tissue. The cholesterol level was significantly ($P < 0.05$) higher in the nodule tissue whereas the low n-6/n-3 ratio diets significantly ($P < 0.05$) decreased the cholesterol level when compared to the SFO dietary group. A similar effect was noticed when the data were analyzed by the separate one-way ANOVA. The GLA diet also significantly ($P < 0.05$) decreased the cholesterol level in the surrounding tissue compared with the SFO surrounding tissue.

(iii) Lipid parameter ratios (PC/PE and Chol/PL). No diet–tissue interaction was observed for the PC/PE ratio. Two-way ANOVA revealed a significant ($P < 0.05$) tissue effect with a lower PC/PE ratio in the nodule tissue. A significant ($P < 0.05$) diet and tissue interaction was observed for the Chol/PL ratio. Analyses by one-way ANOVA showed a significant ($P < 0.05$) diet effect reflected by a decrease in the Chol/PL ratio with the GLA diet when compared with the EPA diet. Separate one-way ANOVA revealed that the Chol/PL ratio was significantly ($P < 0.05$) decreased in the nodules by the GLA and EPA diets compared to the SFO diet (Table 2).

Comparative FA parameters: effect of SFO, SOY, GLA, and EPA diets on the FA content of the PC and PE phospholipid fraction of hepatocyte nodule, surrounding and control tissues (Tables 3 and 4). (i) Saturated FA (16:0, 18:0).

TABLE 3
Comparison of the Fatty Acid Profiles (μg fatty acid/mg protein) in the Phosphatidylcholine (PC) Phospholipid Fraction of the Nodule, Surrounding and Control Liver of Rats Fed a Diet with Varying n-6/n-3 Fatty Acid Ratios[a]

Diet		SFO			SOY			GLA			EPA	
	∀	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding
SATS												
16:0	N	13.96 ± 1.41	10.30 ± 0.91	9.13 ± 2.87	14.78 ± 1.49	11.68 ± 1.36	10.38 ± 0.72	14.59 ± 2.54	9.45 ± 1.51	9.10 ± 2.94	13.62 ± 2.09	9.04 ± 3.02
18:0	N	10.35 ± 2.31a	10.41 ± 1.47	13.25 ± 1.41	7.10 ± 0.81b	9.66 ± 1.02	12.10 ± 0.87	8.68 ± 1.51ab	9.65 ± 1.71	11.91 ± 3.04	8.24 ± 1.01ab	7.71 ± 2.49
Total	N	24.31 ± 3.23	20.71 ± 1.66	22.37 ± 3.88	21.88 ± 2.28	21.34 ± 2.21	22.47 ± 1.48	23.28 ± 3.76	19.10 ± 3.05	21.02 ± 5.45	21.87 ± 3.06	16.75 ± 5.50
MUFA												
16:1	Y	0.80 ± 0.13^{A}	0.49 ± 0.17^{B}	0.40 ± 0.11^{B}a	0.73 ± 0.09^{A}	0.43 ± 0.11^{B}	0.27 ± 0.07^{C}b	0.98 ± 0.20^{A}	0.45 ± 0.10^{B}	0.17 ± 0.05^{C}b	0.95 ± 0.18^{A}	0.44 ± 0.15^{B}
18:1	Y	7.53 ± 0.99^{A}	4.69 ± 0.88^{B}	4.78 ± 0.60^{B}a	6.04 ± 0.56^{A}	4.81 ± 0.50^{B}	3.73 ± 0.37^{C}b	7.14 ± 1.24^{A}	4.76 ± 0.70^{B}	3.37 ± 0.61^{C}b	7.08 ± 0.90^{A}	3.72 ± 1.34^{B}
Total	Y	8.33 ± 1.03^{A}	5.18 ± 0.91^{B}	5.18 ± 0.72^{B}a	6.78 ± 0.64^{A}	5.25 ± 0.60^{B}	4.01 ± 0.39^{C}b	8.12 ± 1.35^{A}	5.21 ± 0.73^{B}	3.54 ± 0.60^{C}b	8.03 ± 1.05^{A}	4.16 ± 1.49^{B}
n-6												
18:2	N	8.89 ± 1.38	5.50 ± 1.07	5.30 ± 1.36	8.99 ± 1.22	6.30 ± 0.45	5.02 ± 0.58	8.24 ± 1.46	5.91± 0.87	5.03 ± 1.11	10.28 ± 1.32	5.77 ± 2.62
18:3	Y	0.23 ± 0.05a	0.24 ± 0.05ab	0.32 ± 0.05a	0.14 ± 0.02^{A}b	0.22 ± 0.02^{B}ab	0.17 ± 0.04^{A}b	0.26 ± 0.05a	0.28 ± 0.05a	0.20 ± 0.03b	0.19 ± 0.02a	0.18 ± 0.07b
20:3	Y	0.23 ± 0.05^{A}	0.33 ± 0.10A^{B}a	0.41 ± 0.10^{B}	0.24 ± 0.04^{A}	0.32 ± 0.02^{B}a	0.36 ± 0.08^{B}	0.32 ± 0.06^{A}	0.57 ± 0.09^{B}b	0.36 ± 0.09^{A}	0.30 ± 0.06	0.41 ± 0.16ab
20:4	N	18:32 ± 2.85a	15.57 ± 2.38	19.10 ± 1.75a	11.47 ± 1.21b	14.56 ± 1.37ab	16.10 ± 1.15b	14.12 ± 2.48ab	15.84 ± 2.65a	15.83 ± 1.80b	13.36 ± 1.68b	11.97 ± 2.38b
22:4	N	0.41 ± 0.1a	0.56 ± 0.14a	0.44 ± 0.11	0.08 ± 0.01b	0.19 ± 0.11b	0.11 ± 0.03b	0.12 ± 0.02b	0.13 ± 0..03b	0.12 ± 0.04b	0.09 ± 0.01b	0.07 ± 0.03b
22:5	Y	1.24 ± 0.32^{A}a	2.43 ± 0.32^{B}a	2.33 ± 0.18^{B}a	0.05 ± 0.02^{A}b	0.16 ± 0.05^{B}b	0.19 ± 0.02^{B}b	0.08 ± 0.02b	0.12 ± 0.03b	0.16 ± 0.07b	0.05 ± 0.02^{A}b	0.09 ± 0.02^{B}b
Total	N	28.42 ± 5.39a	25.21 ± 4.47a	27.90 ± 3.07a	20.97 ± 2.30b	22.08 ± 2.16ab	21.94 ± 1.79b	23.14 ± 4.04ab	22.85 ± 3.67ab	21.69 ± 4.88b	24.26 ± 2.94ab	17.54 ± 6.12b
n-3												
18:3	Y	004 ± 0.01a	0.05 ± 0.01a	0.05 ± 0.02a	0.13 ± 0.02b	0.10 ± 0..02b	0.08 ± 0.01b	0.08 ± 0.01c	0.08 ± 0.02bc	0.05 ± 0.01a	0.08 ± 0.04bc	0.06 ± 0.02ac
20:5	Y	ND	ND	ND	0.16 ± 0.03a	0.11 ± 0.02a	0.15 ± 0.05	0.25 ± 0.05^{A}b	0.27 ± 0.11^{A}b	0.09 ± 0.05^{B}	0.30 ± 0.04b	0.27 ± 0.12b
22:5	Y	0.02 ± 0.01a	0.03 ± 0.01a	0.03 ± 0.01a	0.34 ± 0.05b	0.45 ± 0.12b	0.47 ± 0.10b	0.60 ± 0.15c	0.65 ± 0.15b	0.44 ± 0.12b	0.52 ± 0.06c	0.46 ± 0.24b
22:6	Y	0.17 ± 0.04^{A}a	0.24 ± 0.06^{AB}a	0.30 ± 0.06^{B}a	1.03 ± 0.20^{A}b	2.61 ± 1.02^{B}b	3.45 ± 0.47^{B}b	1.96 ± 0.44^{A}c	3.04 ± 0.54^{B}b	3.28 ± 1.10^{B}b	1.78 ± 0.37^{A}c	2.49 ± 0.65^{B}b
Total	Y	0.23 ± 0.06^{A}a	0.31 ± 0.08^{AB}a	0.37 ± 0.07^{B}a	1.66 ± 0.25^{A}b	3.27 ± 1.16^{B}b	4.15 ± 0.55^{B}b	2.90 ± 0.58^{A}c	4.04 ± 0.78^{B}b	3.86 ± 1.23^{B}b	2.69 ± 0.46c	3.28 ± 0.97b
PUFA	N	28.66 ± 5.45	25.53 ± 4.54	28.27 ± 3.13	22.63 ± 2.47	25.36 ± 1.72	26.10 ± 2.24	26.04 ± 4.51	26.89 ± 4.43	25.55 ± 6.01	26.95 ± 3.31	20.82 ± 7.07
LCPUFA	N	19.49 ± 0.43a	19.74 ± 3.30	22.61 ± 1.21	13.36 ± 1.33b	18.74 ± 1.25	20.83 ± 1.50	17.47 ± 2.80ab	20.62 ± 3.22	20.27 ± 4.46	16.40 ± 1.96ab	14.81 ± 4.09
FA ratios												
20:4 N/S		1.18 ± 0.09a		——	0.79 ± 0.12b		—	0.87 ± 0.14b		—	1.12 ± 0.15a	
20:4/20:5	Y	3.23 ± 0.08a	3.20 ± 0.08a	3.28 ± 0.04a	1.87 ± 0.06^{A}b	2.12 ± 0.11^{B}b	2.04 ± 0.11^{AB}b	1.75 ± 0.04^{A}bc	1.80 ± 0.15^{A}c	2.27 ± 0.24^{B}b	1.65 ± 0.02c	1.63 ± 0.06c
Δ6 S/P	Y	5.86 ± 0.73^{A}a	2.11 ± 0.31^{B}	1.96 ± 0.34^{B}	8.11 ± 1.77^{A}b	2.18 ± 0.36^{B}	1.50 ± 0.15^{B}	4.01 ± 0.73^{A}c	1.97 ± 0.04^{B}	1.60 ± 0.18^{B}	5.52 ± 0.79^{A}ac	2.24 ± 0.56^{B}
n-6/n-3	Y	123.21 ± 10.67^{A}a	81.42 ± 6.70^{B}a	76.85 ± 11.30^{B}a	12.79 ± 1.64^{A}b	7.94 ± 2.13^{B}b	5.32 ± 0.50^{B}b	8.07 ± 1.02^{A}c	5.69 ± 0.36^{B}b	5.80 ± 1.08^{B}b	9.12 ± 0.92^{A}c	5.28 ± 0.41^{B}b
P/S	Y	1.17 ± 0.10a	1.23 ± 0.17ab	1.29 ± 0.21	1.03 ± 0.06^{A}b	1.19 ± 0.06^{B}a	1.16 ± 0.03^{B}	1.12 ± 0.09^{A}ab	1.42 ± 0.15^{B}b	1.23 ± 0.15^{AB}	1.24 ± 0.05a	1.24 ± 0.03ab

[a]Values are means ± SD of 5 or 6 replications. ∀, A dietary group–tissue type interaction, analyzed by two-way ANOVA, Y = a significant ($P < 0.05$) diet–tissue interaction and N = no interaction. Initial statistical analyses included two-way ANOVA testing for interaction effects between diet and tissue type, followed by one-way ANOVA testing for overall diet effects across all tissue types and testing for overall tissue effects across all dietary groups. Separate one-way ANOVA was performed to test for significance between different tissue types separately within the same dietary group, as indicated by superscript uppercase letters within a row ($P < 0.05$). One-way ANOVA was also used to test separately for differences within the same tissue type across the different dietary groups, as indicated by lowercase letters ($P < 0.05$) in a row. SFO = sunflower dietary oil, SOY = soybean dietary oil, GLA = sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 dietary oil, EPA = sunflower/eicosapentaenoic acid-50 dietary oil, SATS = saturated fatty acids, MUFA = monounsaturated fatty acids, LC PUFA, long-chain PUFA, ND = not detected, 20:4/20:5 = log ratio of 20:4n-6 to 20:5n-3, n-6/n-3 = n-6 to n-3 fatty acid ratio, P/S = polyunsaturated to saturated FA ratio, Δ6 S/P=ratio of the substrates to products of the Δ-6-desaturase enzyme, 20:4 N/S = 20:4n-6 nodule to surrounding tissue ratio.

TABLE 4

Comparison of the Fatty Acid Profiles (μg fatty acid/mg protein) in the Phosphatidylethanolamine (PE) Phospholipid Fraction of the Nodule, Surrounding and Control Liver of Rats Fed a Diet with Varying n-6/n-3 Fatty Acid Ratios[a]

Diet		SFO			SOY			GLA			EPA	
	∀	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding
SATS												
16:0	N	2.98 ± 0.63a	3.06 ± 0.60	2.43 ± 0.72	3.77 ± 0.44ab	3.14 ± 0.55	2.72 ± 0.45	3.92 ± 0.63b	3.01 ± 0.50	2.46 ± 0.72	3.73 ± 0.48ab	2.65 ± 1.27
18:0	N	8.18 ± 1.20	4.17 ± 0.85	5.67 ± 1.69	6.40 ± 0.85	3.84 ± 0.30	4.73 ± 0.44	6.84 ± 1.92	4.97 ± 1.04	4.89 ± 1.96	7.13 ± 0.98	3.43 ± 1.43
Total	N	11.16 ± 1.44	7.24 ± 1.05	8.10 ± 1.31	10.17 ± 1.24	6.98 ± 0.66	7.45 ± 0.88	10.77 ± 2.08	7.98 ± 1.36	7.34 ± 2.48	10.86 ± 1.31	6.08 ± 2.66
MUFA												
16:1	N	0.13 ± 0.03	0.09 ± 0.04	0.07 ± 0.04	0.14 ± 0.03	0.08 ± 0.04	0.05 ± 0.02	0.15 ± 0.03	0.07 ± 0.01	0.03 ± 0.01	0.16 ± 0.03	0.05 ± 0.02
18:1	Y	3.97 ± 0.50^{A}a	1.56 ± 0.36^{B}	1.76 ± 0.36^{B}a	2.93 ± 0.24^{A}b	1.62 ± 0.30^{B}	1.05 ± 0.15^{C}b	3.00 ± 0.58^{A}b	1.79 ± 0.25^{B}	1.02 ± 0.27^{C}b	3.36 ± 0.46^{A}ab	1.22 ± 0.55^{B}
Total	Y	4.10 ± 0.50^{A}a	1.66 ± 0.34^{B}	1.83 ± 0.33^{B}a	3.07 ± 0.25^{A}b	1.70 ± 0.33^{B}	1.09 ± 0.16^{C}b	3.15 ± 0.58^{A}b	1.86 ± 0.26^{B}	1.05 ± 0.27^{C}b	3.52 ± 0.46^{A}ab	1.27 ± 0.57^{B}
n-6												
18:2	N	2.61 ± 0.34	1.27 ± 0.35	1.36 ± 0.43	2.35 ± 0.17	1.46 ± 0.67	0.98 ± 0.16	2.06 ± 0.26	1.51 ± 0.18	0.99 ± 0.21	2.55 ± 0.34	1.25 ± 0.68
18:3	Y	0.02 ± 0.01^{A}	0.02 ± 0.01^{A}	0.04 ± 0.01^{B}	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01^{A}	0.03 ± 0.01^{B}	0.02 ± 0.01^{AB}	0.01 ± 0.01	0.01 ± 0.01
20:3	Y	0.10 ± 0.02^{AB}	0.09 ± 0.04^{A}a	0.17 ± 0.07^{B}a	0.08 ± 0.04	0.05 ± 0.03a	0.08 ± 0.02b	0.09 ± 002^{A}	0.15 ± 0.03^{B}b	0.08 ± 0.03^{A}b	0.08 ± 0.01	0.09 ± 0.05ab
20:4	Y	10.25 ± 1.54^{A}a	5.66 ± 1.49^{B}ab	7.78 ± 2.92^{AB}	6.56 ± 0.72^{A}b	4.60 ± 0.75^{B}ab	5.20 ± 0.59^{B}	7.93 ± 1.41^{A}b	6.87 ± 1.34^{AB}a	5.36 ± 2.05^{B}	7.93 ± 1.07^{A}b	4.23 ± 1.83^{B}b
22:4	Y	1.04 ± 0.20^{A}a	0.63 ± 0.20^{B}a	0.69 ± 0.35^{AB}a	0.22 ± 0.02b	0.21 ± 0.08b	0.15 ± 0.02b	0.21 ± 0.08^{A}b	0.24 ± 0.06^{AB}b	0.15 ± 0.05^{B}b	0.21 ± 0.04^{A}b	0.12 ± 0.05^{B}b
22:5	N	2.38 ± 0.45a	2.13 ± 0.45a	2.74 ± 1.09a	0.10 ± 0.02b	0.16 ± 0.04b	0.14 ± 0.01b	0.14 ± 0.02b	0.14 ± 0.04b	0.12 ± 0.06b	0.09 ± 0.02b	0.07 ± 0.02b
Total	N	16.40 ± 2.49^{A}a	9.80 ± 2.40^{B}a	12.78 ± 4.83^{AB}a	9.32 ± 0.82b	6.68 ± 1.44ab	6.55 ± 0.79b	10.43 ± 1.85b	8.92 ± 1.62ab	6.73 ± 2.39b	10.87 ± 1.46b	5.78 ± 2.63b
n-3												
18:3	N	0.03 ± 0.01a	0.02 ± 0.01a	0.02 ± 0.01a	0.08 ± 0.01b	0.06 ± 0.01b	0.04 ± 0.01b	0.05 ± 0.02c	0.04 ± 0.01ab	0.02 ± 0.01a	0.05 ± 0.01c	0.03 ± 0.02ab
20:5	Y	ND	ND	ND	0.09 ± 0.01^{A}a	0.05 ± 0.02^{B}a	0.08 ± 0.04^{AB}	0.14 ± 0.03^{A}b	0.15 ± 0.03^{A}b	0.05 ± 0.02^{B}	0.19 ± 0.03b	0.13 ± 0.07b
22:5	Y	0.05 ± 0.01^{A}a	0.03 ± 0.01^{B}a	0.04 ± 0.02^{AB}a	0.65 ± 0.09^{A}b	0.34 ± 0.12^{B}b	0.39 ± 0.08^{B}b	0.99 ± 0.30^{A}c	0.83 ± 0.19^{A}c	0.40 ± 0.17^{B}b	0.94 ± 0.14^{A}c	0.47 ± 0.29^{B}b
22:6	Y	0.35 ± 0.06^{A}a	0.23 ± 0.08^{B}a	0.37 ± 0.16^{AB}a	2.32 ± 0.23^{A}b	2.10 ± 0.76^{A}b	3.07 ± 0.39^{B}b	3.63 ± 0.56c	3.91 ± 0.75c	3.26 ± 1.49b	3.45 ± 0.52^{A}c	2.31 ± 0.76^{B}b
Total	Y	0.42 ± 0.07^{A}a	0.28 ± 0.09^{B}a	0.43 ± 0.19^{AB}a	3.14 ± 0.31^{AB}b	2.55 ± 0.93^{A}b	3.58 ± 0.48^{B}b	4.80 ± 0.88c	4.94 ± 0.98c	3.74 ± 1.66b	4.62 ± 0.69^{A}c	2.94 ± 1.11^{B}b
PUFA	N	16.83 ± 2.56a	10.08 ± 2.49ab	13.21 ± 5.00	12.46 ± 1.05b	9.23 ± 1.87a	10.13 ± 1.25	15.23 ± 2.71ab	13.86 ± 2.57b	10.47 ± 4.04	15.49 ± 2.06ab	8.72 ± 3.73a
LCPUFA	Y	14.17 ± 1.06^{A}a	8.77 ± 1.99^{B}ab	11.80 ± 1.10^{B}	10.01 ± 0.90^{A}b	7.70 ± 1.15^{B}a	9.10 ± 0.98^{AB}	13.23 ± 2.17a	12.28 ± 2.19b	9.44 ± 3.41	12.88 ± 1.59^{A}a	7.42 ± 2.77^{B}a
FA ratios												
20:4 N/S		1.81 ± 0.31a		——	1.43 ± 0.27ab		—	1.15 ± 0.25b		—	1.87 ± 0.24a	
20:4/20:5	Y	3.01 ± 0.06^{A}a	2.74 ± 0.10^{B}a	2.87 ± 0.13^{AB}a	1.89 ± 0.02b	1.99 ± 0.08^{A}b	1.83 ± 0.12^{B}b	1.76 ± 0.02^{A}c	1.65 ± 0.05^{B}c	2.00 ± 0.10^{C}b	1.63 ± 0.02^{A}d	1.53 ± 0.03^{B}d
Δ6 S/P	Y	1.37 ± 0.11^{A}a	0.82 ± 0.13^{B}	0.68 ± 0.10^{B}a	1.37 ± 0.13^{A}a	0.83 ± 0.18^{B}	0.48 ± 0.03^{C}b	0.86 ± 0.08^{A}b	0.65 ± 0.03^{B}	0.49 ± 0.08^{C}ab	1.06 ± 0.09^{A}b	0.75 ± 0.21^{B}
n-6/n-3	N	39.03 ± 2.49a	36.11 ± 5.01a	31.67 ± 7.64a	2.98 ± 0.24b	2.61 ± 1.47b	1.84 ± 0.09b	2.17 ± 0.09b	1.82 ± 0.11b	1.86 ± 0.17b	2.36 ± 0.20b	1.92 ± 0.21b
P/S	Y	1.51 ± 0.12a	1.39 ± 0.26	1.61 ± 0.38	1.23 ± 0.10b	1.32 ± 0.20	1.36 ± 0.04	1.43 ± 0.17^{A}a	1.74 ± 0.20^{B}	1.43 ± 0.26^{AB}	1.43 ± 0.05a	1.45 ± 0.12

[a]Values are means ± SD of 5 or 6 replications. ∀, A dietary group–tissue type interaction, analyzed by two-way ANOVA, Y = a significant ($P < 0.05$) diet–tissue interaction and N = no interaction. Initial statistical analyses included two-way ANOVA testing for interaction effects between diet and tissue type, followed by one-way ANOVA testing for overall diet effects across all tissue types and testing for overall tissue effects across all dietary groups. Separate 1-way ANOVA was performed to test for significance between different tissue types separately within the same dietary group, as indicated by superscript uppercase letters within a row ($P < 0.05$). One-way ANOVA was also used to test separately for differences within the same tissue type across the different dietary groups, as indicated by lowercase letters ($P < 0.05$) in a row. SFO = sunflower dietary oil, SOY = soybean dietary oil, GLA = sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 dietary oil, EPA=sunflower/eicosapentaenoic acid-50 dietary oil, SATS=saturated fatty acids, MUFA=monounsaturated fatty acids, ND=not detected, 20:4/20:5=log ratio of 20:4n-6 to 20:5n-3, n-6/n-3 = n-6 to n-3 fatty acid ratio, LCPUFA = long-chain PUFA, P/S = polyunsaturated to saturated fatty acid ratio, Δ6 S/P = ratio of the substrates to products of the Δ6-desaturase enzyme, 20:4 N/S = 20:4n-6 nodule to surrounding tissue ratio.

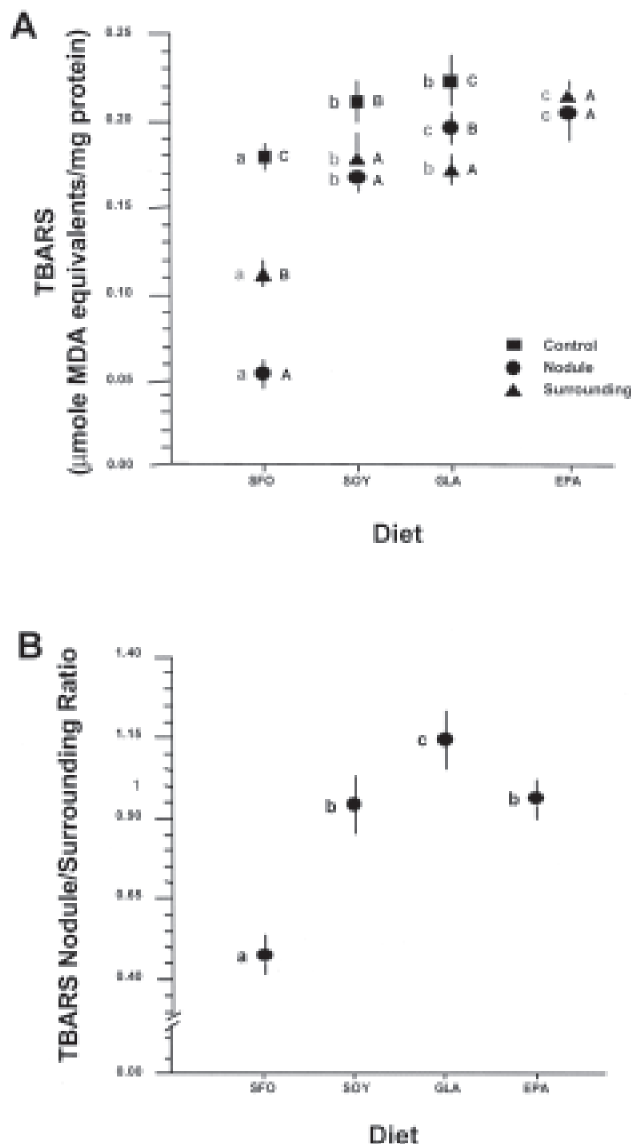

FIG. 2. The TBARS level (µmole MDA equivalents/mg protein) was determined in hepatocyte nodules, surrounding and control tissue samples from rat livers modulated with diets containing sunflower oil (SFO), soybean oil (SOY), sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 (GLA) and sunflower/eicosapentaenoic acid-50 (EPA) as fat sources (Fig. 2A). Two-way ANOVA indicated a significant diet-tissue interaction. One-way ANOVA showed that the overall TBARS level was significantly ($P < 0.05$) increased by the low n-6/n-3 ratio diets compared with the SFO-fed group. Over all the diets, the lowest TBARS level ($P < 0.05$) was observed in the nodules compared with the control tissue. Statistical analyses by one-way ANOVA of the separate tissue types and dietary groups were also done. This was performed to test for significance between different tissue types within the same dietary group, as indicated by uppercase letters, and for differences within the same tissue type, but compared between the different dietary groups, as indicated by lowercase letters ($P < 0.05$). Control (a,b); surrounding (c–e); nodules (f–h). Figure 2B represents the change in ratio between nodule and surrounding tissue (nodule/surrounding ratio) compared with a theoretically value of 1 (i.e., no difference between nodule and surrounding tissue). Statistical analysis by one-way ANOVA was performed to test for significance ($P < 0.05$) between different dietary groups, as indicated by lowercase letters.

PC fraction. No significant diet–tissue interactions were observed. Two-way ANOVA showed a significant ($P < 0.05$) tissue effect for 16:0, 18:0, and the total saturated FA (SATS), as well as a diet effect ($P < 0.05$) for 18:0. Overall, the levels of 16:0 and the total SATS were higher in the nodule tissue than in the surrounding and control, except for 18:0 which was higher in the control tissue. Separate one-way ANOVA showed that the level of 18:0 was also significantly ($P < 0.05$) decreased in the nodules by the SOY diet when compared with the SFO diet (Table 3).

PE fraction. There were no diet–tissue interactions. Two-way ANOVA revealed a significant ($P < 0.05$) tissue effect for 16:0, 18:0, and total SATS. Overall, the levels of 16:0, 18:0, and total SATS were higher in the nodules than in surrounding and control tissues. Separate one-way ANOVA revealed that 16:0 was significantly ($P < 0.05$) increased in the nodules by the GLA diet when compared with the SFO diet (Table 4).

(ii) Monounsaturated FA (16:1, 18:1). PC fraction. A significant ($P < 0.05$) diet-tissue interaction was observed for 16:1, 18:1, and the total monounsaturated FA (MUFA). Analyses by one-way ANOVA showed a significant ($P < 0.05$) tissue effect for these FA and the total MUFA. Overall, these FA levels were highest in the nodules compared with surrounding and control tissue. Separate one-way ANOVA indicated that the SOY and GLA diets significantly ($P < 0.05$) decreased the levels of these FA in the control tissue when compared with the SFO diet (Table 3).

PE fraction. A significant ($P < 0.05$) diet–tissue interaction was observed for 18:1 and the total MUFA only. One-way analyses revealed a significant ($P < 0.05$) tissue effect with the highest levels observed in the nodule tissue compared with the surrounding and control. Two-way ANOVA for 16:1 indicated a significant ($P < 0.05$) tissue effect with the highest level observed in the nodules. Separate one-way ANOVA revealed that 18:1 and total MUFA were significantly ($P < 0.05$) decreased in the nodule and control tissues by the SOY and GLA diets when compared with the SFO diet (Table 4).

(iii) n-6 PUFA (18:2, 18:3, 20:3, 20:4, 22:4, 22:5). PC fraction. Significant ($P < 0.05$) diet–tissue interactions were observed for 18:3, 20:3, and 22:5. One-way ANOVA showed a significant ($P < 0.05$) diet effect for 18:3 and 22:5, whereas a diet and tissue effect ($P < 0.05$) was observed for 20:3. Overall, the SOY and EPA diets decreased 18:3 when compared with the SFO and GLA fed groups. The GLA diet also increased 20:3 when compared with the other diets, and 22:5 was decreased by the low n-6/n-3 ratio diets. The tissue effect of 20:3 was reflected by a lower level in the nodule tissue. Separate one-way ANOVA indicated that the SOY diet significantly ($P < 0.05$) decreased 18:3 in the nodule tissue while 22:5 was decreased by the low n-6/n-3 ratio diets (Table 3).

Two-way ANOVA revealed significant ($P < 0.05$) tissue effects for 18:2, a diet effect for the total n-6 PUFA level, and both diet and tissue effects for 20:4 and 22:4. Overall, the 18:2 level was the highest in the nodule tissue, whereas 20:4

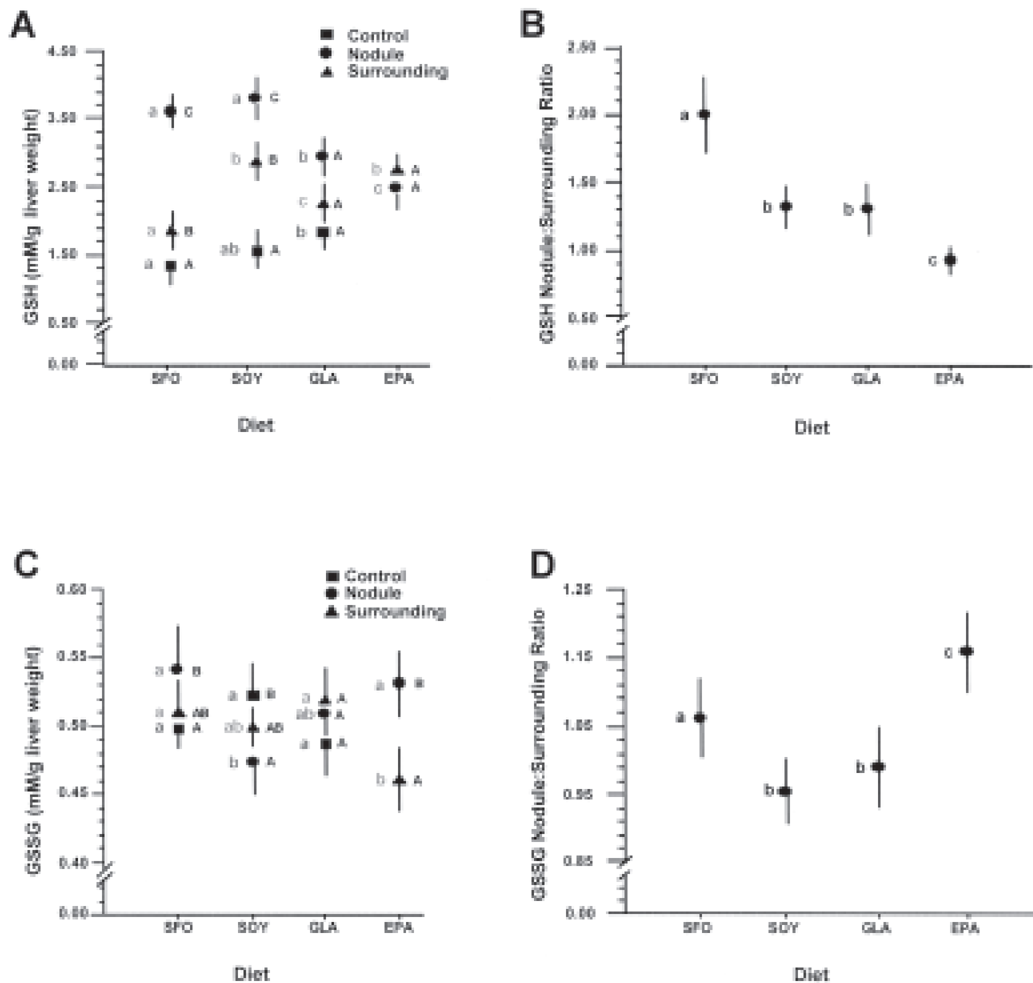

FIG. 3. The GSH (Fig. 3A) and GSSG (Fig. 3B) levels were determined in hepatocyte nodules, surrounding and control tissue from rats fed diets containing sunflower oil (SFO), soybean oil (SOY), sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 (GLA), and sunflower/eicosapentaenoic acid-50 (EPA) as fat sources. Two-way ANOVA indicated a significant ($P < 0.05$) diet–tissue interaction for GSH and GSSG, although only a significant ($P < 0.05$) effect due to tissue was observed for GSH. Over all the diets, the GSH level was significantly ($P < 0.05$) higher in the nodule tissue. Statistical analyses by one-way ANOVA of the separate tissue types and dietary groups were also done. These were performed to test for significance between different tissue types within the same dietary group, as indicated by uppercase letters, and for differences within the same tissue type, but compared between the different dietary groups, as indicated by lowercase letters ($P < 0.05$). Control (a,b); surrounding (b–e); nodules (d–h).Figures 3C and 3D indicate the change in ratio in GSH and GSSG between nodule and surrounding tissue, respectively. Statistical analysis by one-way ANOVA was performed to test for significance ($P < 0.05$) between different dietary groups, indicated by lowercase letters.

and 22:4 were decreased. With regard to the diet, 20:4 was decreased by the SOY and EPA diets compared to the SFO and GLA dietary groups, whereas 22:4 and the total n-6 PUFA levels were decreased by the low n-6/n-3 ratio diets. A similar effect was noticed when conducting the separate one-way ANOVA on the levels of 20:4 and 22:4 in the nodules, surrounding and control tissues (Table 3).

PE fraction. Significant ($P < 0.05$) diet–tissue interactions were observed for 18:3, 20:3, 20:4, and 22:4. One-way ANOVA showed a significant ($P < 0.05$) diet effect for 20:3 and 22:4, whereas a diet and tissue effect was observed for 18:3 and 20:4. 20:3 was decreased by the SOY diet while 22:4 was decreased by the low n-6/n-3 ratio diets when compared to the SFO diet. Overall, 18:3 was decreased by the SOY and EPA diets, while the level was lower in the nodule tissue when compared to the control. The SOY diet decreased the 20:4 level when compared to the SFO diet but, overall, this FA remained higher in the nodule tissue compared with surrounding and control. 22:4 was decreased by the low n-6/n-3 ratio diets when compared to the SFO fed group.

Two-way ANOVA revealed a significant ($P < 0.05$) tissue effect for 18:2, a diet effect for 22:5 and a diet–tissue effect for the total n-6 PUFA. Overall, 18:2 was the highest in the nodule tissue. With regard to diet, 22:5 was decreased by the low n-6/n-3 ratio diets compared with the SFO-fed group. Although the total n-6 PUFA level was decreased by the low n-6/

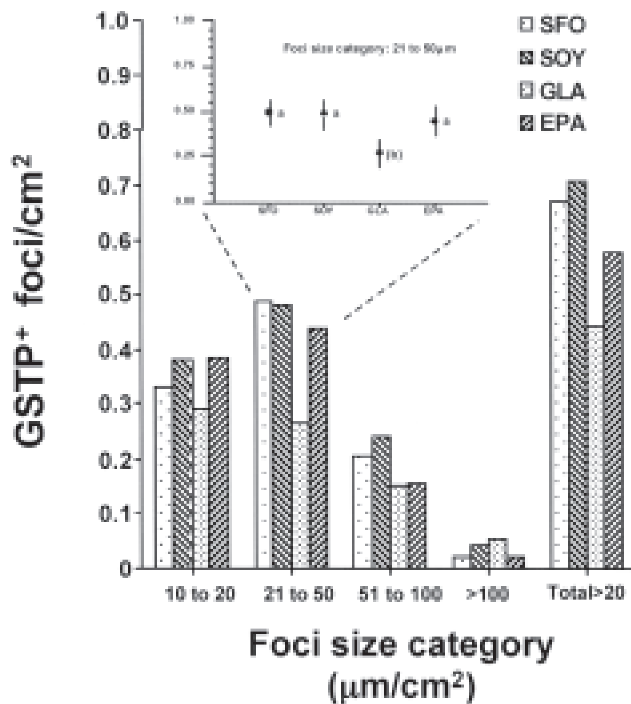

FIG. 4. Effect of diets containing sunflower oil (SFO), soybean oil (SOY), sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 (GLA), and sunflower/eicosapentaenoic acid-50 (EPA) as fat sources on number of GSTP$^+$ foci in liver sections. The number and size of the GSTP$^+$ foci were quantified by microscope (4x objective) and categorized according to the internal diameter of the foci as follows: 10 to 20, 21 to 50, 51 to 100, >100 μm/cm^2 and total >20. Statistical analysis by one-way ANOVA was performed to test for significance between different dietary groups within a foci size category. The graph insert shows the number of GSTP$^+$ foci in the 21 to 50 μm/cm^2 foci size category of the different dietary groups. The letter in parentheses (b) indicates a marginally significant ($P < 0.1$) effect.

n-3 ratio diets, overall the level remained the highest in the nodules. When considering the separate one-way ANOVA, 20:4 was decreased in the nodules while 22:4 and 22:5 were decreased in all the tissues by the low n-6/n-3 ratio diets. This resulted in a significant decrease in the total n-6 FA in the nodules (Table 4).

(iv) n-3 PUFA (18:3, 20:5, 22:5, 22:6). PC fraction. Significant ($P < 0.05$) diet–tissue interactions were observed for 18:3, 20:5, 22:5, and 22:6 as well as for the total n-3 PUFA. One-way ANOVA showed a significant ($P < 0.05$) diet and tissue effect for 18:3, 20:5 and 22:6, but only a diet effect ($P < 0.05$) for 22:5 and the total n-3 PUFA. The 18:3, 20:5, and 22:6 levels were increased by the low n-6/n-3 ratio diets compared with the SFO-fed group. Overall, 18:3 and 20:5 were higher in the nodule tissue, whereas 22:6 was lower when compared with surrounding and control tissue. The overall levels of 22:5 and total n-3 PUFA were increased by the low n-6/n-3 ratio diets.

Separate one-way ANOVA indicated that 18:3 was increased by the low n-6/n-3 ratio diets in the nodule, with the highest level obtained with the SOY diet. 20:5 was increased significantly ($P < 0.05$) by the GLA and EPA diets when compared with the SOY diet. 22:5, 22:6, and total n-3 PUFA were significantly increased by the low n-6/n-3 ratio diets in the nodules, with the highest levels obtained with the GLA and EPA diets (Table 3).

PE fraction. Two-way ANOVA showed a significant ($P < 0.05$) diet and tissue effect for 18:3. The low n-6/n-3 ratio diets increased the 18:3 level when compared with the SFO-fed group, with the highest level achieved by the SOY diet. Overall, 18:3 was highest in the nodule tissue. Significant ($P < 0.05$) diet–tissue interactions were observed for 20:5, 22:5, 22:6, and the total n-3 PUFA. One-way ANOVA showed significant ($P < 0.05$) effects due to the diet and tissue for 20:5 and 22:5, but only a diet effect for 22:6 and the total n-3 PUFA. The low n-6/n-3 ratio diets increased the levels of 20:5, 22:5, 22:6, and the total n-3 PUFA. However, of the three diets, the level of 22:5 was lowest in the SOY-fed group, whereas 22:6 and the total n-3 PUFA were the highest in the GLA-fed group. Over all the tissue, 20:5 and 22:5 were the highest in the nodules when compared to the control tissue. Separate one-way ANOVA showed a significant ($P < 0.05$) increase of the n-3 FA in all the tissues when compared to the SFO diet. The highest incorporation was obtained with the GLA and EPA diets (Table 4).

(v) PUFA and long-chain PUFA (LCPUFA). PC fraction. No significant diet–tissue interactions were observed. Two-way ANOVA only revealed significant ($P < 0.05$) effects due to the diet and tissue for LCPUFA. Over all the tissue, the LCPUFA level was decreased by the SOY and EPA diets when compared with the SFO diet in contrast to the GLA diet, where the tissue level of LCPUFA was not affected. The level of the LCPUFA was significantly ($P < 0.05$) lower in the nodule and surrounding tissue when compared with the control. Separate one-way ANOVA indicated that the LCPUFA was decreased in the nodules only by the SOY diet when compared to the SFO diet (Table 3).

PE fraction. Two-way ANOVA for PUFA revealed a significant ($P < 0.05$) diet and tissue effect. The PUFA level was lowered by the SOY diet when compared with the SFO and GLA diets. Overall, PUFA were the highest in the nodule tissue compared with surrounding and control. A significant ($P < 0.05$) diet–tissue interaction was observed for the LCPUFA while the one-way ANOVA indicated a significant diet and tissue effect. Over all the tissue, the GLA diet effected a higher level of LCPUFA when compared with the SOY diet, while the level was higher in the nodule tissue compared to the surrounding and control. Separate one-way ANOVA indicated that the SOY diet significantly ($P < 0.05$) decreased the levels of LCPUFA and PUFA in the nodules (Table 4).

(vi) Membrane FA ratios (Tables 3 and 4). 20:4/20:5, Δ6 S/P, n-6/n-3, P/S ratios. PC fraction. A significant ($P < 0.05$) diet–tissue interaction was observed for the 20:4n-6 to 20:5n-3 FA ratio (20:4/20:5), Δ6-desaturase FA substrate to product ratio (n-6 S/P), total n-6 to total n-3 PUFA ratio (n-6/n-3), and the polyunsaturated to saturated FA ratio (P/S). One-way ANOVA indicated a significant ($P < 0.05$) diet effect for the

20:4/20:5 and n-6/n-3 ratios, a tissue effect for the Δ6 S/P ratio and a diet and tissue effect for the P/S ratio. Over all the tissues, the 20:4/20:5 and n-6/n-3 ratios were decreased by the low n-6/n-3 ratio diets, with the lowest 20:4/20:5 ratio observed in the EPA dietary group. The Δ6 S/P ratio was overall the highest in the nodule tissue. The SOY diet significantly decreased the P/S ratio when compared with the GLA diet. Overall, the ratio was lower in the nodule tissue.

Separate one-way ANOVA indicated that the 20:4/20:5 and n-6/n-3 ratios were decreased in the nodules by the low n-6/n-3 ratio diets, with the highest decrease obtained with the GLA and EPA diets. The Δ6 S/P ratio was increased by the SOY diet but decreased by the GLA diet when compared with the SFO diet. The P/S ratio was decreased only by the SOY diet (Table 3).

PE fraction. A significant ($P < 0.05$) diet–tissue interaction was observed for the 20:4/20:5, Δ6 S/P, and the P/S ratios. One-way ANOVA indicated a significant ($P < 0.05$) diet effect for the 20:4/20:5 and P/S ratios and a diet and tissue effect for the Δ6 S/P ratio. Over all the tissues, the 20:4/20:5 ratio was decreased by the low n-6/n-3 ratio diets, with the EPA diet exhibiting the lowest ratio. The P/S ratio was decreased by the SOY diet when compared with the SFO and GLA diets. The Δ6 S/P ratio was decreased by the GLA diet compared with the SFO diet, while overall the ratio was the higher in the nodule tissue. Separate one-way ANOVA indicated that the 20:4/20:5 ratio was decreased in the nodules with the low n-6/n-3 ratio diets, with the lowest ratio obtained with the EPA diet. The Δ6 S/P ratio was decreased in the nodules by the GLA and EPA diets. The P/S ratio was reduced in the nodules by the SOY diet (Table 4).

The 20:4n-6 nodule to surrounding ratio (20:4n-6 N/S) was significantly ($P < 0.05$) decreased by both the SOY and GLA diets in PC and the GLA diet ($P < 0.05$) in PE.

Lipid peroxidation (Fig. 2). Significant ($P < 0.05$) diet and tissue interactions were revealed for the TBARS level. For the diet, one-way ANOVA showed that the overall TBARS level was significantly ($P < 0.05$) increased by the low n-6/n-3 ratio diets compared with the SFO diet (Fig. 2A). Over all the diets, the lowest TBARS level ($P < 0.05$) was observed in the nodules compared with the control tissue.

Separate one-way ANOVA showed that the TBARS level in the nodules of the low n-6/n-3 ratio diets was significantly ($P < 0.05$) increased to a similar level as the respective surrounding tissue (Fig. 2A). This resulted in a significant ($P < 0.05$) increase in the TBARS nodule/surrounding ratio in the low n-6/n-3 ratio diets with the highest ($P < 0.05$) ratio observed in the GLA diet (Fig. 2B). The low n-6/n-3 ratio diets exhibited a shift in the ratio toward an equilibrium of 1 between the nodule and surrounding tissue (Fig. 2B). The TBARS level in the nodules of the EPA and GLA diets was significantly higher than the SOY diet (Fig. 2A).

Glutathione (GSH and GSSG; Fig. 3). Both the GSH and GSSG levels showed a significant ($P < 0.05$) diet–tissue interaction, although this was related to a significant ($P < 0.05$) tissue effect in the case of GSH only. Over all the diets, the GSH level was significantly ($P < 0.05$) higher in the nodule tissue.

Separate one-way ANOVA showed that the GSH level was significantly higher ($P < 0.05$) in the nodules of the SFO dietary group compared with the respective surrounding and control tissue (Fig. 3A). A similar pattern was also observed with the SOY diet, although the level was significantly increased ($P < 0.05$) in the surrounding tissue. The EPA and GLA diets significantly reduced ($P < 0.05$) the GSH levels in the nodules (Fig. 3A), while the GLA diet significantly ($P < 0.05$) increased the level in control tissue. These changes resulted in a significant ($P < 0.05$) reduction in the GSH nodule to surrounding ratio (Fig. 3B) with the low n-6/n-3 ratio diets. The lowest GSH nodule to surrounding ratio was observed with the EPA diet, while the SOY and GLA diets exhibited similar ratios. With respect to GSSG, only the SOY diet significantly ($P < 0.05$) reduced the level in the nodules (Fig. 3C). The GSSG nodule to surrounding ratio (Fig. 3D) was significantly ($P < 0.05$) lowered by the SOY and GLA diets and significantly ($P < 0.05$) increased with the EPA diet compared to the SFO diet.

Induction of GSTP$^+$ foci (Fig. 4). No significant effect on the induction of GSTP$^+$ foci was observed with the SOY and EPA diets as compared to the SFO diet (Fig. 4). The GLA diet markedly lowered foci in all the size categories when compared to the SFO and SOY diets, except for the size category >100 μm/cm^2. The GLA diet marginally ($P = 0.052$) reduced the 21 to 50 μm/cm^2 focal size category when compared to the SFO, SOY, and EPA diets (Fig. 4 insert). This size category constituted 70 to 76% of the total focal count in these diets compared with the 64% obtained with the GLA diet.

DISCUSSION

Alterations in lipid metabolism are associated with cancer development affecting the function and growth of neoplastic cells (6,10). It appears that the regulatory mechanisms related to normal lipid metabolism are disrupted, thereby altering the growth and survival of preneoplastic cells (10,39). The interaction between cholesterol, phospholipids, and FA is of importance in maintaining the integrity and functioning of cell membranes (40,41). A study by Blom *et al.* (42) reported that, in response to cholesterol loading, there was an increase in the phospholipid species containing PUFA, resulting in an increased membrane unsaturation. In the present study, increased levels of cholesterol and PE in hepatocyte nodules were associated with changes in the SATS, MUFA, and PUFA. The low n-6/n-3 FA ratio diets mainly altered the LCPUFA content of the major phospholipid fractions as well as the cholesterol level and Chol/PL ratio in the nodule tissue, while the PC and PE phospholipid concentrations were decreased. These changes were shown to affect the membrane fluidity of hepatocyte nodules in a previous study (10). Alterations in these parameters could be of importance when considering the potential role of lipid rafts in controlling certain cell signaling events by modulating the activity of raft proteins (43,44).

PUFA are known to affect various cellular processes including proliferation and/or apoptosis through the formation of oxidation products and prostaglandins (11,45). In the pres-

ent study, the low n-6/n-3 ratio diets significantly increased the n-3 FA content, especially 20:5n-3 and 22:6n-3, with a concomitant decrease in the n-6 PUFA levels. The regulation of 20:4n-6 in cancer cells by dietary FA intervention utilizing 20:5n-3, a precursor of the 3-series prostaglandins, has been suggested as it competes for the cyclooxygenase enzyme. The 20:4n-6 to 20:5n-3 FA ratio is therefore of importance, indicating a shift in the type of prostaglandin synthesized (46). The low n-6/n-3 ratio diets significantly decreased 20:4n-6 and increased 20:5n-3 in PC and PE resulting in a decreased 20:4/20:5 ratio. The kinetics of changes in these parameters differed between the diets with the EPA diet inducing the largest effect. However, as 20:4n-6 has been implicated in the induction of apoptosis *via* the stimulation of ceramide release (47,48), excessive reduction of 20:4n-6 could impact negatively on the apoptotic rate in the nodules in counteracting the enhanced cell proliferation in this tissue type. Therefore, control over the level of 20:4n-6 by dietary intake of 20:5n-3 and 22:6n-3 can be exercised, due to their feedback inhibition on the activity of the Δ5- and Δ6-desaturases (8,12). Dietary GLA also modulates the levels of 20:3n-6 and 20:4n-6, thereby influencing prostaglandin synthesis (49,50). In the present study, the GLA diet modulated the 20:4/20:5 ratio by stabilizing the replacement/decrease of 18:3n-6, 20:3n-6, and 20:4n-6 by the SOY diet and 18:3n-6 and 20:4n-6 by the EPA diet. The characteristic difference in the 20:4n-6, PUFA, and LCPUFA pattern between the nodule and surrounding tissue was equalized by the GLA diet in PE. GLA decreased the 20:4n-6 nodule/surrounding ratio to approximately 1 by bypassing the impaired Δ-6-desaturase. This could compensate for the increased level of 20:4n-6 due to the persistent high concentration of PE in the nodule tissue. In contrast, the EPA diet had a comparable nodule to surrounding ratio to the SFO diet, as the replacement of 20:4n-6 by the n-3 PUFA was similar in the nodule and surrounding tissue. Therefore, changes in the levels of both 18:3n-6 and 20:3n-6, together with a shift in the 20:4 nodule/surrounding and 20:4/20:5 ratios in hepatocyte nodules, are likely to direct prostaglandin synthesis away from 20:4n-6. In addition, the n-3 PUFA inhibit the phospholipase-induced release of 20:4n-6 (51,52), while 20:5n-3 has a potentiating effect on the growth regulatory effects of 20:3n-6 presumably *via* the production of the 1-series prostaglandins and 15-OH-dihomo-GLA, which inhibits cell proliferation (11). This potentiating effect is manifested by the lack of interference of n-3 PUFA in the elongation of 18:3n-6 to 20:3n-6, which will favor the formation of PGE_1, thereby further modulating PGE_2 formation (11).

High LCPUFA and lipid peroxidation levels have been shown to be important mechanisms in the inhibition of cancer cell proliferation (15,53). Impairment of the Δ6-desaturase enzyme could therefore play an important role in maintaining low LCPUFA levels, contributing to a low oxidative status in cancer tissue, with respect to hepatocyte nodules (10,12). Changes in cholesterol, phospholipids, and FA unsaturation level of PC have been shown to be important for the optimal functioning of the Δ6-desaturase enzyme (54,55). In the present study the dysfunctional desaturase enzyme was not affected by the low n-6/n-3 ratio diets, indicated by the persistent elevated levels of 18:1n-9 and 18:2n-6 in the nodule PC and PE fractions and persistent lower level of 22:6n-3 in the nodule tissue. The increase in lipid peroxidation can therefore be attributed to the increase in the n-3 LCPUFA, specifically 22:6n-3, which is known to be a good substrate for lipid peroxidation (56). Although 22:6n-3 was increased by the low n-6/n-3 ratio diets, it remained lower in the nodules in comparison to the surrounding and control tissue. This is probably due to the impaired Δ-6-desaturase enzyme, which also catalyzes the conversion of 22:5n-3 to 22:6n-3. Of interest is that the level of 18:1n-9, reported to be an effective antioxidant (16), was decreased in PE by the SOY and GLA diets which could contribute to the increased lipid peroxidation level in the nodules. 18:1n-9 also promotes cell proliferation and is a negative regulator of apoptosis (57).

The regulation of the oxidative status by GSH is of importance with regard to tumor growth (58). A higher GSH level in breast cancer tumors is associated with an increased level of cell proliferation (59). GSH is also elevated in a number of drug-resistant tumor cell lines and tumor cells isolated from patients resistant to drug therapy (60). In the present study, the GSH level was significantly increased in the nodules of the SFO- and SOY-fed dietary rats, but was decreased in the nodules of the GLA and EPA dietary groups, which coincided with the increased TBARS formation. A study by Kokura *et al.* (61) showed a similar effect with respect to an increased lipid peroxidation and decreased GSH levels in tumor cells of rats dosed with 20:5n-3. The decreased GSH, associated with the EPA and GLA diets, can be attributed to the high level of dietary 20:5n-3, which lies downstream of the Δ6-desaturase. In contrast, the SOY diet did not decrease the GSH level in the nodules, presumably, as stated above, due to the source of dietary n-3 FA, i.e., 18:3n-3, which lies upstream of the Δ6-desaturase. As mentioned earlier, the nodules of the SOY dietary group had lower n-3 FA levels in contrast to the GLA and EPA groups (Tables 3 and 4). The increased lipid peroxidation mainly targeted the hepatocyte nodules with a far less significant impact on the oxidative status in the surrounding and control tissue. In the case of the GLA diet, the increased oxidative status in the nodule tissue contributed to the reduced number of $GSTP^+$ foci and/or nodules in the foci category comprising the largest number of foci. This decrease can be ascribed to the so-called "GLA effect", which seems to stabilize the level of 20:4n-6, when considering the 20:4n-6 nodule/surrounding ratio. In addition to the potential modulating effect of 18:3n-6, *via* 20:3n-6, and 20:5n-3 on prostaglandin synthesis in tumor cells, these PUFA, including 20:4n-6, also elicit responses by directly influencing intracellular signaling pathways and transcription factor activity (6,8,12,23). Other FA such as 18:1n-9 have also been shown to exhibit antiapoptotic effects and stimulate cell proliferation in MDA-MB-231 breast cancer cells. This effect was found to be linked to 18:1n-9 stimulation of PI 3-kinase (PI3-K) activity, a key signal transduction enzyme involved in the control of cell

growth (57). Of interest is that both the SOY and GLA diets significantly reduced the level of 18:1n-9 in the PE phospholipid fraction of the nodules.

The decrease in LCPUFA levels, due to impairment of the Δ6-desaturase in hepatocyte nodules, is likely to play an important role in the subsequent modulatory effect on nodule development. In this regard, the role of dietary FA in controlling cellular homeostasis seems to be important in balancing events related to apoptosis and cell proliferation to sustain normal growth. Certain dietary n-6 and n-3 FA, therefore, could create a multiple control mechanism for regulating cancerous growth depending on the dietary n-6/n-3 FA ratio as well as the type of FA constituting this ratio.

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