Production, Mutagenicity, and Immunotoxicity of Gliotoxin

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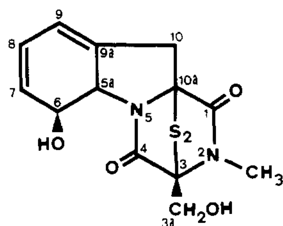
Gliotoxin, an epidithiodioxopiperazine mycotoxin, was isolated for the first time from culture of *Dichotomomyces cejpii* (Mil'ko) Scott. Production was optimized so that the best results were obtained with potato dextrose or soya media under static conditions and with potato dextrose or malt extract media under shake conditions. With or without metabolic activation, this toxin showed no mutagenic activity in *Salmonella typhimurium* strains (TA 98, TA 100, and TA 1537). Immunotoxic assays in vitro showed an immunomodulating effect of gliotoxin on splenocytes. At low doses (0.05–5 μg/L) this toxin had a stimulating effect, particularly without mitogen and with PHA. In contrast, it did not affect Con A stimulated T-lymphocytes. Gliotoxin stimulation of T-helper lymphocytes was higher than that of the suppressor. Higher dose inhibition of lymphocyte proliferation was strongly dose dependent (ID_{50} from 27.5 to 50 μg/L according to mitogen).

INTRODUCTION

Gliotoxin, an epidithiodioxopiperazine mycotoxin, was first isolated from *Gliocladium fimbriatum* (Weindling, 1941) and later from species of *Trichoderma*, *Aspergillus*, and *Penicillium* (Nagarajan, 1984).



Aspergillus fumigatus is a common human and animal pathogen (Howard, 1983). Its pathogenicity can be linked to the production of various toxins including gliotoxin (Glister and Williams, 1944; Müllbacher et al., 1985). The biological effects of this toxin include inhibition of various bacteria (Taylor, 1971), fungi (Johnson et al., 1943), viruses (Larin et al., 1965; Ho et al., 1969), and transplantable tumors (Kidd, 1947). In spite of the broad antibiotic spectrum (Taylor, 1971), its toxicity renders it useless as a therapeutic agent (Johnson et al., 1943; Larin et al., 1965).

During our investigation of some metabolites from *Dichotomomyces cejpii* (Mil'ko) Scott, a component was identified as gliotoxin (Cole and Cox, 1981); this toxin had never been isolated from this fungus. We previously reported uncommon ^1H and ^{13}C NMR assignments (Kaouadji et al., 1990). The present work concerns production of gliotoxin by *D. cejpii*, mutagenic properties, and in vitro immunomodulating effects.

MATERIALS AND METHODS

Fungal Strain, Culture Conditions, and Toxin Production. *D. cejpii* (Mil'ko) Scott (CMPG 893) was isolated from marine sediments (Barcelona, Spain) and accessed by the Laboratory of Cryptogamy (CMPG, Collection Mycologie Pharmacie Grenoble). The strain was stored at 4 °C on solid malt extract medium (1.5%). Eight-day cultures grown at 24 °C were used as inocula. Cultivation proceeded at 22 °C for 8 days in a 10-L batch fermentor (Biolafitte, France) with either shaking (100 rpm) and aeration (1 1.1 min^{-1}) or under static conditions. Liquid culture media were potato dextrose (Difco), Sabouraud, Czapek-Dox, Galzy and Slonimski (1957) with or without corn steep liquor (1.5%), and soya (Smith and Rosazza, 1974). Sterilization was done by autoclaving for 20 min at 121 °C. Isolation and characterization of gliotoxin have previously been reported (Kaouadji et al., 1990). Production was estimated according to the antifungal activity detected by the agar-well diffusion method (Hufford, 1975; Devillers et al., 1989) using *Candida albicans* (Ca) (CMPG 684) and *Candida tropicalis* R_2 IP (CtR2) (Polyen-resistant; Institut Pasteur).

Table I. Antifungal Bioassay Relative to the Production of Gliotoxin by *D. cejpii* Cultivated for 8 Days in Various Conditions (Diameter of Inhibition in Millimeters)[a]

	static		shaking	
medium	CA	CT R2	CA	CT R2
Sab	16	28	0	13
CzD	12	13	0	0
GS	24	33	10	17
GS CSL	14	21	0	10
ME	11	28	15	22
PD	37	45	33	43
Soja	29	38	0	12

[a] CA, *C. albicans*; CT R2, *C. tropicalis* R2; Sab, Sabouraud; CzD, Czapek-Dox; GS, Galzy and Slonimski; CSL, corn steep liquor; ME, malt extract; PD, potato dextrose.

Ames/*Salmonella* Assay. The Ames/*Salmonella* mutagenicity test (Ames et al., 1975) was performed with *Salmonella typhimurium* strains TA 1537, TA 98, and TA 100 (obtained from Dr. Bruce N. Ames, University of California, Berkeley, CA). 2-Aminoanthracene (TA 1537, TA 98, and TA 100) was positive control for metabolic activation using S9 mix. Hycanthone (TA 1537), 2-nitrofluorene (TA 98), and sodium azide (TA 100) were control mutagens not requiring metabolic activation. Determinations were triplicated. A compound was mutagenic when it induced a concentration-dependent increase in the revertant number and the highest number of revertants was more than twice that of the control.

In Vitro Immunotoxic Assays. Stock solution of gliotoxin in ethanol (10 mg/mL) was stored at 4 °C and diluted in phosphate-buffered saline (PBS) medium before use. Reference mitogens were lipopolysaccharide (LPS) from *S. typhimurium* (Difco Laboratories), pokeweed mitogen (PWM), purified phytohemagglutinin (PHA), and concanavalin A type IV (Con A) (Sigma Chemical Co.). Stock solutions of mitogens in PBS were stored at −70 °C. Dilutions were prepared in RPMI 1640 me-

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Table II. Mutagenicity of Gliotoxin in *S. typhimurium* Strains (TA 98, TA 100, TA 1537) without (−S9) and with (+S9) Metabolic Activation

chemical	concn µg/plate	concn nmol/plate	revertants/plate[a] for strain TA 98 −S9	TA 98 +S9	TA 100 −S9	TA 100 +S9	TA 1537 −S9	TA 1537 +S9
none (control)			16	26	113	127	4	7
2-aminoanthracene[b]	2	10.4		1484		1561		115
2-nitrofluorene[b]	2	9.5	262					
hycanthone[b]	50	17.8					319	
sodium azide[b]	20	307.6			3621			
gliotoxin	1	3.07	17	33	110	164	5	7
	0.3	0.92	10	31	112	132	4	4
	0.1	0.31	14	34	116	165	6	6
	0.03	0.09	12	28	115	151	3	4
	0.01	0.03	19	25	97	149	5	3

[a] Mean of three or more determinations. Spontaneous revertant colonies were not subtracted. [b] 2-Nitrofluorene, sodium azide, and hycanthone were positive unactivated control, respectively, for TA 98, TA 100, and TA 1537. 2-Aminoanthracene was positive activated control for all strains.

Table III. Mitogen Responses of Murine (Balb/C) Splenic Cells Treated in Vitro with Various Concentrations of Gliotoxin

gliotoxin, mg/mL	mitogen: none	Con A[b]	PHA[b]	PWM[b]	LPS[b]
0	941.70 ± 90.00	59651.20 ± 2283.90	9457.40 ± 1154.90	6206.90 ± 577.50	8613.10 ± 1569.00
5×10^{-6}	3199.20 ± 435.60	56536.00 ± 1165.40	13334.60 ± 5614.00	5463.80 ± 398.30	13241.50 ± 286.10
P	<0.01	>0.05	>0.05	>0.05	<0.02
5×10^{-5}	2849.60 ± 456.60	55016.10 ± 1620.50	18462.90 ± 3662.90	9333.80 ± 253.20	14895.90 ± 3556.60
P	<0.01	>0.05	<0.05	<0.01	>0.05
5×10^{-4}	3621.70 ± 794.70	50813.80 ± 6147.20	22038.00 ± 3801.40	9784.00 ± 719.70	15700.30 ± 3223.40
P	<0.01	>0.05	<0.01	<0.01	<0.05
5×10^{-3}	2833.20 ± 1621.30	50139.30 ± 494.00	24482.00 ± 3736.30	7345.60 ± 405.40	11909.80 ± 2417.80
P	>0.05	<0.01	<0.01	>0.05	<0.05
5×10^{-2}	292.50 ± 14.50	4675.70 ± 945.50	307.70 ± 61.10	277.70 ± 19.50	294.50 ± 99.40
P	<0.001	<0.001	<0.001	<0.001	<0.01
5×10^{-1}	232.30 ± 33.70	286.10 ± 11.20	194.20 ± 1.10	242.00 ± 73.20	238.00 ± 54.50
P	<0.001	<0.001	<0.001	<0.001	<0.01
5	344.70 ± 136.80	289.30 ± 31.80	204.60 ± 3.60	236.30 ± 29.30	246.40 ± 43.10
P	<0.001	<0.001	<0.001	<0.001	<0.01

[a] Results are expressed in dpm ● standard deviation (SD). Student's *t*-test was significative for $P < 0.05$. [b] Con A, concanavalin A; PHA, phytohemagglutinin; PWS, pokeweed mitogen; LPS, lipopolysaccharide.

dium (Eurobio) supplemented with 10% heat-inactivated and filtered fetal bovine serum (Eurobio), 2 mM glutamine (Eurobio), 50 units of penicillin G/mL, and 50 µg of streptomycin/mL (Boehringer). The final concentrations were PHA and Con A 10 µg/mL, PWM 5 µg/mL and LPS 30 µg/mL.

Spleens aseptically excised from 8–9-week-old female Balb/C mice (20-g body weight) were pooled for each experiment. The cell suspension containing 5×10^5 cells/100 µL was distributed in wells of microtest tissue plate (Costar); 50 µL of mitogen (or 50 µL of RPMI medium for each assay without mitogen) and 50 µL of the diluted toxin (or 50 µL of RPMI medium for the control) were added. Cultures were incubated at 37 °C for 48 h in a 5% CO_2 (in air) humidified atmosphere; 37 kBq of [^{3}H]-thymidine ([^{3}H] TdR, TMM-79C, specific activity 1.66 TBq/mmol CEA) was added to each well, and incubation was continued for an additional 18 h. Cells were collected with a multiple automated sample harvester (Flow) on glass-fiber filters disks (GF/C) (Whatman). After desiccation, the disks were placed in 3 mL of toluene scintillator (Packard), and ^{3}H activity was determined via liquid scintillation counting (Model 2000 Tri-Carb, Packard Instrument Co.). Results were expressed as disintegrations per minute (dpm) of triplicate samples. All data were analyzed by Student-Fischer's *t*-test. A 0.05 level of significance was chosen.

RESULTS AND DISCUSSION

The production of gliotoxin by *D. cejpii* cultivated under various conditions is reported in Table I. After 8 days of fermentation, the best results were obtained with either potato dextrose or soya media under static conditions. Production was generally higher with potato dextrose or malt extract media when shaking procedures were used. Cultivation of *D. cejpii* in synthetic Czapek-Dox medium did not give good results, meaning that nitrate as N source is not suitable for the fungus. Production was higher in synthetic Galzy and Slonimski medium where the N source was $(NH_4)_2SO_4$. The addition of corn steep liquor reduced gliotoxin production, or this compound was produced first and rapidly metabolized.

Mutagenic activity of gliotoxin according to the Ames/*Salmonella* assay is shown in Table II. With or without metabolic activation, this toxin did not show any mutagenic activity toward strains TA 98, TA 100, and TA 1537 for concentrations studied. These results agree with the absence of genotoxic activity of gliotoxin observed both in the Kada test (Boutibonnes et al., 1984) and in the SOS chromotest (Auffray and Boutibonnes, 1986). We have observed bacteriostatic effects on Ames strains TA 98, TA 100, and TA 1537 when gliotoxin concentration was >1 µg/mL.

In vitro immunotoxic assays showed that splenocytes were sensitive to gliotoxin. At low doses, this toxin had a stimulatory effect, particularly without mitogen and with PHA (Figure 1 and Table III). With higher doses, it

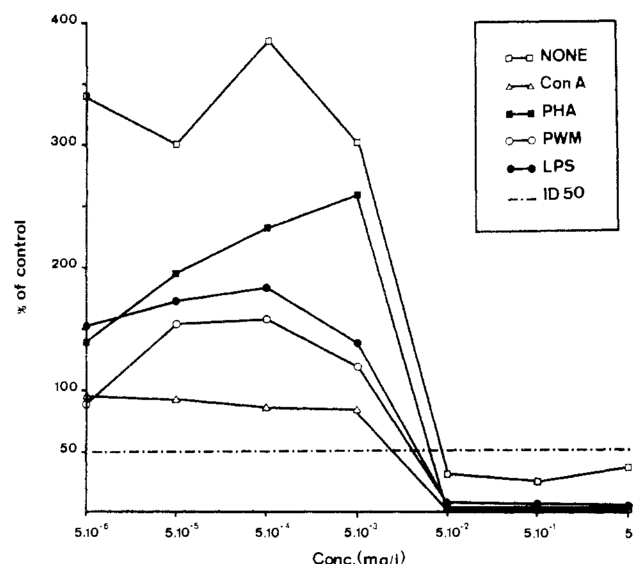

Figure 1. Mitogen responses (in percent of control) of splenic cells treated in vitro with various concentrations of gliotoxin (none, control; Con A, concanavalin A; PHA, phytohemagglutin; PWH, pokeweek mitogen; LPS, lipopolysaccharide; ID_{50}, inhibition dose 50).

strongly inhibited lymphocyte proliferation, in a dose-dependent manner (ID_{50} from 27.5 to 50 μg/L according to mitogen). Without mitogen, from 5 to 500 ng/L gliotoxin increased significantly [^{3}H]thymidine incorporation, while higher doses decreased it (ID_{50} = 50 μg/L). PHA-stimulated lymphocytes were strongly stimulated from 0.05 to 5 μg/L toxin. We also found that gliotoxin had a cytotoxic effect in vitro against MRC_5 cells at 25 μg/L. It is interesting to observe that this mycotoxin had a stimulatory effect toward splenocytes at doses much lower than 25 μg/L.

The data suggest that gliotoxin enhanced the PHA stimulation of T-lymphocytes at low doses (233% and 259%, respectively, at 50 and 500 μg/L). On the contrary, it did not affect Con A stimulated T-lymphocytes. It seems that this toxin stimulated more T-helper than suppressor lymphocytes. At the same concentrations, PWM- and LPS-stimulated B-lymphocytes were weakly but significantly stimulated. At higher doses from 50 μg/L, T- and B-lymphocytes were strongly depressed, whichever the mitogen used.

Our results are in agreement with others concerning gliotoxin (Müllbacher and Eichner, 1984; Müllbacher et al., 1985, 1986). This toxin irreversibly inhibited phagocytosis of thioglycolate-induced peritoneal macrophages after only 10 min of contact at concentrations of 20–50 μg/L (Müllbacher and Eichner, 1984). Other immune or immune-related functions inhibited by gliotoxin at low concentrations were the ability of stimulator cells in mixed lymphocyte reactions to induce cytotoxic T cells and the activation of T and B cells by mitogen (Müllbacher et al., 1986).

It is interesting that Müllbacher et al. (1986) suggested that the bridged disulfide moiety was the most important entity for the immunomodulating activity of gliotoxin and epipolythiodioxopiperazines since the presence of reducing agents suppressed their activity (Trown and Bilello, 1972).

In conclusion, we have shown that the highest production of gliotoxin by *D. cejpii* was obtained in PD medium. The lack of mutagenic activity of this compound has been confirmed on the Ames/*Salmonella* assay. Immunomodulating activity in vitro on splenocytes was highest without mitogen or with PHA. Further research is in progress to optimize gliotoxin production and to point out other biological activities.

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