Cytotoxicity of Fumonisin B_1, Diethylnitrosamine, and Catechol on the SNO Esophageal Cancer Cell Line

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Mycotoxins that commonly contaminate staple food grains pose a health hazard to animals and humans. Fumonisin B_1 (FB_1), a mycotoxin produced by *Fusarium verticillioides*, causes equine leukoencephalomalacia and porcine pulmonary edema and has been implicated in the etiology of esophageal cancer (EC) in the Transkei, South Africa. Various studies have indicated that nitrosamines induce EC, and *F. verticillioides* enhancement of nitrosamine-induced EC in rats has been reported. Dietary catechol (CAT), a constituent of cigarette smoke, was previously found to be a cocarcinogen with methyl-*N*-nitrosamine for inducing esophageal tumors in rats. In the present study we therefore investigated the cytotoxic effects of FB_1, diethylnitrosamine (DEN), and CAT on a human esophageal epithelial cell line (SNO) using the methylthiazol tetrazolium assay. For each treatment, toxin concentrations ranged from 2.165 to 34.64 µM. The results showed that the cytotoxic response of SNO cells was highest in cells treated with 34.64 µM FB_1. SNO cells treated with DEN + FB_1 showed greater cytotoxicity than did cells treated with FB_1 alone, whereas FB_1 appeared to inhibit the cytotoxic effect exerted by CAT alone. The results of this study provide further evidence for the involvement of FB_1 in the etiology of esophageal carcinoma. *Key words:* catechol, cell culture, diethylnitrosamine, fumonisin B_1, methylthiazol tetrazolium assay. *Environ Health Perspect* 110:813–815 (2002). [Online 28 June 2002]
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Approximately 60–90% of human cancers are attributable to environmental factors, particularly chemical carcinogens (*1*). Two important groups of carcinogens that have emerged are the mycotoxins and *N*-nitrosamines.

Fumonisin B_1 (FB_1) is a mycotoxin produced by the fungus *Fusarium verticillioides*, a ubiquitous soil-borne fungus that frequently contaminates maize and maize products. FB_1 is the most abundant and toxic of the known fumonisins and has been associated with many animal diseases, including equine leukoencephalomalacia (*2*), porcine pulmonary edema (*3*), and liver and kidney tumors in rats (*4*). The geographic areas where FB_1 occurs in high concentrations have been associated with high rates of esophageal cancer (EC) in humans (*5*). Although FB_1 has been classified by the International Agency for Research on Cancer as a type 2B carcinogen (*6*) and appears to be an initiator and promotor of carcinogenesis in rats (*7*), we have as yet no convincing evidence that FB_1 is a human carcinogen.

Minute quantities of nitrosamines are present in cigarette smoke, alcoholic beverages, and certain foods, but their cumulative effect over several years could play a role in cancer. *N*-nitrosamines are precarcinogens, giving rise to ultimate carcinogens only after enzymatic activation under acidic conditions in the stomach (*8*). A possible correlation exists between EC and gastrectomy, that is, alkaline reflux into the esophagus. This could account for the mechanism by which ultimate carcinogens of nitrosamines reach the esophagus.

N-nitrosamines are organ specific, and in this regard, asymmetrical nitrosamines (e.g., *N*-nitrosomethylalanine and *N*-nitrosopiperidine) have a tendency to induce tumors of the esophagus (*1*). Mingxin et al. (*9*) found that methylbenzylnitrosamine, nitrososarcosine ethyl ester, and other secondary amines induced carcinoma of the esophagus in rats. Harris et al. (*10*) reported that cultured human esophageal cells activated dimethylnitrosamine into DNA-binding metabolites.

F. verticillioides enhancement of nitrosamine-induced EC in rats has been reported by van Rensburg (*11*). Also, nitrosamines and their precursors have been found in foods from a high-EC area of China (*12*). These findings suggest that nitrosamines may play a role in the etiology of human esophageal carcinogenesis, possibly by acting synergistically with mycotoxins or other metabolites of *F. verticillioides* (*13*).

Excessive exposure to tobacco and alcohol has repeatedly been implicated as a principal factor in carcinoma of the esophagus (*14,15*). Tobacco and tobacco smoke contain many distinct classes of chemical carcinogens and cocarcinogens, including the *N*-nitrosamines, which are important environmental carcinogens. Dietary catechol (CAT), a constituent of cigarette smoke, was previously found to be a cocarcinogen with methyl-*N*-nitrosamine (MNAN) for inducing esophageal tumors in rats. CAT in drinking water was not significantly cocarcinogenic with MNAN, but ethanol and CAT given in the drinking water was cocarcinogenic with MNAN and tumorigenic when given without MNAN (*16*). The increased carcinogenicity by MNAN occurs because ethanol and CAT affect MNAN metabolism.

The human esophageal carcinoma cell line SNO was derived from a well-differentiated squamous cell carcinoma (6.5 cm long and metastatic to the lymph nodes) that was explanted from patient S.N., a 62-year-old Zulu male, in July 1972 (*17*).

There is sufficient evidence of the carcinogenicity of FB_1 in test animals, but not enough data to draw definite conclusions for humans (*18*). We undertook this study to investigate whether FB_1 is an etiologic agent in human esophageal carcinoma, as well as to review the cytotoxic effect of other etiologic factors in this disease. In this study we evaluated the cytotoxicity of FB_1, diethylnitrosamine (DEN), and CAT on the human SNO EC cell line.

Materials and Methods

Materials. We purchased all cell culture media and plasticware from Sterilin (Durban, South Africa). We obtained methylthiazol tetrazolium (MTT) salt, DEN, and CAT from Sigma Chemical Company (St. Louis, MO, USA) and FB_1 from the Programme on Mycotoxins and Experimental Carcinogenesis (Cape Town, South Africa). We purchased all solvents from Merck (Johannesburg, South Africa).

Preparation of toxin stock solutions. We prepared stock solutions of FB_1, DEN, and CAT by dissolving 0.5 mg of each toxin in 30 µL ethanol, 170 µL dimethyl sulfoxide (DMSO), and 4.8 mL complete culture medium (CCM; minimum essential medium supplemented with 5% fetal calf serum, 1% Penstrep fungizone, and 1% L-glutamine), thus yielding a toxin concentration of 100 µM. The control stock solution contained ethanol, DMSO,

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and CCM. For the cytotoxicity assay, we used serial dilutions (2.165–34.64 µM) prepared from the stock solution.

Preparation of the MTT salt. We dissolved MTT salt (5 mg) in 1 mL Hank's balanced salt solution (HBSS) to give a concentration of 5 mg/mL. We then filtered the suspension through a 0.45-µm filter and stored it in the dark at room temperature until use.

The MTT assay. We determined the cytotoxic effect of the toxins on SNO cells using the MTT assay. We washed two confluent monolayers in 75-cm^3 culture flasks in CCM with HBSS, which we then trypsinized to detach cells from the flask. We removed the trypsin and replaced it with 15 mL CCM and gently shook the flasks to dislodge the cells. We assessed cell viability using trypan blue, determined cell numbers using a hemocytometer (*19*), and resuspended cells to give a cell count of 4.5×10^6 cells. We aliquoted the cell suspension (100 µL) into each well of two 96-well tissue culture plates and incubated them at 37°C.

After 24 hr, we replaced the CCM with 100 µL toxin at concentrations ranging from 2.165 to 34.64 µM, using five replicates for each serial dilution. After a 48-hr incubation at 37°C, we removed the toxin and replaced it with 10 µL MTT (5 mg/mL) and 100 µL CCM, and then incubated the mixture for a further 4 hr. We subsequently removed the supernatant and aliquoted 100 µL DMSO into each well to solubilize any resulting formazan crystals. After 1 hr, we determined the optical density of the resulting solution spectrophotometrically using a Bio-Rad (Johannesburg, South Africa) multiwell plate reader at 595 nm and a reference wavelength of 630 nm. We calculated cell viability from the absorbance values obtained.

We then statistically analyzed the results using one-way analysis of variance (ANOVA) for multiple comparisons and Student's *t*-test and the Mann-Whitney rank sum test for two-group comparisons. We constructed graphs to illustrate cell viability after calculation of the standard deviation. We considered data comparisons significant if $p < 0.05$.

Results and Discussion

In this study we evaluated the cytotoxic effect of FB_1 and other etiologic agents (DEN and CAT) on SNO cells using the MTT assay.

We observed minimal cytotoxicity in SNO cells exposed to FB_1 over 48 hr compared with control cells (Figures 1 and 2), with FB_1-treated cells showing a 23% cell mortality after 48-hr incubation with 34.64 µM FB_1 ($p < 0.022$). The low cytotoxic response by SNO cells at the concentrations tested is in agreement with other studies. Cawood et al. (*20*) noted a low cytotoxic response in primary hepatocytes. However, FB_1 has been shown to be cytotoxic to certain mammalian cell lines (*21,22*), suggesting that some tissue may accumulate FB_1 over time or may be more susceptible to the cancer-promoting ability of FB_1. The effects of FB_1 are chronic, as suggested by the late age of onset of EC (between 40 and 60 years). FB_1 is also poorly absorbed (experiments show that 90% of FB_1 is directly excreted), which explains why FB_1 is a slow-acting carcinogen (*23*).

Apart from a chronic effect, the low toxic response over 48 hr by cells treated with FB_1 may suggest that FB_1 is more effective as a cancer promoter than an initiator of EC in humans. This disagrees with studies that show a toxic effect by FB_1 in rat liver and kidney. The presence of an amino group and the location of the hydroxyl on C_{14}/C_{15} may also play an important role in both the toxic and cancer-initiating activities of FB_1. The presence of the amino group facilitates the conjugation of FB_1 via gluteraldehyde to protein carriers (*24*). This would provide a plausible mechanism by which FB_1 exerts its mitogenic effect.

Cytotoxicity depends on the ability of a molecule to bind cellular receptors and/or penetrate the cell membrane (dependent on size, structural conformation, and polarity of the compound). Polarity appears to be an important determinant in the cytotoxic behavior of FB_1. In general, less polar molecules have higher cytotoxicity (*25*). FB_1 is a strongly polar compound (*26*). This explains the low cytotoxic response observed on treatment with FB_1.

Another possibility is that FB_1 acts in synergy with a cocarcinogen that forms part of the etiology of EC. It therefore appears that SNO cells are more susceptible to combined treatment with DEN and FB_1. One-way ANOVA revealed a significant difference between treatment with FB_1 alone compared with treatment with DEN + FB_1 at 34.64 µM ($p < 0.043$), 17.32 µM ($p < 0.008$), 8.66 µM ($p < 0.005$), and 4.33 µM ($p < 0.03$). A cell mortality of 37% occurred upon treatment with DEN + FB_1 (34.64 µM). At the same concentration, we observed only 9% cell mortality upon treatment with DEN alone. Figure 1 shows the differences observed in cell viability after treating SNO cells with FB_1, DEN, and DEN + FB_1.

DEN is part of a widely acting group of potent carcinogens. Nitrosamines are metabolized by mixed-function oxidase enzymes to a chemically active alkylating agent in the rat

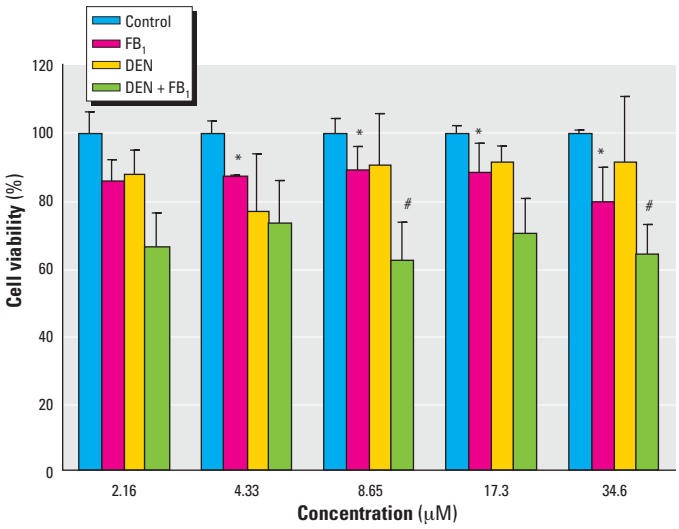

Figure 1. Cell viability of control, FB_1-treated, DEN-treated, and DEN+FB_1-treated SNO cells after 48-hr exposure to the toxins.
Significant difference between *control and FB_1; #FB_1 and DEN + FB_1 ($p < 0.05$, Student's *t*-test and Mann-Whitney rank sum test).

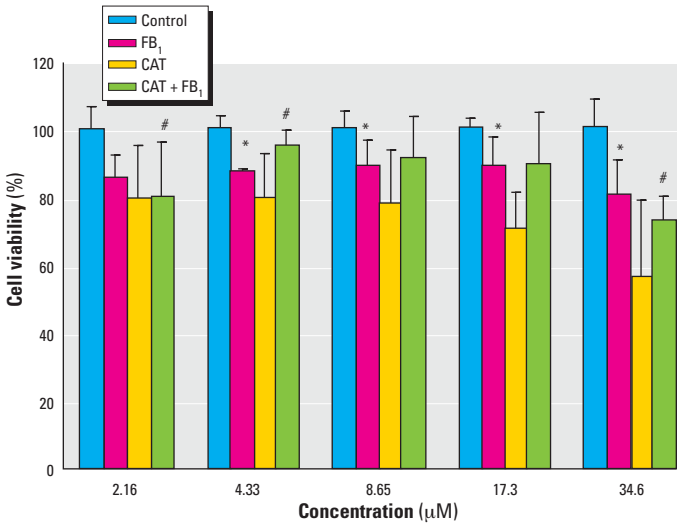

Figure 2. Cell viability of control, FB_1-treated, CAT-treated, and CAT+FB_1-treated SNO cells after 48-hr exposure to the toxins.
Significant difference between *control and FB_1; #FB_1 and CAT + FB_1 ($p < 0.05$, Student's *t*-test and Mann-Whitney rank sum test).

and possibly in the human esophagus (*27*). This reaction produces alkylating intermediates that can form O^6-alkylguanines from DNA guanine. DNA guanine then pairs with thymine rather than cytosine. It is thought that this mispairing produces a mutation that initiates carcinogenesis (*28*). In support of this view, we detected elevated levels of O^6-methylguanine in esophageal DNA extracted from EC patients in China (*29*). When nitrosamines alkylate rat esophageal DNA and thereby initiate cancer, they are presumably activated by microsomes in the rat esophagus because the active metabolites do not travel in the blood from other organs to the esophagus (*24*). The same may apply to nitrosamine induction of EC in humans. The results suggest that DEN acts in concert with FB_1 and may act as a cancer initiator to promote the activity of FB_1.

The cytotoxicities of FB_1, CAT, and CAT + FB_1 are shown in Figure 2. CAT is cytotoxic to SNO cells, producing 44% cell mortality at 34.64 µM ($p < 0.03$). This agrees with epidemiologic studies that have clearly demonstrated a strong positive association of chronic tobacco smoking with EC (*14*). We also observed a significant difference between FB_1 treatment and FB_1 + CAT at 4.33 µM ($p < 0.01$) and 2.165 µM ($p < 0.038$). However, we found no significant differences in cytotoxic activity between treatment with FB_1 alone and FB_1 + CAT (23% and 28% cell death, respectively) when we assessed the entire range of concentrations against each other. When CAT and FB_1 co-occur, there seems to be a stimulation of cell growth or a reduction in mitochondrial dehydrogenase activity. The results of this study suggest that FB_1 inhibits the effect of CAT on the cells, possibly by reducing binding sites for the toxin.

Conclusions

The results show that FB_1 alone is not overtly cytotoxic in humans, but that it may act as a promotor or initiator of carcinogenesis in synergy with certain cocarcinogens. Future studies will involve the treatment and immunolocalization of FB_1 in SNO cells, using light and electron microscopy. In addition, we know that FB_1 alters sphingolipid metabolism. We will therefore expose SNO cells to sphinganine and FB_1 to determine the cytotoxic effect on this cell line.

References and Notes

1. Fong LYY. Environmental carcinogens and dietary deficiencies in esophageal cancer in Asia. In: Cancer of the Esophagus (Pfeiffer CJ, ed). Boca Raton, FL:CRC Press, 1982;41–63.
2. Marasas WFO, Kellerman TS, Gelderblom WCA, Coetzer JAW, Thiel FG, van der Lugt JJ. Leukoencephalomalacia in a horse induced by fumonisin B_1 isolated from *Fusarium moniliforme*. Onderspoort J Vet Res 55:197–203 (1988).
3. Harrison LR, Colvin BM, Greene JT, Newman LE, Cole JR. Pulmonary oedema and hydrothorax in swine produced by fumonisin B_1, a toxic metabolite of *Fusarium moniliforme*. J Vet Diagn Invest 2:217–221 (1990).
4. Gelderblom WCA, Kriek NPJ, Marasas WFO, Thiel PG. Toxicity and carcinogenicity of the *Fusarium moniliforme* metabolite, fumonisin B_1 in rats. Carcinogenesis 12:1247–1251 (1991).
5. Rheeder JP, Sydenham EW, Marasas WFO, Thiel PG, Shepherd GS, van Schalkwyk DJ. *Fusarium moniliforme* and fumonisins in corn in relation to human oesophageal cancer in Transkei. Phytopathology 82:353–357 (1992).
6. IARC. Heterocyclic amines. IARC Monogr Eval Carcinog Risks Hum 56:107–108 (1993).
7. Gelderblom WCA, Marasas WFO, Vleggaar R, Thiel PG, Cawood MF. Fumonisins: isolation, chemical characterisation and biological effects. Mycopathologia 117:11–16 (1992).
8. Louis CJ. Tumours: Basic Principles and Clinical Aspects. Edinburgh:Churchill Livingstone, 1978.
9. Mingxin L, Ping L, Baorong L. Recent progress in research on oesophageal cancer in China. Adv Cancer Res 33:173–249 (1980).
10. Harris CC, Autrup H, Stoner GD, Trump BF, Hillman E, Schafer PW, Jeffrey AM. Metabolism of benzo(*a*)pyrene, *N*-nitrosodimethylamine, and *N*-nitrosopyrrolidine and identification of major carcinogen-DNA adducts formed in cultured human esophagus. Cancer Res 39:4401–4406 (1979).
11. van Rensburg SJ. Esophageal cancer micronutrient malnutrition, and silica fragments. Lancet 2:1098–1099 (1982).
12. Yang CS. Research on oesophageal cancer in China: a review. Cancer Res 40:2633–2644 (1980).
13. Voss KA. Toxins from *Fusarium moniliforme*, a common fungus in corn. Vet Hum Toxicol 32:57–63 (1990).
14. Ming Xin L, Shi Jun Cheng. Etiology of carcinoma of the oesophagus. In: Carcinoma of the Oesophagus and Gastric Cardia (Huang GJ, Kai WY, eds). Berlin:Springer-Verlag, 1984;26–51.
15. Rose EF. Esophageal cancer in the Transkei—the pattern and associated risk factors. In: Cancer of the Esophagus (Pfeiffer CJ, ed). Boca Raton, FL:CRC Press, 1982;19–28.
16. Mirvish SS, Weisenburger DD, Henrichs SH, Nickols J, Hinman C. Effect of catechol and ethanol with and without methylamylnitrosamine on oesophageal carcinogenesis in the rat. Carcinogenesis 15(5):883–887 (1994).
17. Bey E, Alexander J, Whitcutt JM, Hunt JA, Gear JHS. Carcinoma of the esophagus in Africans: establishment of a continuously growing cell line from a tumor specimen. In Vitro 12(2):107–114 (1976).
18. Norred WP. Fumonisins—mycotoxins produced. Environ Health 38:309–328 (1993).
19. Freshney IR. Culture of Animal Cells: A Manual of Basic Technique. 1st ed. New York:Alan RS Incorporated, 1983.
20. Cawood ME, Gelderblom WCA, Alberts IF, Snyman SD. Interaction of ^{14}C-labelled fumonisin B mycotoxins with primary rat hepatocyte cultures. Food Chem Toxicol 32(7):627–632 (1994).
21. Abbas HK, Gelderblom WCA, Cawood ME, Shier WT. Biological activities of fumonisins, mycotoxins from *Fusarium moniliforme*, in jimsonweed and mammalian cell cultures. Toxicon 31:345–353 (1993).
22. Shier WT, Abbas HK, Mirocha CJ. Toxicity of the mycotoxins fumonisins B_1 and B_2 and *Alternaria alternata* f. sp. *lycopersici* toxin (AAL) in cultured mammalian cells. Mycopathologia 116:97–104 (1991).
23. Dutton MF. Fumonisins, mycotoxins of increasing importance: their nature and their effects. Pharmacol Ther 70:137–161 (1996).
24. Azcona-Oliviera JI, Abouzied MM, Plattner RD, Pestka JJ. Production of monoclonal antibodies to the mycotoxins fumonisins B_1, B_2 and B_3. J Agric Food Chem 40:531–534 (1992).
25. Gelderblom WCA, Cawood ME, Snyman SD, Vleggaar R., Marasas WFO. Structure-activity relationships of fumonisins in short-term carcinogenesis and cytotoxicity assays. Food Chem Toxicol 31(6):407–414 (1993).
26. Diaz GJ, Boermans HJ. Fumonisin toxicoses in domestic animals: a review. Vet Hum Toxicol 36(6):548–555 (1994).
27. Mirvish SS, Huang Q, Chen SC, Birt DF, Clark GW, Hinder RA, Smyrk TC, de Meester TR. Metabolism of carcinogenic nitrosamines in the rat and human oesophagus and induction of oesophageal adenocarcinoma in rats. Endoscopy 25(9):627–631 (1993).
28. Xia QJ, Zhan Y. Fungal invasion in oesophageal tissue and its possible relation to oesophageal carcinoma. Chin Med J 58(7):392–396 (1978).
29. Umbenhauer D, Wild CP, Momtesano R, Saffhill R, Boyle JM, Huh N, Kirstein U, Thomale J, Rajewsky MF, Lu SH. O^6-methyldeoxyguanine in oesophageal DNA among individuals at high risk of oesophageal carcinoma. Int J Cancer 36(6):661–665 (1985).