Cancer initiation by fumonisin B₁ in rat liver – role of cell proliferation

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Abstract

Fumonisin B₁ (FB₁), a carcinogenic mycotoxin produced by the fungus Fusarium verticillioides in corn, causes cancer initiation in rat liver in a similar manner to genotoxic carcinogens although apparently with different kinetics. The present experiment was designed to evaluate the role of regenerative cell proliferation, effected by partial hepatectomy (PH) and carbontetrachloride (CCl₄) and direct mitogen-induced hyperplasia, induced by lead nitrate (PbNO₃), on FB₁-induced cancer initiation. Initiation was effected over a period of 14 days by gavage administration of FB₁ at different daily doses ranging from 0.14 to 3.5 mg FB₁/100 g body weight while the stimuli for cell proliferation were introduced 7 days after the start of the FB₁ treatment. Based on the proliferative stimulus used, cancer promotion was effected 3 weeks after completion of the initiating treatment by 2-acetylaminofluorene (2-AAF) treatment followed by PH or carbon tetrachloride CCl₄ on day 4. Cancer initiation by FB₁ was associated with a hepatotoxic effect and an increase in lipid peroxidation. In contrast to compensatory liver cell proliferation induced by PH and CCl₄, mitogen-induced hyperplasia (PbNO₃) failed to enhance the cancer initiating potential of FB₁ suggesting that cancer induction by a non-genotoxic carcinogen is supported by regenerative cell proliferation. Cognizance of the enhancing role of cell proliferation during cancer initiation by FB₁ is required in assessing the risks posed by this mycotoxin to humans. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Fumonisin B₁; Cancer initiation; Cell proliferation; Risk assessment

1. Introduction

Hepatocyte proliferation is known to be an important parameter in the development of liver cancer induced by genotoxic carcinogens in the rat [1,2]. During cancer initiation, regenerative hepatocyte proliferation is presumably required to ‘fix’ the mutational event. Proliferation is also a major driving force in the clonal expansion of initiated cells to form hepatocyte nodules during cancer promotion. With genotoxins, the efficacy of initiation is enhanced either by regenerative cell proliferation as induced by partial hepatectomy (PH) or a necrogenic dosage of carbon tetrachloride (CCl₄). This is in sharp contrast with the
inefficacy of cell proliferation induced by mitogens such as lead nitrate (PbNO₃) [3,4]. At present it is not known whether a similar distinct effect on cancer initiation will be obtained with non-genotoxic chemical carcinogens.

Fumonisin B₁ (FB₁), a mycotoxin produced by *Fusarium verticillioides* (= *F. moniliforme*), was characterized as an active liver cancer-promoter isolated from corn cultures of the fungus [5]. Subsequent studies on the dosimetry of FB₁-induced cancer initiation indicated that prolonged exposure at relatively high and toxic dietary dosages is required [6]. It would appear that the absolute level of events (DNA mutations, etc.), resulting in initiation, occur at a far slower rate when compared to genotoxic carcinogens. One possible contribution to the slow kinetics could be the inhibitory effect of FB₁ on cell proliferation [7] resulting in an inhibition and/or delay in the cancer initiation process [1]. In this regard, pretreatment of rats with FB₁ decreased the efficacy of cancer initiation by diethylnitrosamine (DEN) in Sprague-Dawley rats [8]. Recently, FB₁ was shown to induce apoptosis in the liver [9], a protective mechanism whereby genetically damaged cells are removed, decreasing the population of initiated cells in the liver [10].

FB₁ is generally regarded as a non-genotoxin, in that it lacks activity in mutagenicity [11] and genotoxicity assays [6,12] and appears not to bind directly to DNA. Studies concerning the cancer initiating activity of FB₁ indicated that a cytotoxic/proliferative threshold exists for cancer initiation in rat liver and levels that fail to induce a toxic effect, lack cancer initiating activity [6]. This was further supported in a long-term study indicating that low dietary levels that cause only mild toxic changes fail to induce hepatocellular cancer in rats [13]. A recent study by Mehta et al. [14] in Sprague-Dawley rats also suggested that compensatory cell proliferation in response to cellular toxicity is a prerequisite for initiation. This is in agreement with the hypothesis set forward by Cohen and Ellwein [15] that, for non-genotoxic carcinogens, a cytotoxic/proliferative threshold is likely to exist for cancer induction. A recent study by Abel et al. [16] proposed that induction of oxidative stress and the resultant lipid peroxidation as secondary events of the FB₁-induced hepatotoxic effects, could possibly explain the delayed cancer initiating activity of FB₁ as compared to genotoxic carcinogens. A parallel was drawn with the cancer initiating potential of a choline deficient diet where hepatocyte cell death and lipid peroxidation also precede the initiation event [17]. It would appear that the hepatotoxicity, as proposed previously [13], could therefore be regarded as an initial event in FB₁-induced hepatocarcinogenesis.

In view of the critical role of regenerative cell proliferation during cancer initiation with genotoxic carcinogens in rat liver [18], the present study investigated the role of different modulators of cell proliferation on the cancer initiating potential of FB₁.

2. Materials and methods

2.1. Animals

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

2.2. Chemicals and solutions

2-acetylamino-fluorene (2-AAF), bromodeoxyuridine (BrdU), 2-thiobarbituric acid and PbNO₃ were obtained from Sigma Chemical Co. (Lyon, France). FB₁ was purified according to the method of Cawood et al. [19]. All solvents used were of analytical grade. The 2-AAF was prepared fresh by dissolving 300 mg in DMSO (1 ml) and sunflower oil (29 ml) to obtain a solution of 10 mg/ml. FB₁ was first dissolved in a small volume of 0.1 M NaOH and then made up to the desired volume with distilled water (pH 5.5). Antibodies (rabbit anti rat) against the placental glutathione S-tranferase (GSTP) were obtained from DAKO.

2.3. Treatments

2.3.1. Initiation by FB₁

Cancer initiation was effected by a repeated gavage treatment according to the method described by Gelderblom et al. [6]. Rats were treated on a daily basis with different doses of FB₁, yielding a total
dose of 2, 6, 20, 30 and 50 mg/100 g body weight (bw) over a period of 14 days (Fig. 1). These total dosages represent a daily dosage of 0.14; 0.42; 1.43; 2.1 and 3.5 mg FB₁/100 g bw, respectively. The body weight was recorded on a daily basis while the relative liver weight of a subgroup of animals (three per group), killed after the 14 day treatment, were determined and preserved for thiobarbituric acid reactive substances (TBARS) determination (see below). Promotion was effected 3 weeks later and consisted

Fig. 1. Experimental protocols for studying the role of different stimuli of cell proliferation on the cancer initiating potential of FB₁. Rats were treated with different doses of FB₁ by gavage over a period of 14 days followed by the promoting stimuli after 3 weeks consisting of 2-AAF (3 × 20 mg /kg body weight) on 3 consecutive days and either PH or CCl₄ on day 4. Rats were killed after a further 2 weeks. Stimuli for cell proliferation were introduced 7 days after commencement of the FB₁ treatment. Control groups without the FB₁-initiating treatment were also included.
of three gavage dosages of 2-AAF (20 mg/kg) on 3 consecutive days followed by PH on day 4. The latter was performed under ether anesthesia according to the original method described by Higgins and Anderson [20]. The drinking water of the rats was supplemented with 5% glucose for 24 h postoperatively. Rats were sacrificed 2 weeks after the promoting treatment and liver tissue sections preserved in buffered formalin for GSTP and BrdU staining where applicable. Eight to ten animals were used in each of the treatment groups.

Different cell proliferative stimuli were introduced halfway during the 14-day FB1 gavage treatment to monitor the role of cell proliferation on the cancer initiating potency.

2.3.1.1. Effect of PbNO3 and CCl4. Cancer initiation was effected by gavage treatment of the rats (eight animals per group) with FB1 over a period of 14 days as described above (Fig. 1). The highest dosage (50 mg FB1/100 g bw) was not included due to its marked effect on body weight gain. After 7 days of the FB1 treatment, rats of the FB1-treated groups as well as a control received a single dose of either CCl4 (0.2 ml/kg bw by gavage) or PbNO3 (100 µmol/kg; i.v. – femoral vein). Promotion was effected by a 2-AAF/PH treatment, 3 weeks after the initiation treatment and the experiment was terminated as described above.

2.3.1.2. The effect of PH. Initiation was effected by gavage treatment of the rats over a period of 14 days to obtain the desired total FB1 dose indicated in Fig. 1. Once again the highest dose of FB1 was not included due to its marked effect on body weight gain (50 mg FB1/100 g bw). After 7 days of the FB1 treatment, rats of the FB1-treated groups as well as a control received a single dose of either CCl4 (0.2 ml/kg bw by gavage) or PbNO3 (100 µmol/kg; i.v. – femoral vein). Promotion was effected by a 2-AAF/PH treatment, 3 weeks after the initiation treatment and the experiment was terminated as described above.

2.3.2. TBARS

To monitor the effect of FB1 on the level of oxidative damage, the liver of the rats were collected following the 14 day initiating treatment (three animals per group), frozen in liquid nitrogen and stored at −80°C. TBARS were determined in the liver homogenates according to the method described by Esterbauer and Cheeseman [21]. In short, a liver homogenate (10%) was prepared in 1.15% KCl in 3 mM EDTA solution containing 0.01% BHT as an antioxidant. After a further 13× dilution with the above solution, a subsample (1 ml) was mixed with two volumes of ice cold 10% trichloroacetic acid (TCA) to precipitate the proteins. After centrifugation (3000 rev./min for 10 min), the supernatant (2 ml) was mixed with an equal volume of the TBA reagent (0.67% in distilled water) and incubated at 100°C for 10 min. The mixture was allowed to reach room temperature, the absorbance was measured at 532 nm and the lipid peroxidation expressed as nmole malondialdehyde (MDA) equivalents/mg protein using the molar extinction coefficient of 1.56 × 105 M−1 cm−1.
for MDA. As the assay is not specific for MDA the term TBARS is used to described the reaction product.

2.6. Autopsies and histochemical analyses

Rats were sacrificed under ether anesthesia and the livers were observed macroscopically for any abnormalities. Tissue sections of all the major liver lobes were preserved in buffered formalin for H&E and GSTP analyses, respectively. GSTP staining was performed according to the method of Ogawa et al. [22] using the avidin-biotin-peroxidase complex (ABC) and affinity-purified biotin-labeled goat anti-rabbit IgG serum (Vector Laboratories, Burlingame, CA). Paraffin wax sections (5 μm) of the formalin preserved material were washed with petroleum benzene and a graded alcohol series before staining with the reagents in the ABC kit. GSTP-antiserum was used at a dilution of 1:800. Sections were counterstained with Carazzi’s haematoxylin to provide blue stained nuclei with the GSTP1 cells showing a reddish-brown pigmentation. Negative controls, omitting the primary antibody, were included to test for the specificity of anti-GSTP antibody binding. The number and size of GSTP1 foci were monitored by light microscopy (10–20x magnification). The GSTP1 foci were further categorized into lesions <10 cells (mini foci) and >10 cells per focus and expressed as number per cm². BrdU was detected by the ABC method using a monoclonal anti-BrdU antibody (Sigma Chemical Company) and the labeling indices scored by counting at least 1000 cells randomly per liver section (×40).

2.7. Statistical analyses

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

3. Results

3.1. Effect on body weight gain

FB1 significantly reduced the body weight gains at doses of 20 mg/100 g bw and higher over a period of 14 days (Fig. 2A). The relative liver weight was also significantly decreased in the 30 and 50 mg FB1/100 g treated groups.

When compared to the body weight gain of control rats over a 14 day period, treatment with the different cell proliferative stimuli, PH and PbNO3 administered at day 7 resulted in a significant reduction (P < 0.05) in body weight gain (control group in Fig. 2A vs control groups in Fig. 2B). No significant effect on the body weight gain was noticed with the CCl4 gavage treatment. When treated with the different
doses of FB1 in combination with the cell proliferative stimuli, there were further significant (P < 0.01) reductions in the body weight gains at doses 20 and 30 mg FB1/100g with CCl4 and at 6, 20 and 30 mg FB1/100g with PH (Fig. 2B) compared to the proliferative stimuli alone (control, Fig. 2B). However, with PbNO3, FB1 doses up to 30 mg/100 g bw did not lead to any significant reduction in body weight gain compared to the PbNO3 alone.

### 3.2. Induction of GSTP positive lesions

The results indicated that treatment with FB1 significantly induced the formation of GSTP positive lesions (minifoci (<10 cells/focus) and foci (>10 cells/focus)) at the highest dose of 50 mg FB1/100 g bw, which is equivalent to a daily dosage of 3.5 mg FB1/100 g bw (Table 1). Treatment of the rats with PbNO3 during the initiating treatment period did not alter the cancer initiating potential of FB1 up to a dosage of 30 mg per 100 g bw. However, the formation of GSTP positive foci (>10 cells/focus) was markedly (not significantly) and significantly (P < 0.05) increased at the doses of 20 and 30 mg FB1/100g bw, respectively, when using CCl4 (Table 1). With PH as the proliferative stimulus and AAF/CCl4 as the promoting regimen (Table 2), a significant increase in the number of GSTP positive foci (>10 cells/focus) was observed only at the 30 mg FB1/100g bw dose. Analysis of the total number of GSTP positive lesions (minifoci and foci), illustrated in

### Table 1

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<tr>
<th>Treatment</th>
<th>GSTP positive lesions (no/cm²)</th>
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<tbody>
<tr>
<td>FB1 (mg/100 g bw)</td>
<td>&lt;10 cells/focus (mini foci)</td>
</tr>
<tr>
<td>FB1 (Ctrl)*</td>
<td>PbNO3</td>
</tr>
<tr>
<td>Solvent</td>
<td>0.02 ± 0.03a</td>
</tr>
<tr>
<td>2</td>
<td>0.02 ± 0.04a</td>
</tr>
<tr>
<td>6</td>
<td>0.01 ± 0.02a</td>
</tr>
<tr>
<td>20</td>
<td>0.01 ± 0.03a</td>
</tr>
<tr>
<td>30</td>
<td>0.03 ± 0.04a</td>
</tr>
<tr>
<td>50</td>
<td>0.21 ± 0.33a</td>
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* Values are means of 5–8 animals per treatment group. Means followed by the same letter (column) do not differ statistically. When letter differs then P < 0.05, and when cases differ then P < 0.01. *Normal cancer initiating protocol was used followed by the AAF/PH selection regimen. nd, not determined; Ctrl, control.

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSTP positive lesions (no/cm²)</th>
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<tbody>
<tr>
<td>FB1 (mg/100 g bw)</td>
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<td>6</td>
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<td>20</td>
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<td>30</td>
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* Values are means of 5–8 animals per treatment group. Means followed by the same letter (column) do not differ statistically. When letter differs then P < 0.05, and when cases differ then P < 0.01. *Normal initiating protocol was used followed by the AAF/CCl4 promoting regimen; Ctrl, control.
Fig. 3. showed that both the CCl₄ and PH, stimuli for regenerative cell proliferation, enhanced the induction of GSTP¹ and therefore the cancer initiating potential of the higher doses of FB₁. No effect was obtained with PbNO₃ at any of the FB₁ doses used.

### 3.3. Hepatocyte proliferation

The liver of the control rats hardly showed any labeling with BrdU, whilst levels of labeling, of approximately 9.5, 20, and 50%, were measured in the liver of the rats treated with PbNO₃, CCl₄, and PH, respectively (Fig. 4). Under the present experimental conditions PbNO₃ exhibited approximately 2- and 5-fold lower proliferative indices than CCl₄ and PH, respectively.

### 3.4. Formation of TBARS

A significant ($P < 0.001$) increase of approximately 2-fold in the level of TBARS was detected in the liver of rats that received the 50 mg FB₁/100 g bw dose (Fig. 5).

### 3.5. Histopathological changes

Histological changes in the liver have been described elsewhere [6,7] and were mainly evident in the 30 and 50 mg FB₁/100 g bw dose groups. Briefly, these included degenerative changes such as single cell necrosis (apoptosis), hydrophic cell swelling and hyaline droplet accumulation. Mild proliferation in oval cells and increased mitotic figures were also noticed in the 50 mg FB₁/100g body weight dose.

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**Fig. 3.** The dose dependent cancer initiating effect of FB₁ in rat liver (A) and the modulating role of PbNO₃ (B), CCl₄ (C) and PH (D) on FB₁-induced cancer initiation. The proliferative stimuli were introduced 7 days after the initiating treatment commenced. AAF/CCl₄ was used as the promoting sequence when PH was used as the proliferative treatment during initiation (D). Values are the mean of 8–10 rats per group. *$P < 0.05$, for comparisons between FB₁/proliferative stimuli and FB₁/Ctrl in the same panel. Ctrl, control.
group. After 2-AAF/PH selection, 1–3 nodules (2–5 mm) were visible macroscopically in the livers of these rats.

4. Discussion

FB1-induced cancer initiation is likely to proceed via a similar pathway to that described for the genotoxic class of carcinogens with respect to the induction of initiated hepatocytes and their subsequent resistance to the mitoinhibitory effect of 2-AAF resulting in their development into hepatocyte nodules [6,23]. The only apparent difference appears to be in the induction kinetics of the initiated hepatocyte which, in contrast to genotoxic carcinogens, is only induced after a prolonged exposure of at least 2 weeks [6]. The biochemical phenotype of the FB1-induced nodules is not known, although it is likely to be similar to that induced by genotoxic chemicals [24] as the nodules also stain positively with two histochemical markers for preneoplasia, i.e. gamma glutamyl transpeptidase and GSTP [6]. A unique feature of early preneoplastic and cancerous lesions induced by fumonisins in rat liver is the over stabilization of cyclin D1, suggesting that an epigenetic mechanism could be involved [25]. The mechanism involved in the genesis of these FB1-induced initiated cells is not known at present but a recent study suggests that oxidative damage as a result of chronic FB1-induced hepatic effects could be involved [16]. Three other studies also indicated that FB1 causes lipid peroxidation in membranal environments [26], rat liver nuclei [27] and cells in culture [28]. In the present study a significant increase in lipid peroxidation, as determined by the TBARS assay, occurred in the liver of rats exposed to the high FB1 dose (50 mg FB1/100 g bw). Marked hepatotoxicity as well as cancer initiation was also observed at this dose level, suggesting a close relationship between hepatotoxicity and cancer initiation as hypothesized in earlier studies [23]. Whether oxidative damage is also responsible for the genotoxic effects of FB1 reported by Knasmuller et al. [29] in primary hepatocytes is not known at present. Nevertheless, the majority of studies to date indicate that FB1 is better classified as a non-genotoxic hepatocarcinogen.

Cancer initiation, resulting from treatment with genotoxic carcinogens, is enhanced by PH and CCl4 treatments which induce regenerative cell proliferation, but not by chemicals such as PbNO3, ethylbromide, etc which induce direct hyperplasia [3,30]. However, it is not known whether increased cell proliferation also plays a determining role in the initiation effected by non-genotoxic chemicals such as the fumonisins. With respect to FB1, it was hypothesized that the level of cell proliferation, as a
result of hepatotoxicity, plays a critical role during initiation, but that it is counteracted by the inhibitory effect of FB1 on cell proliferation in normal liver [6,7]. An important balance therefore seems to exist that, as a function of time, will determine whether cell proliferation will exceed a critical level to sustain cancer initiation by the fumonisins [6]. It was therefore of interest to determine whether an increase in cell proliferation during the cancer initiating treatment would support the induction of initiated cells.

Only the two agents inducing regenerative cell proliferation, namely PH and CCl4, significantly enhanced the cancer initiating effect of FB1 and even then only at the highest FB1 dose used (30 mg FB1/100 g bw). The CCl4-induced proliferation also showed a marginal effect on cancer initiation in the rats that received 20 mg FB1/100 g bw. No direct comparison between the effect of these two regenerative stimuli, namely PH and CCl4, can be made as different promoting regimens (AAF/CCl4 vs AAF/PH) were used. In contrast to PH and CCl4, the mitogen-induced hyperplastic effect of PbNO3 failed to enhance the cancer initiating potential of FB1. Of the three proliferative stimuli, PH was the most effective followed by CCl4 and PbNO3 in enhancing the BrdU labeling index in the liver. Whether these differences in the rate of cell proliferation could have an effect on initiation is not known at present. However, PH and CCl4 introduced 7 days after commencing the FB1 initiating treatment, did result in a further reduction in body weight gain (Fig. 2B), presumably due to an enhanced susceptibility to FB1-induced hepatotoxic effects. A recent study indicated that FB1 was more toxic in regenerating liver following PH [31]. It can be argued that these agents exert their effects on the cancer initiating potency of FB1 both by enhancing the hepatotoxicity of FB1 as well as increased regenerative cell proliferation. In contrast, PbNO3 treatment combined with FB1 revealed no significant interaction in terms of reduced body weight gain (Fig. 2B). As a relation exists between the reduction in body weight gain and FB1-induced hepatotoxic effects [6], it would appear that mitogen-induced cell proliferation induced by PbNO3 not only failed to enhance FB1-induced initiation but also did not enhance the hepatotoxic effects of FB1. Lemmer et al. [32] indicated that dietary iron protects against a reduction in the relative liver weight induced by FB1. Of relevance was the finding that dietary iron, a known mitogen in the liver [33], reduced the cancer initiating potency of FB1 under the specified experimental conditions.

Differences therefore seem to exist between regenerative and mitogen-induced cell proliferation with respect to the biological effects of FB1 in the liver. The interaction between CCl4 and FB1, both of which induce oxidative damage in the liver, is of interest with respect to initiation. Cell death induced by CCl4 in zone 4 (central vein) of the lobule resulted from excessive lipid peroxidation in hepatocyte membranes [34]. However, in the case of FB1, lipid peroxidation appears to be a secondary event following cell death and has been implicated as a causative factor in cancer initiation [16]. As the combined effect of the cell proliferative stimuli and the FB1 treatment on oxidative damage was not measured during the initiating treatment, it is not known whether lipid peroxidation contributed to the enhanced cancer initiating potency of FB1.

The differences in the type of cell proliferation induced by PH and/or CCl4 versus that caused by mitogens such as PbNO3 and their respective role in the initiating events induced by genotoxic carcinogens have been well established [4,35]. In the case of regenerative cell proliferation, the liver is in the process of regaining its critical mass following injury and/or removal of a part by PH. When carcinogen exposure is timed to coincide with the majority of cells passing through the S- or DNA synthesis phase of the cell cycle, a maximum number of initiated cells is produced. However, a single and/or multiple dose of FB1 in conjunction with PH, failed to cause initiation as compared to the effect of genotoxins [23]. This finding indicated that the kinetics of the initiating step induced by FB1 is different from the latter compounds. With respect to mitogen-induced hyperplasia, the liver is stimulated to produce increased tissue mass but the original size is restored after removal of the stimulus due to the elimination of the excess cells through the process of apoptosis [4]. As initiated cells seem to be prone to undergo apoptosis [10] it could well be that, in conjunction with the delayed cancer initiating potential of FB1, the initiated cells are more readily removed from the liver. It is known that FB1 also directly induces apoptosis in the liver and hence this could be a further
restriction on the survival of initiated cells [9]. A recent study indicated that pre-treatment of rats with dietary FB1 significantly reduced the persistence of DEN-induced GSTP + hepatocytes in the liver [8].

The present study suggests that regenerative cell proliferation either supports the process of initiation and/or renders the liver more susceptible to FB1-induced hepatotoxicity, which facilitates the process of cancer initiation. As in the case of initiation with genotoxic carcinogens, mitogen-induced hyperplasia did not enhance initiation by FB1. The fact that regenerative cell proliferation enhances the cancer initiating potency of FB1 has to be taken into account, as in the case of genotoxins, when determining tolerance levels and establishing risk assessment parameters for the fumonisins in humans. This is of particular interest as the fumonisins co-occur naturally in corn with aflatoxin B1 in areas where people are commonly infected with hepatitis B virus [36].

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