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Effects of purified altertoxins I, II, and III in the metabolic communication V79 system

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EFFECTS OF PURIFIED ALTERTOXINS I, II, AND III IN THE METABOLIC COMMUNICATION V79 SYSTEM

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Purified Alternaria alternata *altertoxins I, II, and III were evaluated for comparative cytotoxicity and ability to inhibit gap junction communication in the Chinese hamster lung metabolic cooperation assay. The noncytotoxic test range for each altertoxin was determined for the metabolic communication assays: altertoxin I, 1, 2, 3, 4, 5 μg/ml; altertoxin II, 0.02, 0.008, 0.006, 0.004, 0.002, 0.0008 μg/ml; and altertoxin III, 0.2, 0.1, 0.08, 0.06, 0.04 μg/ml. Altertoxin II was the most cytoxic in the V79 system, followed by altertoxins III and I. The last cytotoxic of the three, altertoxin I, weakly disrupted metabolic communication at two concentrations (4 and 5 μg/ml). Altertoxins III and II did not significantly inhibit gap junction communication more than the weak tumor promoter 4-O-methyl ether tetradecanoylphorbol 13-acetate.*

INTRODUCTION

Alternaria species are common spoilage fungi that contaminate fruit, vegetables, and grains with their numerous toxic metabolites (King and Schade, 1984; Robeson et al., 1984; Watson, 1984). Three metabolites produced by *Alternaria alternata,* altertoxins I, II, and III, have been isolated and proposed to have hydroxyperylenequinone structures. All three compound are mutagenic in the *Salmonella typhimurium* assay, suggesting a potential danger to exposed populations (Stack and Prival, 1986).

Alternaria toxic metabolites may be found in minute amounts in food supplies of developed nations. Repeated consumption of contaminated foods may result in chronic toxic effects (King and Schade, 1984) or an increased incidence of tumors and blood disorders (Hadidane et al., 1985).

Chronic exposure to substances at low concentrations may enhance the establishment of semifunctional cells by promotion of cell clones to various disease states (Archer, 1985; Trosko and Chang, 1980; Trosko et al., 1983). Development of abnormal cell clones may be explained by at

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least one proposed cellular mechanism. Semifunctional or initiated cells that have been removed from the environmental influence of normal cells may proliferate by the promotional effects of disruption of metabolic communication between adjacent cell gap junctions (Trosko et al., 1982; Zhong-Xiang et al., 1986).

The effects of altertoxins I, II, and III were observed in the Chinese hamster lung V79, $HGPRT^+$ $HGPRT^-$ metabolic cooperation assay to determine if the altertoxins could eliminate cell-cell communication via gap junction. This system of gap-junctional communication has been effectively used to identify promotional chemicals that disrupt intracellular communication (Trosko et al., 1982; Yotti et al., 1979; Tsushimoto et al., 1982).

MATERIALS AND METHODS

Cell Culture and Maintenance

Wild type V79, 2-amino-6-mercaptopurine (6-thioguanine, 6-TG)-sensitive ($6\text{-}TG^s$, $HGPRT^+$); mutant V79, 6-TG-resistant ($6\text{-}TG^r$, $HGPRT^-$); and gap-junction mutant (MC^-, $HGPRT^-$) Chinese hamster lung fibroblast cells for cytotoxicity and metabolic cooperation assays were generously provided by Dr. J. E. Trosko, Michigan State University, East Lansing, Mich. Cells were stored in liquid nitrogen at −120°C after the first subculture. Revived cells were cultured in modified Eagle's medium, 1 m*M* sodium pyruvate and 3% fetal bovine serum. The medium used for experimental trials was further supplemented with an antibiotic mix of penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). Cells were subcultured twice weekly. A new set of cells ($6\text{-}TG^s$, $6\text{-}TG^r$, and MC^-) was revived for use from frozen storage every 2 mo. All cell cultures were grown at 37 ± 0.5°C in a humidified incubator with an atmosphere containing 5% CO_2.

Chemicals

Altertoxins I, II, and III that were prepared, isolated, and purified from *A. alternata* grown on cooked rice were kindly provided by Michael Stack, Food and Drug Administration, Division of Contaminants Chemistry, Washington, D.C. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA), 4-*O*-methyl ether tetradecanoylphorbol 13-acetate (4-O-MTPA), and 2-amino-6-mercaptopurine (6-thioguanine, 6-TG) were purchased from Sigma Chemical Co., St., Louis, Mo.

Cytotoxicity Assay for the Metabolic Cooperation Assay

The determination of the optimal concentration range for the metabolic cooperation assay and the assay itself were conducted according to the procedure of Trosko et al. (1981) and Aylsworth et al. (1984). Tissue

culture plates, 60 mm, containing 100 MC^-, $HGPRT^-$, gap junction mutants, and 4×10^5 $6\text{-}TG^s$, $HGPRT^+$ wild-type cells planted 4 h previously were exposed to a range of altertoxin dilutions (50 μl/plate) prepared in acetone/ethanol (50:50 v/v). Five or six plates were used for each altertoxin dilution, along with TPA (4 ng/ml), the strong tumor promoter control, and 4-O-MTPA (4 ng/ml), the weak tumor promoter control (Trosko et al., 1981; Mass et al., 1984; Keisari et al., 1984; Leach and Blumberg, 1985). 6-TG (10 μg/ml) was added to each plate after addition of alterotoxins and controls. Plates were incubated at 37°C for 7–8 d. Old medium was removed every 3 d and replaced with fresh 3% serum-supplemented medium containing only 6-TG. Plates were washed with phosphate-buffered saline, fixed with methanol, and stained with 1% crystal violet at the end of the incubation period. Surviving MC^- colonies of 50 or more cells were counted and the cytotoxic range that permitted 70% survival was determined.

Metabolic Cooperation Assay

Dilutions of altertoxins in the selected ranges of 70–95% cytotoxicity were distributed in 50 μ aliquots (10–12 plates/dilution) into plates seeded 4 h earlier with 100 $6\text{-}TG^r$, $HGPRT^-$, and 4×10^5 $6\text{-}TG^s$, $HGPRT^+$. 6-TG was added to each plate 30 min after the addition of altertoxins and controls.

Statistical Analysis

The number of $6\text{-}TG^r$ colonies surviving was expressed as percent of survivors in appropriate solvent control. These percentages were analyzed by taking the arcine (percent/100) half transform to ensure normality. An analysis of variance was computed to compare altertoxin dilutions with TPA and 4-O-MTPA controls (Ostle and Mesing, 1975). The difference in means was determined at the $\alpha = 0.05$ level by Duncan's test (Duncan, 1955). Curves were plotted to show the 95% confidence intervals around the TPA and 4-O-MTPA controls (Ostel and Mesing, 1975). Standard deviation bands were plotted around the means for each toxin dilution.

RESULTS

Cytotoxicity Assay for Metabolic Cooperation

The cytotoxicity range that allowed 70–95% MC^- colonial survival was determined for each altertoxin (Table 1). Altertoxin II was the most cytotoxic, followed by altertoxins III and I. The 70–95% colonial survival of altertoxins II and III falls within a concentration range of 3 logs, whereas that of altertoxin I was only 1 log. The cytotoxic activities of altertoxins II and III are at least 100 times that of altertoxin I.

TABLE 1. Comparison of Cytotoxicity in Altertoxins I, II, and III

Concentration (μg/ml)	Altertoxin I colonial survival (%)[a]	Altertoxin II colonial survival (%)	Altertoxin III colonial survival (%)
5	73		
4	79		
3	79		
2	82		
1	96		
0.2			78
0.1			80
0.08			85
0.06			89
0.04			86
0.02		80	95
0.008		74	96
0.006		74	
0.004		93	
0.002		93	
0.0008		94	

[a]Percentage of colonial survival based on solvent controls survival.

Metabolic Cooperation Assay

The result from the metabolic cooperation assay for the three altertoxins are plotted as percentages of 6-TGr colonial survival in Fig. 1.

Altertoxin I was significantly more disruptive to metabolic communication at all concentrations, when compared with the weak promoter 4-O-MTPA ($\alpha = 0.05$). The greatest disruption was seen at concentrations of 4 and 5 μg/ml. Concentrations of 4 and 5 μg/ml of altertoxin I were also significantly different from the solvent controls in the cytotoxicity assay, although they were within the acceptable 70–95% cytotoxicity range. The concentrations of 2 and 3 μg/ml did not differ significantly from the solvent and 4-O-MTPA controls of the metabolic cooperation assay.

All concentrations of altertoxins II and III that were within the 70–95% cytotoxicity range were not significantly different from the solvent and 4-O-MTPA in the metabolic communication assay. Altertoxins II and III did not disrupt metabolic communication under the experimental conditions described in the Methods section.

DISCUSSION

Many metabolites of *Alternaria* have been shown to be highly toxic in vivo and in vitro (Christensen et al., 1968; Pero et al., 1973). We evaluated the comparative cytotoxic activity of altertoxins I, II, and III with the 100 $MC^- + 4 \times 10^5$ 6-TG^s cytotoxicity assay. This assay is appropriate

for these types of compounds because the number of available cells for toxin exposure is equivalent to the number of cells available in the metabolic cooperation assay, that is, 100 6-TG^r + 4 × 10^5 6-TG^s. Cytotoxicity is expected to be comparable in both systems. Altertoxin II was the most cytotoxic of the three altertoxins. The cytotoxic curves of all the altertoxins were dose-responsive.

Our cytotoxicity data are comparable to the results of Pero et al. (1973). Using the HeLa S3 monolayer, they found altertoxin II preparation to be more cytotoxic at 0.5 μg/ml (ID50) than altertoxin I preparation at 20 μg/ml (ID50). They did not report on altertoxin III. The cytotoxic activity of altertoxin I in the MC$^-$ assay of μg/ml and of 0.02 μg/ml for altertoxin II compared well with the 100-fold difference in the HeLa S3 monolayer.

The more cytotoxic altertoxins II and III did not inhibit metabolic communication. The effects of altertoxin I at concentrations of 4 and 5 μg/ml in the V79 system were significantly different from those of the solvent and weak tumor promoter. These last two concentrations also

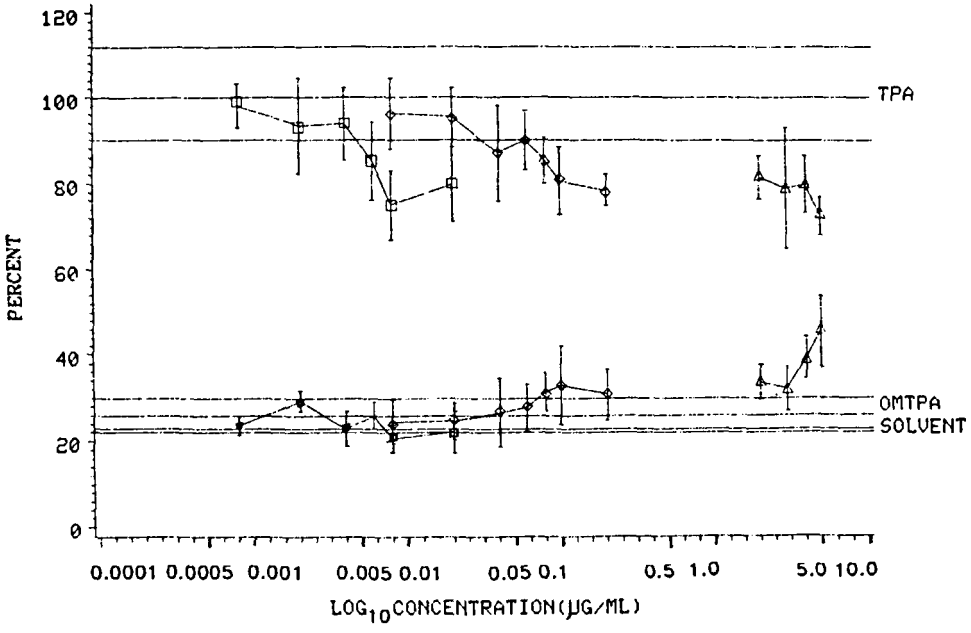

FIGURE 1. Cytotoxicity curves for altertoxin II (□), altertoxin III (◇), and altertoxin I (△), placed in order of decreasing cytotoxicity, are shown in the upper plot. Cytotoxic activity for the concentrations tested for each altertoxin lies within the 70–96% cytotoxicity range (7–95% cell survival). Metabolic cooperation curves are represented by the lower curves, which may be compared with the nonpromoting solvent control, weak tumor-promoting 4-O-MTPA, and strong tumor-promoting TPA. Bars show mean ± 1 SD. The 95% confidence limits are shown around TPA, 4-O-MTPA, and controls.

presented a significant difference in cytotoxic response as compared with solvent controls. This cytotoxicity did not diminish the disruptive effects on cellular communication. The polybromated biphenyl 3,3′,4,4′,5,5′-hexabromobiphenyl has been shown to be nonpromotional at noncytotoxic doses and promotional at cytotoxic concentrations (Jensen et al., 1983).

We conclude that all altertoxins display dose-responsive cytotoxicity and that altertoxin I can weakly disrupt metabolic communication. The altertoxins that can cause significant cellular death or interfere with cell-cell communication may adversely affect populations exposed to contaminated food supplies.

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