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Fumonisin Inhibition of *de Novo* Sphingolipid Biosynthesis and Cytotoxicity Are Correlated in LLC-PK$_1$ Cells

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Fumonisins are a group of structurally related compounds produced by *Fusarium moniliforme.* Recently, it has been shown that fumonisins B_1 and B_2 are the first naturally occurring inhibitors of sphingosine and sphinganine *N*-acyltransferase (ceramide synthase) in rat primary hepatocytes (Wang *et al. J. Biol. Chem.* **266**, 14,486–14,490, 1991). These enzymes are key components in the pathways for *de novo* sphingolipid biosynthesis and sphingolipid turnover. The results of the present study show that fumonisins B_1 and B_2 inhibit proliferation and are cytotoxic to LLC-PK$_1$ cells. Concentrations of fumonisin B_1 and B_2 between 10 and 35 μM inhibited cell proliferation, whereas higher concentrations (>35 μM) killed cells. Inhibition of cell proliferation and cell death were preceded by a lag period of at least 24 hr during which cells appeared to be functioning normally. Cells exposed to fumonisin B_1 exhibited normal growth kinetics and morphology soon after fumonisin B_1 was removed; thus, the effects of fumonisin B_1 were reversible. The EC_{50} for alterations in sphingolipid biosynthesis was 10 to 15 μM. Inhibition of *de novo* sphingolipid biosynthesis occurred before inhibition of cell proliferation or cytotoxicity, and the dose response for the decrease in the [^{3}H]sphingosine to [^{3}H]sphinganine ratio at 7 hr closely paralleled the dose response for effects on proliferation and cytotoxicity at 3–5 days. In addition, the level of free sphinganine, and to a lesser extent sphingosine, increased in fumonisin-treated cells in a dose-dependent manner. During the 24-hr lag period preceding inhibition of cell proliferation, the free sphinganine content increased by 12,800% in cells exposed to 35 μM fumonisin B_1. Whereas a mechanistic relationship between the inhibition of *de novo* sphingolipid biosynthesis and inhibition of proliferation and cell death has not been demonstrated, the results of this study support the hypothesis that inhibition of *de novo* sphingolipid biosynthesis is an early event in the toxicity of fumonisins to LLC-PK$_1$ cells.

Fumonisins (i.e., B_1 and B_2; Fig. 1) are a group of naturally occurring (Sydenham *et al.*, 1990; Ross *et al.*, 1991; Voss *et al.*, 1989), structurally related compounds produced by *Fusarium moniliforme,* one of the most commonly occurring fungi on agricultural commodities including corn grown throughout the world (Marasas *et al.*, 1984b). There are currently at least three diseases associated with the consumption of fungal culture materials from *F. moniliforme* or feeds containing corn contaminated with *F. moniliforme:* equine leukoencephalomalacia (ELEM) (Kellerman *et al.*, 1972), porcine pulmonary edema syndrome (Kriek *et al.*, 1981), and human esophageal cancer (Marasas *et al.*, 1988a). In addition, *F. moniliforme* culture materials can cause liver cancer in rats (Marasas *et al.*, 1984a; Jaskiewicz *et al.*, 1987). Equine leukoencephalomalacia and porcine pulmonary edema are animal health problems of increasing concern in the United States where corn is an important component of animal feeds (Ross *et al.*, 1991; Harrison *et al.*, 1990).

The compound produced by *F. moniliforme* which is responsible for ELEM is fumonisin B_1 (Marasas *et al.*, 1988b; Kellerman *et al.*, 1990). The clinical signs of porcine pulmonary edema have been reproduced in pigs dosed intravenously with pure fumonisin B_1 (Harrison *et al.*, 1990). Additionally, pure fumonisin B_1 has been shown to cause liver cancer in rats (Gelderblom *et al.*, 1991). In short-term carcinogenesis studies with rats, fumonisins B_1, B_2, and B_3 exhibited cancer-initiating activity (Gelderblom *et al.*, 1992). Fumonisins neither are mutagenic in the *Salmonella* test nor do they induce unscheduled DNA synthesis in primary rat hepatocytes (Gelderblom *et al.*, 1989; Norred *et al.*, 1990).

Recently, it was shown that fumonisins B_1 and B_2 are the first discovered naturally occurring inhibitors of sphingosine and sphinganine *N*-acyltransferase (ceramide synthase) in rat primary hepatocytes (Wang *et al.*, 1991). These enzymes are key components in the pathways for *de novo* sphingolipid biosynthesis (Fig. 2) and sphingolipid turnover (Merrill, 1991). Fumonisin inhibition of *de novo* sphingolipid biosynthesis, blocking amide linkage of fatty acids to sphinganine (sphinganine *N*-acyltransferase), results in an increase in the amount of sphinganine and a decrease in sphingosine (Wang *et al.*, 1991). Prolonged inhibition of sphinganine *N*-acyltransferase decreases the total sphingolipid content of rat hepatocytes (Wang *et al.*, 1991). The toxicity associated

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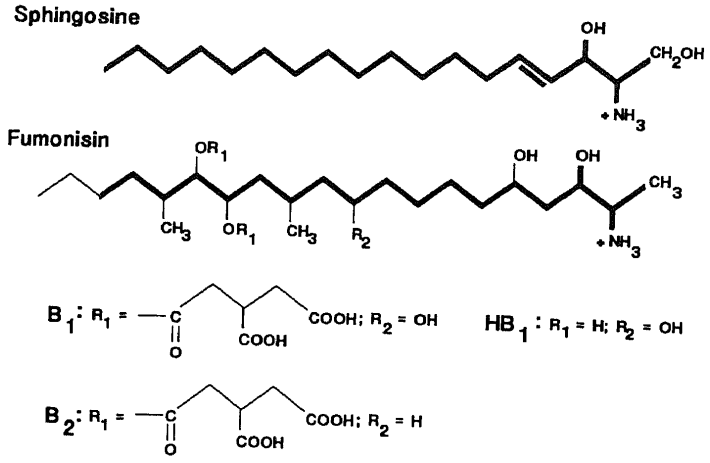

FIG. 1. Structures of fumonisins B_1, B_2, the acid hydrolysis product of FB_1 (HB_1), and sphingosine.

with inhibition of *de novo* sphingolipid biosynthesis is unknown. Fumonisins do not appear to be acutely toxic to primary rat hepatocytes (Norred *et al.*, 1991); there was no increase in cell death at concentrations up to 10 mM fumonisin B_1 for 2 hr. Wang *et al.* (1991) found no apparent increase in cell death in hepatocytes exposed to fumonisins for 4 days at a concentration (1 μM) which inhibited sphingosine biosynthesis by 95% and reduced total sphingolipid content by 50%.

Preliminary *in vivo* studies with rats indicate that fumonisin B_1 inhibits ceramide synthases *in vivo* when gavaged at 25 mg/kg for 2 days (Wang *et al.*, 1991) with no apparent toxic consequences. However, considering the important role of sphingolipids in cells (Hannun and Bell, 1989; Merrill, 1991), it is unlikely that disruption of *de novo* sphingolipid biosynthesis would not have profound effects on cell function.

The purpose of the present work was to study the effects of fumonisins using a proliferating cell line. The LLC-PK_1 pig kidney cell line was chosen because it is easily maintained in culture (Hull *et al.*, 1976) and it is well characterized with regards to its biochemistry, physiology, and response to toxic agents. The specific objectives of our studies with LLC-PK_1 cells were to determine: (i) the cytotoxicity of fumonisins to LLC-PK_1 cells, (ii) the effect of fumonisin B_1 on *de novo* sphingolipid biosynthesis, and (iii) the dose–response relationship between the cytotoxicity and effects on *de novo* sphingolipid biosynthesis.

MATERIALS AND METHODS

Cells. Renal epithelial cells (LLC-PK_1, CRL 1392, passage 196) were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown and maintained as previously described (Riley *et al.*, 1987). For all experiments with LLC-PK_1 cells, the cells were subcultured at approximately 3000 viable cells/cm^2 in 24-well plates (2 cm^2), 25-cm^2 flasks, or 50-cm^2 dishes containing DMEM/Ham's F12 (1:1) with 1 or 5% fetal calf serum at 37°C and 5% CO_2. Media containing 1% fetal calf serum will not support cell growth for as long as cells grown in growth medium containing 5% fetal calf serum. Cells were allowed to attach and grow for 24 hr prior to addition of fumonisins.

Proliferation assay. The effect of fumonisins on cell growth was determined by measuring the changes in protein content in 2-cm^2 wells seeded with LLC-PK_1 cells as described above. Solutions of growth medium containing various concentrations of fumonisins, dissolved in growth medium, were added to the cultures on Day 1 (24 hr after seeding). The solutions were changed on Day 3 and every other day thereafter until the experiment was terminated by aspirating the solutions and rinsing each well three times with Dulbecco's phosphate-buffered saline (Dulbecco and Vogt, 1954). The remaining materials in each well were digested in 0.2 N NaOH and protein level was determined (Lowry *et al.*, 1951). Additional details for specific experiments are described in figure legends.

De novo sphingolipid biosynthesis. The incorporation of [^{3}H]serine into sphinganine (S_a) and sphingosine (S_o) was measured as previously described for rat hepatocytes (Wang *et al.*, 1991). Briefly, subconfluent densities of LLC-PK_1 cells grown in 25-cm^2 culture flasks or 50-cm^2 culture dishes were exposed to various concentrations of fumonisin B_1 for 5 or 3 hr in growth medium and then 2 or 4 additional hours in phosphate-buffered saline plus fumonisin B_1 and [^{3}H]serine (40 or 80 μCi). After 7 hr exposure to fumonisin B_1, the cells were scraped off the surface of the culture flasks or dishes, 25 to 50 μg of carrier sphinganine, sphingosine, and sphingomyelin were added, and the lipids were extracted and acid hydrolyzed to liberate the free long-chain bases. The acid-hydrolyzed lipids were then separated by thin-layer chromatography on Kodak silica gel sheets (Rochester, NY) developed with $CHCl_3$:MeOH:2 N NH_4OH (40:10:1, v/v/v). The ^{3}H label in acid-hydrolyzed lipids was measured by radiometric scanning with a Bioscan System 200 (Washington, DC). Sphinganine and sphingosine were visualized by spraying with ninhydrin (0.2 g/100 ml ethanol). The areas containing radioactivity that migrated with authentic standards were cut from the appropriate regions of the thin-layer sheets and quantified by liquid scintillation counting.

Free sphinganine and sphingosine content of cells. The relative amounts of sphinganine and sphingosine in base-treated cell extracts were determined by HPLC as previously described (Merrill *et al.*, 1988).

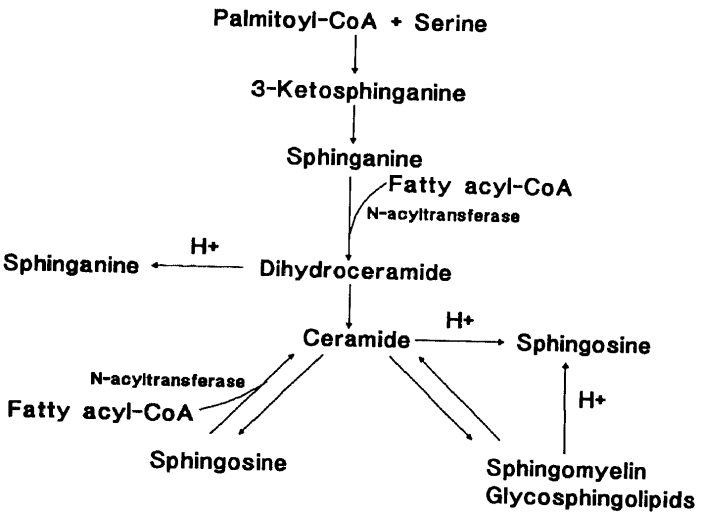

FIG. 2. Flow chart showing the pathways for *de novo* sphingolipid biosynthesis (endoplasmic reticulum) and the pathway for turnover of complex sphingolipids (plasma membrane). Free long-chain bases (e.g., 3-ketosphinganine, sphinganine, and sphingosine) are intermediates or precursors in these pathways and therefore do not normally accumulate in cells (Merrill, 1991). Incorporation of [^{3}H]serine into sphingosine and sphinganine is quantified following acid hydrolysis (depicted by H^+) of the N-acyl products (dihydroceramides, ceramides). Ceramides form the backbone of sphingomyelin and glycosphingolipids. Sphingosine contained within these complex sphingolipids is also released by acid hydrolysis. The steps inhibited by fumonisins are shown; sphingosine and sphinganine *N*-acyltransferase.

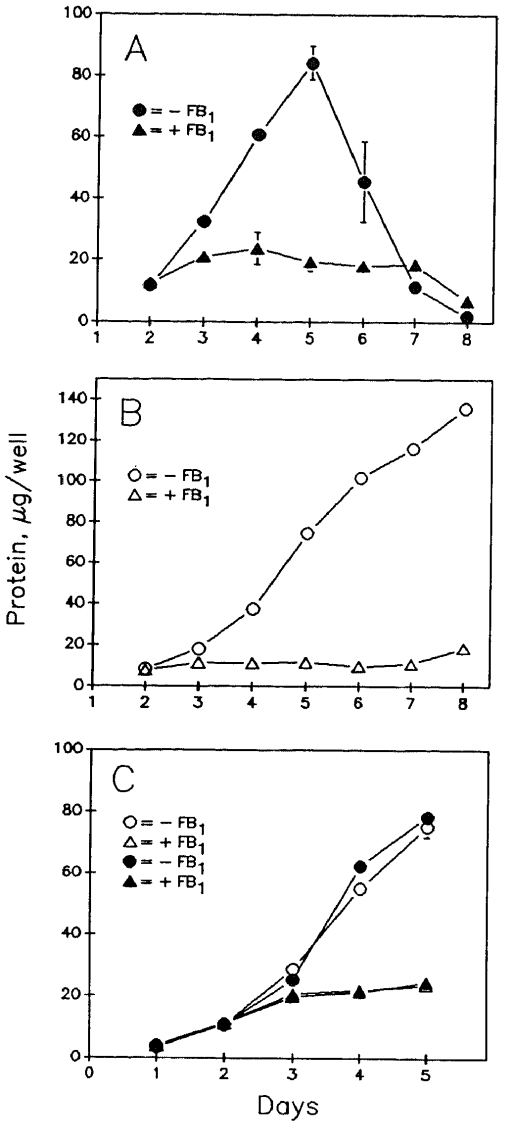

FIG. 3. Inhibition of LLC-PK$_1$ cell proliferation (based on protein content) and the recovery of normal growth kinetics following exposure for 8 days to 35 μM fumonisin B$_1$ (FB$_1$). (A) Growth medium with 1% fetal calf serum with and without 35 μM fumonisin B$_1$ and (B) with 5% fetal calf serum with and without 35 μM fumonisin B$_1$. (C) Recovery of normal growth kinetics by cells harvested from wells represented in (A), Day 8. Briefly, following 8 days in growth medium plus 1% fetal calf serum with and without 35 μM fumonisin B$_1$, wells (three each) were rinsed and trypsinized as previously described (Riley *et al.*, 1987). Suspended cells were seeded into 25-cm^2 flasks and grown for 4 days on growth medium plus 5% fetal calf serum. After 4 days the cultures were trypsinized and viable cells counted using a hemocytometer and trypan blue. Cells derived from control wells (Fig. 3A, −FB$_1$) and cells from treated wells (Fig. 3A, +FB$_1$) were seeded at 1000 cells/cm^2 into 25-cm^2 flasks and allowed to grow to confluence using growth medium plus 5% fetal calf serum. These cells were used as the seed stock for the experiment depicted in (C); open circles and triangles are cells originally harvested from wells represented by (A), −FB$_1$, and solid circles and solid triangles are those harvested from wells represented by (A), +FB$_1$. Growth medium with 5% fetal calf serum with and without 35 μM fumonisin B$_1$ was changed on Days 1 and 3. Values are means plus or minus 95% confidence interval for one experiment with four replicates per day per treatment.

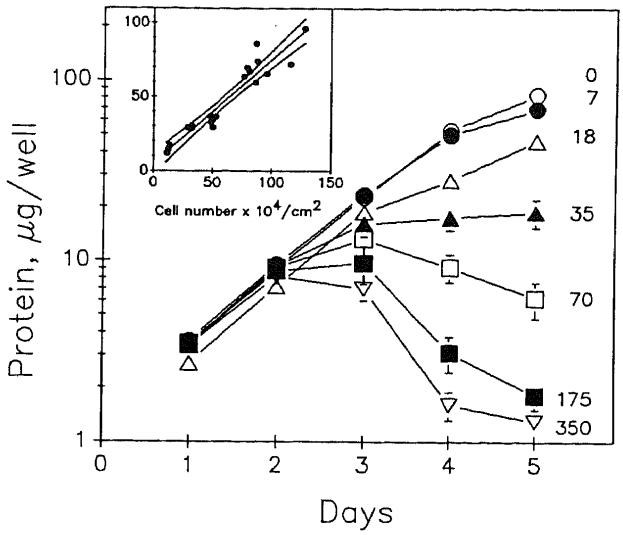

FIG. 4. Fumonisin B$_1$ cytotoxicity as measured by the daily change in protein content of 2-cm^2 wells. The micromolar concentration of fumonisin B$_1$ is given to the right of each growth curve. Each plotted value is the mean from three or four experiments, with three or four replicates per dose per day per experiment. Inset is the relationship between cell number and total protein. Cell number was determined, following trypsin treatment, by counting viable cells (trypan blue excluding) using a hemocytometer. The least-squares regression line, 95% confidence intervals, and the actual values for samples taken at 0 to 6 days (mean of three replicates/day) are given.

Fumonisins and reagents. Fumonisin B$_1$ was either purchased from the Division of Food Sciences and Technology, CSIR (Pretoria, South Africa) or isolated from *F. moniliforme*-inoculated corn (MRC 826) by reverse phase (C18) and normal phase (silica gel) chromatography; identity was confirmed by HPLC and GC-MS. Fumonisin B$_2$ was also purchased from CSIR. The purity of the South African fumonisin B$_1$ and B$_2$ was estimated to be greater than 95%, and the material isolated from MRC 826 culture materials was 70% based on a combination of HPLC and GC-MS (Plattner *et al.*, 1990; Wilson *et al.*, 1990). The 70% fumonisin B$_1$ was used only in the experiments described in Fig. 5. Reference standards were provided by Pieter Thiel (South African Medical Research Council, P.O. Box 19070, Tygerberg 7505, South Africa). L-[G-^{3}H]Serine (37.8 Ci/mmol) was purchased from NEN Research Products (Boston, MA) and sphingolipid standards were purchased from Sigma (St. Louis, MO) or prepared synthetically (Merrill *et al.*, 1989).

Statistics. Unless indicated, all values are expressed as means plus or minus the 95% confidence intervals. Differences between treatments were analyzed statistically with the *t* statistic for two means. Least-squares regression analysis was accomplished using SAS/STAT (SAS Institute, Inc., 1985). Where indicated, data were fitted to linear (first-order) and quadratic regression models, and the best fit was decided on the basis of a comparison of the *t* statistics for the model parameters.

RESULTS

Fumonisins inhibit proliferation and are cytotoxic to LLC-PK$_1$ cells. Fumonisin B$_1$ (35 μM) significantly decreased the rate of increase in protein content of LLC-PK$_1$ cells grown in growth medium that contained either 1 or 5% fetal calf serum (Figs. 3A and 3B). In LLC-PK$_1$ cells seeded at low density, the increase in cell number/cm^2 (cell proliferation) and protein/cm^2 was well correlated (Fig. 4, inset). Inhibition of proliferation as measured by an increase in protein content

occurred between 10 and 35 μM fumonisin B_1 (Fig. 4). The rate of increase in protein content exhibited zero-order kinetics after 3 days at 35 μM fumonisin B_1 and became negative at concentrations above 35 μM (Figs. 3 and 4), indicating cytotoxicity. Untreated cells (controls) began to die after 5 days when grown in growth medium with 1% fetal calf serum (Fig. 3A). Cells treated with levels of fumonisin B_1 (0.5 to 10 μM) which were not cytotoxic grew at the same rate as controls but also began to die after 5 days, suggesting that fumonisin B_1 is not mitogenic in LLC-PK_1 cells. There was a lag period of approximately 24–48 hr preceding the inhibition of cell proliferation after treatment with fumonisin B_1 (Fig. 3 and 4). During this lag period cells appeared normal. After 24–48 hr, cells treated with 35 μM fumonisin B_1 began to develop a fibroblast-like appearance (loss of cell–cell contact, elongated, spindle shaped). Blebbing was never observed. Cells floating in the culture media were dead as evidenced by the inability to exclude trypan blue (data not shown). However, cells which remained attached were able to recover and grow after the removal of the fumonisin B_1. Cultures which were allowed to grow to confluence after exposure to fumonisin B_1 had a normal epithelial morphology and formed domes (fluid-filled blisters indicative of transepithelial movement of sodium). When these cells were subcultured they grew at the same rate and were morphologically identical to untreated cells. Cells which survived fumonisin treatment were equally sensitive (compared to untreated cells) to fumonisin B_1 inhibition of cell proliferation in subsequent experiments (Fig. 3C), indicating that surviving cells were not conferred with resistance to fumonisin B_1.

Fumonisins B_1 and B_2 were equally toxic to LLC-PK_1 cells (Fig. 5). Using the protein content of the wells at 5 days (relative to protein content of concurrent controls), the EC_{50} for the fumonisin B_1 effect on protein content was between 20 and 30 μM.

Fumonisin B_1 inhibits de novo biosynthesis of sphingosine. As the fumonisin B_1 concentration was increased the incorporation of [^{3}H]serine into sphingosine decreased and incorporation into sphinganine increased (Fig. 6). The ratio of sphingosine to sphinganine has been found to be a sensitive indicator of alterations in *de novo* sphingolipid biosynthesis (Wang *et al.*, 1991). A plot of the ratio of [^{3}H]sphingosine to [^{3}H]sphinganine revealed that the 7-hr EC_{50} for this index was approximately 30 μM (Fig. 7). This is approximately the same concentration of fumonisin B_1 which decreased the protein content in wells of treated cells by 50% and inhibited cell proliferation approximately 100% (35 μM).

The sensitivity of LLC-PK_1 cells to fumonisin-induced alterations in *de novo* sphingolipid biosynthesis may be somewhat greater than that indicated in Fig. 7. Incubation of LLC-PK_1 cells for 2 hr in [^{3}H]serine was based on preliminary studies in which we found that the ratio of [^{3}H]sphingosine/[^{3}H]sphinganine in untreated LLC-PK_1 cells increased over the first 2 hr of labeling to a ratio of approximately 2.0 (data not shown). This ratio remained relatively constant up to 16 hr. In the experiments summarized in Figs. 6 and 7, ratios in control cultures ranged from 1.0 to

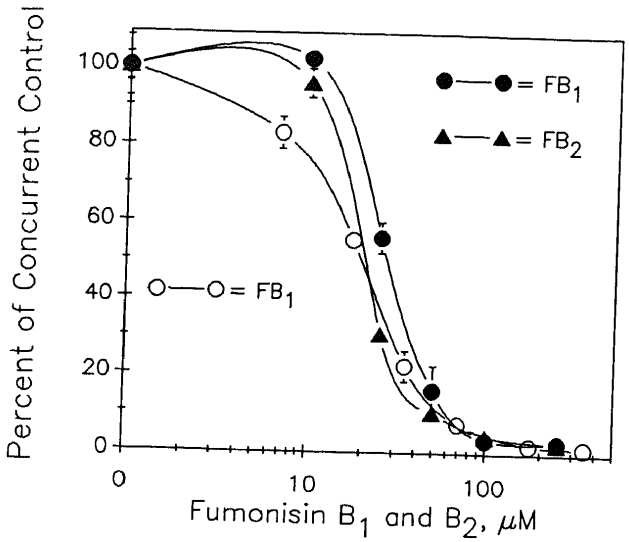

FIG. 5. Dose–response curves comparing the effects of fumonisin B_1 and fumonisin B_2 based on the 5-day protein values obtained as described in the legend to Fig. 4. Values are expressed as a percentage of concurrent controls. The results with fumonisin B_1 represent two to four experiments with four replicates per dose per experiment. The results for fumonisin B_2 are from a single experiment with four replicates per dose. Solid symbols represent fumonisins B_1 and B_2 (>95% pure) purchased from the Division of Food Sciences and Technology, CSIR (Pretoria, South Africa). The results with fumonisin B_1 depicted by the open circles were obtained using fumonisin B_1 isolated from corn cultures (MRC 826) and estimated to be 70% pure based on a combination of HPLC and GC-MS.

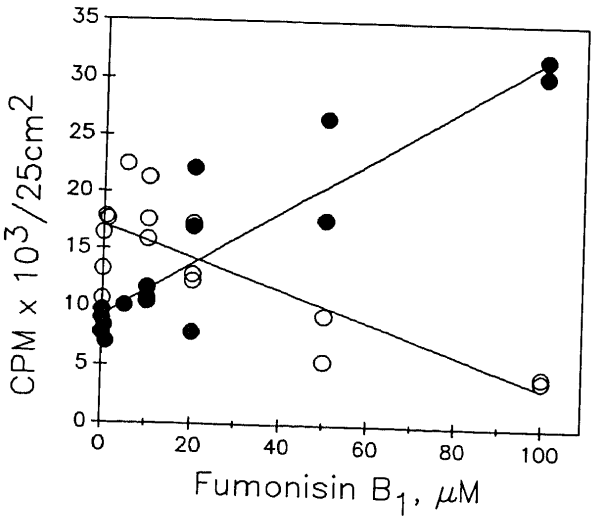

FIG. 6. Dose response for [^{3}H]serine incorporation into sphinganine (solid circles) and sphingosine (open circles) in 3-day-old cells and after 7 hr exposure to fumonisin B_1 including 2 hr with [^{3}H]serine. The results of three experiments with a total of 15 observations were pooled for regression analysis. Data were fitted to linear (first-order) and quadratic regression models and the best fit was decided on the basis of comparison of the *t* statistics for the model parameters.

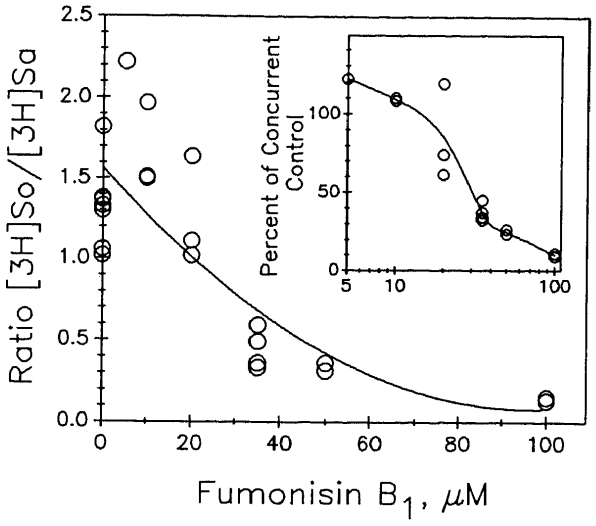

FIG. 7. Change in the ratio of [^{3}H]sphingosine to [^{3}H]sphinganine (S_o/S_a) in fumonisin B_1-treated LLC-PK_1 cells. The results of four experiments with a total of 23 observations were pooled for regression analysis. Data were fitted to linear (first-order) and quadratic regression models and the best fit was decided on the basis of comparison of the t statistics for the model parameters. Inset is the dose response (log scale) for alterations in the [^{3}H]sphingosine/[^{3}H]sphinganine ratio expressed as a percentage of the concurrent control ratio.

1.8, suggesting that the samples were taken before steady state labeling was attained. This would result in an underestimation of the inhibitory potential of fumonisin B_1 in LLC-PK_1 cells. To test this possibility, several experiments were conducted with 4 hr (vs 2 hr) in the presence of [^{3}H]serine but with the total of 7 hr exposure to fumonisin B_1 maintained. Use of a 4-hr labeling period increased the ratio in control cells but had no effect on the fumonisin-treated cells (Fig. 8). At 35 μM for 2 and 4 hr the ratios ex-

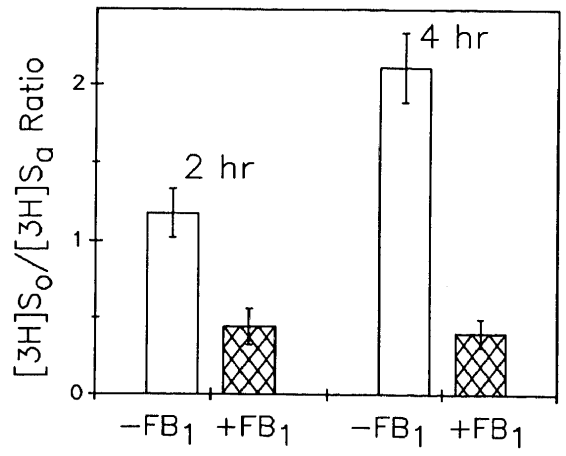

FIG. 8. Comparison of [^{3}H]sphingosine/[^{3}H]sphinganine ratios in control and fumonisin B_1-treated cells after 7 hr total exposure to fumonisin B_1 (35 μM) including either 2 or 4 hr of labeling with [^{3}H]serine. Values are the means (n = 4) plus or minus the 95% confidence intervals from a single experiment.

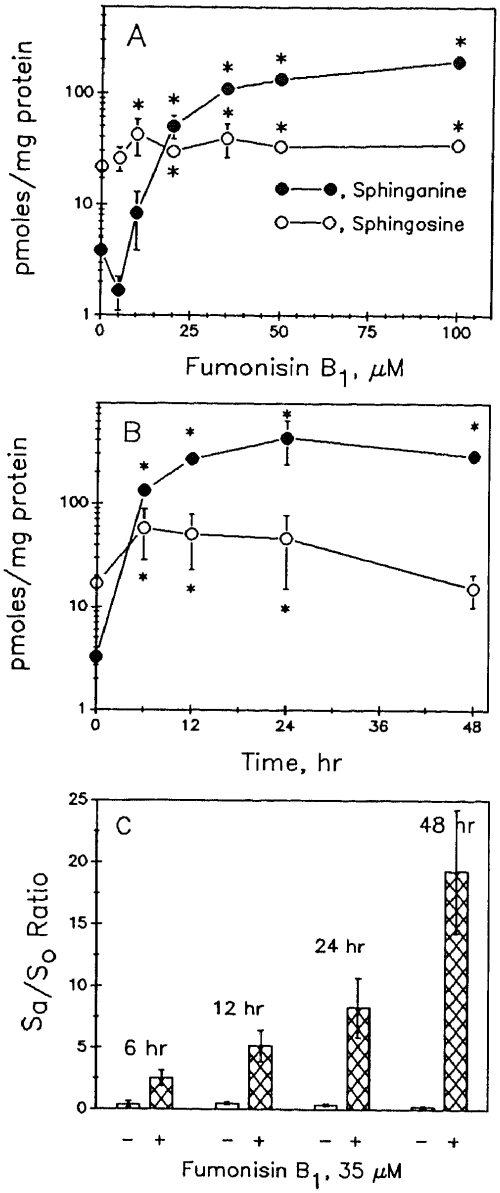

FIG. 9. Comparison of the free sphinganine and free sphingosine content in control and fumonisin B_1-treated cells, determined by HPLC (Merrill *et al.*, 1988). (A) Dose–response curve at 7 hr; (B) after 6, 12, 24, and 48 hr exposure to 35 μM fumonisin B_1; and (C) the data from (B) plotted as the ratio sphinganine/sphingosine. Values are the means (n = 3 to 12) plus or minus the 95% confidence interval from one to three experiments. Asterisks indicate values which are significantly different ($p < 0.05$) from control values.

pressed as a percentage of concurrent controls were 37 ± 6% and 19 ± 5% (n = 4), respectively (calculated from Fig. 8).

A consequence of inhibition of sphinganine *N*-acyltransferase in the *de novo* pathway is an increase in the amount of free sphinganine. Analogously, inhibition of sphingosine *N*-acyltransferase in the turnover pathway should cause accumulation of sphingosine. The dose response for the increase in free sphinganine (Fig. 9A) closely paralleled the dose response for inhibition of *de novo* biosynthesis (Fig. 7). The free sphinganine content of cells was increased 128- and

87-fold after 24 and 48 hr, respectively (Fig. 9B). Free sphingosine was elevated 3.4-, 3.0-, and 2.7-fold at 6, 12, and 24 hr, respectively (Fig. 9B). Free sphingosine returned to control values after 48 hr, but sphinganine levels remained elevated (Fig. 9B). A simple measure of the magnitude of fumonisin's effect on sphingolipid metabolism is the change in the free sphinganine to free sphingosine ratio (Fig. 9C). After 24 and 48 hr exposure to 35 μM fumonisin B_1 the ratio was increased 21- and 84-fold, respectively, over that in control cultures (calculated from Fig. 9B).

DISCUSSION

Unlike studies with primary rat hepatocytes (Wang *et al.*, 1991; Norred *et al.*, 1991), in this work the toxicity of fumonisin B_1 was easily observed in proliferating LLC-PK_1 cells. Inhibition of proliferation of LLC-PK_1 cells occurred between 10 and 35 μM fumonisin B_1, and cytotoxicity occurred after 3 days exposure to concentrations greater than 35 μM. Other proliferating cell lines are also sensitive to fumonisins. The rat hepatoma (H4TG) and dog kidney cell lines (MDCK) are highly sensitive to inhibition of cell proliferation by fumonisins B_1 and B_2 (Shier *et al.*, 1991). Thus, in these cultured cell lines, the ability to undergo cell division appears to be a prerequisite for the occurrence of cytotoxicity. A corollary to this is the fact that there is at least a 24-hr lag period from the time that cells are exposed to fumonisin B_1 and the inhibition of proliferation, irrespective of the fumonisin B_1 concentrations. The doubling time for LLC-PK_1 cells (when fetal calf serum is not limiting) is approximately 24 hr (Riley *et al.*, 1985). In a separate experiment (data not shown), exposure of confluent monolayers of LLC-PK_1 cells to fumonisin B_1 up to 1 mM for 29 hr had no effect on domes (indicative of transepithelial sodium transport and functional tight junctions) or the normal epithelial-like morphology compared to control cultures. Taken together, these results suggest that either cell division, the buildup of some toxic principle, or depletion of an essential cellular constituent is required for toxicity in LLC-PK_1 cells.

De novo sphingolipid biosynthesis in LLC-PK_1 cells is significantly inhibited before inhibition of cell proliferation or cytotoxicity is observed, suggesting that inhibition of *de novo* sphingolipid biosynthesis is an early event in the process leading to cytotoxicity. The data presented in Fig. 8 indicate that a 2-hr labeling period was not long enough for the ^3H label from serine to reach a steady state in the sphingosine pool of untreated cells (controls). As a result, the inhibitory potential of fumonisin B_1 in LLC-PK_1 cells is approximately twice that determined from the data generated by the 2-hr [^{3}H]serine labeling protocol; the estimated EC_{50} based on a 4-hr labeling protocol would be 10 to 15 μM. In primary rat hepatocytes the EC_{50} is around 0.1 μM (Wang *et al.*, 1991). Thus, it appears that LLC-PK_1 cells are much less sensitive to fumonisin-induced alterations in sphingolipid biosynthesis than primary rat hepatocytes. However, cytotoxicity has not been observed in hepatocytes but is observed in dividing cell lines. The reason(s) for this relative difference in sensitivity is unknown at this time. It is possible that cytotoxicity is more easily observed in dividing cells since complex sphingolipids, such as sphingomyelin, are turned over slowly and thus would be more rapidly depleted in dividing populations (compared to nondividing populations) when *de novo* biosynthesis is inhibited.

Fumonisins are clearly cytotoxic to LLC-PK_1 cells. However, a mechanistic relationship between the inhibition of *de novo* sphingolipid biosynthesis and inhibition of proliferation and cell death has not been demonstrated. Is altered sphingolipid metabolism an early event leading ultimately to the death of LLC-PK_1 cells, or is altered sphingolipid metabolism just a benign early effect? Our current hypothesis is that the inhibition of *de novo* sphingolipid biosynthesis and the toxicity of fumonisins to LLC-PK_1 cells are related. There are numerous possible explanations. For example, fumonisin inhibition of sphinganine *N*-acyltransferase causes a dramatic increase in free sphinganine concentration in both LLC-PK_1 cells and rat hepatocytes (Wang *et al.*, 1991). High intracellular concentrations of free sphinganine can be cytotoxic in some cell lines (Merrill, 1983; Stevens *et al.*, 1990). In LLC-PK_1 cells the levels of free sphingosine also increase even though *de novo* biosynthesis is inhibited. The source of the increase in free sphingosine could be due to inhibition of the turnover pathway (sphingosine *N*-acyltransferase) without increased catabolism. Free long-chain bases are now known to be important modulators of intracellular signalling systems (e.g., protein kinase C, enzymes of diacylglycerol and phosphatidic acid metabolism, and the tyrosine kinase activity of the EGF receptor) (Hannun and Bell, 1989; Merrill, 1991). Future studies will attempt to identify sites in LLC-PK_1 cells which are affected by elevated levels of free long-chain bases and the consequences of inhibition on the biosynthesis of specific complex sphingolipids.

In summary: (i) fumonisins B_1 and B_2 are cytotoxic to LLC-PK_1 cells, (ii) inhibition of cell proliferation and cell death are preceded by a lag period of at least 24 hr, during which cells appear to be functioning normally, (iii) the toxic effects of fumonisin B_1 are reversible, (iv) inhibition of *de novo* sphingolipid biosynthesis precedes toxicity, (v) the dose response for the decrease in the [^{3}H]sphingosine to [^{3}H]sphinganine ratio at 7 hr is similar to the dose response for toxicity at 3–5 days, (vi) sphinganine levels become greatly elevated after only 6 hr exposure to 35 μM fumonisin B_1, and (vii) LLC-PK_1 cells are much less sensitive than primary rat hepatocytes to fumonisin inhibition of *de novo* sphingolipid biosynthesis.

REFERENCES

Dulbecco, R., and Vogt, M. (1954). Plaque formation and isolation of pure lines with poliomyelitis viruses. *J. Exp. Med.* **98**, 167–182.

Gelderblom, W. C. A., Kriek, N. P. J., Marasas, W. F. O., and Theil, P. G. (1991). Toxicity and carcinogenicity of the *Fusarium moniliforme* metabolite, fumonisin B_1, in rats. *Carcinogenesis* **12,** 1247–1251.

Gelderblom, W. C. A., Marasas, W. F. O., Theil, P. G., Semple, E., and Farber, E. (1989). Possible non-genotoxic effects of active carcinogenic components produced by *Fusarium moniliforme. Proc. Am. Assoc. Cancer Res.* **30,** 144.

Gelderblom, W. C. A., Marasas, W. F. O., Vleggaar, R., Thiel, P. G., and Cawood, M. E. (1992). Fumonisins: Isolation, chemical characterization and biological effects. *Mycopathologia,* in press.

Hannun, Y. A., and Bell, R. M. (1989). Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science* **243,** 500–507.

Harrison, L. R., Colvin, B., Greene, J. T., Newman, L. E., and Cole, J. R. (1990). Pulmonary edema and hydrothorax in swine produced by fumonisin B_1, a toxic metabolite of *Fusarium moniliforme, J. Vet. Diagn. Invest.* **2,** 217–221.

Hull, R. N., Cherry, W. R., and Weaver, G. W. (1976). The origin and characteristics of a pig kidney cell strain, LLC-PK_1. *In Vitro* **12,** 670–677.

Jaskiewicz, K., van Rensburg, S. J., Marasas, W. F. O., and Gelderblom, W. C. A. (1987). Carcinogenicity of *Fusarium moniliforme* culture material in rats. *J. Natl. Cancer Inst.* **78,** 321–325.

Kellerman, T. S., Marasas, W. F. O., Pienaar, J. G., and Naude, T. W. (1972). A mycotoxicosis of equidae caused by *Fusarium moniliforme* Sheldon, A preliminary communication. *Onderstepoort J. Vet. Res.* **39,** 205–208.

Kellerman, T. S., Marasas, W. F. O., Thiel, P. G., Gelderblom, W. C. A., Cawood, M., and Coetzer, J. A. W. (1990). Leukoencephalomalacia in two horses induced by oral dosing of fumonisin B_1. *Onderstepoort J. Vet. Res.* **57,** 269–275.

Kriek, N. P. J., Kellerman, T. S., and Marasas, W. F. O. (1981). Comparative study of the toxicity of *Fusarium verticillioides* (= *Fusarium moniliforme*) to horses, primates, pigs, sheep and rats. *Onderstepoort J. Vet. Res.* **48,** 129–131.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

Marasas, W. F. O., Jaskiewicz, K., Venter, F. S., and van Schalkwyk, D. J. (1988a). *Fusarium moniliforme* contamination of maize in oesophageal cancer areas in Transkei. *S. Afr. Med. J.* **74,** 110–114.

Marasas, W. F. O., Kellerman, T. S., Gelderblom, W. C. A., Coetzer, J. A. W., Thiel, P. G., and van der Lugt, J. J. (1988b). Leukoencephalomalacia in a horse induced by fumonisin B_1 isolated from *Fusarium moniliforme. Onderstepoort J. Vet. Res.* **55,** 197–203.

Marasas, W. F. O., Kriek, N. P. J., Fincham, J. E., and van Rensburg, S. J. (1984a). Primary liver cancer and oesophageal basal cell hyperplasia in rats caused by *Fusarium moniliforme. Int. J. Cancer* **34,** 383–387.

Marasas, W. F. O., Nelson, P. E., and Toussoun, T. A. (1984b). *Toxigenic Fusarium Species: Identity and Mycotoxicology,* pp. 1–328. The Pennsylvania State Univ. Press, University Park/London.

Merrill, A. H., Jr. (1983). Characterization of serine palmitoyl-transferase activity in Chinese hamster ovary cells. *Biochim. Biophys. Acta* **754,** 284–291.

Merrill, A. H., Jr. (1991). Cell regulation by sphingosine and more complex sphingolipids. *J. Bioenerg. Biomembr.* **23,** 83–104.

Merrill, A. H., Jr., Nimkar, S., Menaldino, D., Hannun, Y. A., Loomis, C., Bell, R. M., Tyagi, S. R., Lambeth, J. D., Stevens, V. L., Hunter, R., and Liotta, D. C. (1989). Structural requirements for long chain base inhibition of protein kinase C in vitro and for the cellular effects of these compounds. *Biochemistry* **28,** 4360–4366.

Merrill, A. H., Jr., Wang, E., Mullins, R. E., Jamison, W. C., Nimkar, S., and Liotta, D. C. (1988). Quantitation of free sphingosine in liver by high-performance liquid chromatography. *Anal. Biochem.* **171,** 373–381.

Norred, W. P., Bacon, C. W., Plattner, R. D., and Vesonder, R. F. (1991). Differential cytotoxicity and mycotoxin content among isolates of *Fusarium moniliforme. Mycopathologia* **115,** 37–43.

Norred, W. P., Plattner, R. D., Vesonder, R. F., Hayes, P. M., Bacon, C. W., and Voss, K. A. (1990). Effect of *Fusarium moniliforme* metabolites on unscheduled DNA synthesis (UDS) in rat primary hepatocytes. *Toxicologist* **10,** 165.

Plattner, R. D., Norred, W. P., Bacon, C. W., Voss, K. A., Peterson, R., Shakelford, D. D., and Weisleder, D. A. (1990). A method of detection of fumonisins in corn samples associated with field cases of equine leukoencephalomalacia. *Mycologia* **82,** 698–702.

Riley, R. T., Goeger, D. E., Showker, J. L., Cole, R. J., and Dorner, J. (1987). Age and growth related changes in cyclopiazonic acid-potentiated lipophilic cation accumulation by cultured cells and binding to freeze-thaw lysed cells. *J. Biochem. Toxicol.* **2,** 251–264.

Riley, R. T., Norred, W. P., Dorner, J., and Cole, R. J. (1985). Increased accumulation of the lipophilic cation tetraphenylphosphonium by cyclopiazonic acid-treated renal epithelial cells. *J. Toxicol. Environ. Health* **15,** 779–788.

Ross, P. F., Rice, L. G., Plattner, R. D., Osweiler, G. D., Wilson, T. M., Owens, D. L., Nelson, H. A., and Richard, J. L. (1991). Concentrations of fumonisin B_1 in feeds associated with animal health problems. *Mycopathologia* **114,** 129–135.

SAS Institute Inc. (1985). *SAS/STAT Guide for Personal Computers.* Cary, NC.

Shier, W. T., Abbas, H. K., and Mirocha, C. J. (1991). Toxicity of mycotoxins fumonisins B_1 and B_2 and *Alternaria alternata* f.sp. *lycopersica* toxin (AAL) in cultured mammalian cells. *Mycopathologia,* **116,** 97–104.

Stevens, V. L., Nimkar, S., Jamison, W. C., Liotta, D. A., and Merrill, A. H., Jr. (1990). Characteristics of the growth inhibition and cytotoxicity of long-chain (sphingoid) bases for Chinese hamster ovary cells: Evidence for involvement of protein kinase C. *Biochim. Biophys. Acta* **1051,** 37–45.

Sydenham, E. W., Thiel, P. G., Marasas, W. F. O., Shephard, G. S., van Schalkwyk, D. J., and Koch, K. R. (1990). Natural occurrence of some *Fusarium* mycotoxins in corn from low and high esophageal cancer prevalence areas of the Transkei, Southern Africa. *J. Agric. Food Chem.* **38,** 1900–1903.

Voss, K. A., Norred, W. P., Plattner, R. D., and Bacon, C. W. (1989). Hepatotoxicity and renal toxicity in rats of corn samples associated with field cases of equine leukoencephalomalacia. *Fd. Chem. Toxicol.* **27,** 89–96.

Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., and Merrill, A. H., Jr. (1991). Inhibition of sphingolipid biosynthesis by fumonisins: Implications for diseases associated with *Fusarium moniliforme. J. Biol. Chem.* **266,** 14,486–14,490.

Wilson, T. M., Ross, P. F., Rice, L. G., Osweiler, G. D., Nelson, H. A., Owens, D. L., Plattner, R. D., Reggiardo, C., Noon, T. H., and Pickrell, J. W. (1990). Fumonisin B_1 levels associated with an epizootic of equine leukoencephalomalacia. *J. Vet. Diag. Invest.* **2,** 213–216.