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Immunology

Immunosuppression *in vitro* by a metabolite of a human pathogenic fungus

(gliotoxin/*Aspergillus fumigatus*/cytotoxic T cells)

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ABSTRACT **Gliotoxin, a metabolite of *Aspergillus fumigatus*, inhibits phagocytosis of macrophages at concentrations of 20–50 ng/ml. Pretreatment of stimulator cells in mixed lymphocyte cultures with gliotoxin (100 ng/ml) abrogates induction of alloreactive cytotoxic T cells. The presence of gliotoxin 48 hr after initiation of cytotoxic T-cell induction has no effect. Inhibition of cytotoxic T-cell induction by gliotoxin at low concentrations, acting on the stimulator cells, can be overridden by concanavalin A-activated cell supernatants. Gliotoxin does not induce immediate cell-surface antigen modification on target cells. The possible role of gliotoxin in the etiology of *A. fumigatus*-related diseases is discussed.**

Fungal metabolites have been extensively investigated for their antimicrobial activities since the fortuitous discovery of penicillin by Fleming. Little is known, however, apart from general toxicological studies on eukaryotic cells, about their effect on specific tissue such as the hemopoietic system. One fungal metabolite, cyclosporin A, has recently gained worldwide attention as a potent immunosuppressive agent. Cyclosporin A has been investigated as an immunoregulatory agent in clinical practice, such as organ transplantation, as well as a tool in fundamental immunological research (1). It appears to act on the T lymphocyte, especially the T helper subset responsible for release of interleukin 2 after antigen recognition (1). Problems associated with cyclosporin A are nephrotoxicity, relatively high concentrations required for activity, and its low solubility in aqueous solutions.

Aspergillus fumigatus, a member of the order Eurotiales, has been shown to be associated with a number of lung diseases in vertebrates including man (2–4). *A. fumigatus* produces a variety of toxic substances including gliotoxin, a compound containing an epidithiadioxopiperazine ring, which possesses antimicrobial activity *in vitro* (5). However, little is known regarding the effect of any of these fungal metabolites *in vivo* and their possible role in the etiology of *A. fumigatus*-related diseases.

We now report on the immunosuppressive and anti-phagocytic activity of gliotoxin *in vitro* and its possible role in the pathogenesis of *A. fumigatus*-induced diseases.

MATERIALS AND METHODS

Animals. CBA/H, BALB/c, and C57BL/10 mice were obtained from the breeding unit of the John Curtin School and were used when >6 weeks old.

Gliotoxin. Gliotoxin was a generous gift of R. Gallagher (Ruakura Animal Research Station, Hamilton, New Zealand) and A. Taylor (Atlantic Research Laboratory, Halifax, Canada). Gliotoxin was dissolved in absolute ethanol at 1 mg/ml and stored in 25-μl aliquots at −70°C.

Target Cells. Thioglycollate-induced peritoneal macrophages (TGM), concanavalin A (Con A)-activated lymphocytes, L929, BW5147, P815 tumor cells, and secondary mouse embryo fibroblasts were grown and labeled with neutral red or with ^{51}Cr as described in detail elsewhere (6).

Preparation of Con A-Activated Cell Supernatants. The preparation and assay of Con A-activated cell supernatants has been described (7).

Mixed Lymphocyte Cultures (MLCs). Responder spleen cell suspensions (2×10^6 cells per ml) were cocultured for 5 days at 37°C in humidified 5% CO_2/95% air with either 2×10^6 allogeneic spleen cells (inactivated by 2000 R from a ^{60}Co source) or 1×10^6 allogeneic TGM in 5 ml of Eagle's minimal essential medium F15 (GIBCO) containing 5% fetal calf serum and 10^{-4} M 2-mercaptoethanol.

Cytotoxicity Assay. The ^{51}Cr release assay for cytotoxic cells using P815, L929, BW5147, Con A blasts, and TGM has been described (6). In brief, aliquots of harvested MLCs in F15 medium containing 5% fetal calf serum were titrated in 3-fold dilution steps in 96-well round-bottom microtiter trays. ^{51}Cr-labeled targets (2×10^4) were added in 100-μl aliquots to each well, and the plates were incubated for 4 hr at 37°C in a humidified 5% CO_2 atmosphere. One-hundred-microliter fractions of supernatants were removed and radioactivity was determined.

Antibody and Complement-Mediated Lysis. This procedure has been described (6). The anti-H-2^k alloantiserum was a gift of I. F. C. McKenzie (University of Melbourne).

RESULTS

The Effect of Gliotoxin on Adherence of Cells to Plastic. During the course of development of the neutral red assay for measurement of T-cell cytotoxicity (6), we observed that supernatants of *A. fumigatus*-contaminated cultures caused total loss of macrophage adherence to the plastic microtiter plates. The fungal metabolite responsible for this phenomenon has been identified as gliotoxin (unpublished results). To investigate the cell specificity of action of gliotoxin, TGM, L929 cells, and BALB/c fibroblasts were stained with neutral red in suspension and added in 100-μl aliquots to serial 2-fold dilutions of gliotoxin. The cells were allowed to adhere for 5 hr at 37°C and nonadherent cells were washed off. Residual cells were lysed and neutral red content was estimated colorimetrically using an ELISA autoreader as described (6). TGM did not adhere at gliotoxin concentrations as low as 20–50 ng/ml (Fig. 1). Fibroblasts and L929 cells, on the other hand, were susceptible to gliotoxin at concentrations of 100–500 ng/ml. This differential susceptibility has been consistently observed between the different cells used in a large number of independent experiments.

Macrophages presumably adhere to plastic by a different mechanism than do fibroblasts and L929 cells—namely, by

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Abbreviations: MLC, mixed lymphocyte culture; Con A, concanavalin A; TGM, thioglycollate-induced peritoneal macrophages.

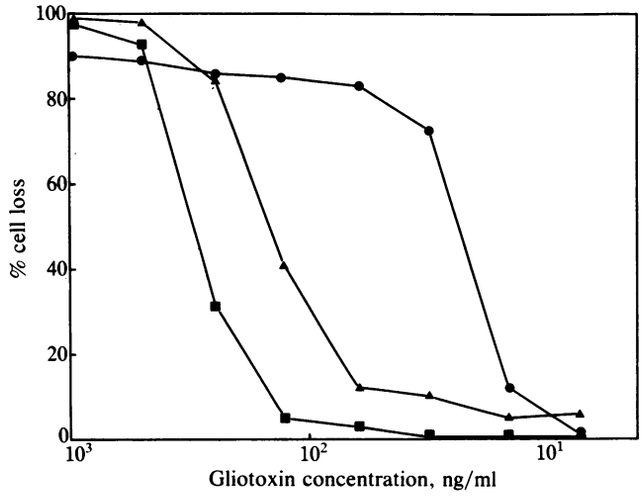

FIG. 1. Cell adherence to plastic in the presence of gliotoxin. Aliquots of 2×10^4 neutral red-labeled L929 (■), BALB/c fibroblasts (▲), or CBA-TGM (●) were added to microtiter wells containing gliotoxin at concentrations of 10^3 to 5×10^0 ng/ml. Cell adherence after 5 hr was measured at 540 nm in an ELISA microreader.

phagocytosis. The effect of gliotoxin on TGM phagocytosis was therefore investigated. Uptake of India ink, carbonyl iron and latex beads as seen under light and electron microscopy was inhibited at gliotoxin concentrations identical to those required to prevent TGM adherence to plastic (data not shown).

The Effect of Gliotoxin on the Generation of Alloreactive Cytotoxic T Cells *in Vitro*. We observed that *in vitro* mixed lymphocyte reactions when contaminated by *A. fumigatus*, although yielding live lymphocytes as ascertained by trypan blue exclusion tests, did not, however, kill the appropriate targets in cytotoxic T-cell assays. Thus, we tested the possibility that low gliotoxin concentrations may also alter the stimulatory capacity of macrophages and spleen cells. BALB/c spleen cells were cocultured with either irradiated C57BL/10 spleen cells or nonirradiated C57BL/10 TGM. Either stimulator population was left untreated or was treated with gliotoxin at 1000 ng/ml and 100 ng/ml for 1 hr (Table 1). Treatment of stimulator cells with gliotoxin at 1000 ng/ml totally inhibited the generation of alloreactive cytotoxic T cells. Treatment of stimulator cells with gliotoxin at 100 ng/ml was equally as effective on TGM stimulator cells but only partially effective on spleen cells.

Kinetics of Action of Gliotoxin on Generation of Alloreactive Cytotoxic T Cells *in Vitro*. To investigate at which stage of generation of alloreactive cytotoxic T cells gliotoxin is active, individual cultures of BALB/c anti-C57BL/10 and BALB/c anti-CBA were set up. Gliotoxin was added to individual 5-ml cultures at days 0, 1, 2, 3, and 4 to give a final concentration of 100 ng/ml. The gliotoxin remained for the duration of the culture period (subject to loss of gliotoxin due to either possible instability of the compound or metabolic degradation). The cultures were harvested on day 5 and cytotoxicity was estimated on C57BL/10 TGM and EL4 tumor cell targets for the BALB/c anti-C57BL/10 MLCs and on CBA, TGM, and BW tumor cell targets for the BALB/c anti-CBA MLCs (Fig. 2). No cytotoxicity was observed from cultures in which gliotoxin was present from day 0. Addition of gliotoxin 24 hr later still markedly inhibited the generation of alloreactivity in the BALB/c anti-CBA MLCs and to a much lesser extent in the BALB/c anti-C57BL/10 MLCs. Addition of gliotoxin 48 hr and 72 hr later had no effect; thus, gliotoxin acts very early in the induction of alloreactive cytotoxic T cells.

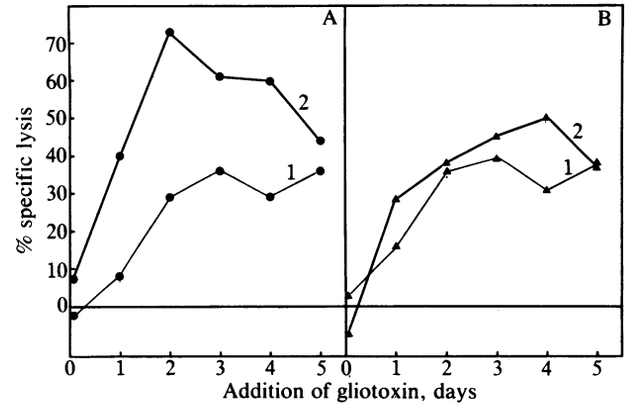

FIG. 2. Alloreactivity of BALB/c anti-CBA (curves 1) cytotoxic T cells tested on BW (*A*) and CBA-TGM (*B*) and alloreactivity of BALB/c anti-C57BL/10 (curves 2) tested on EL4 (*A*) and C57BL/10-TGM targets (*B*). Individual MLCs were given gliotoxin to a final concentration of 100 ng/ml at days 0–4 of culture.

Con A-Activated Lymphocyte Supernatant Activity on Gliotoxin-Treated Allogeneic Spleen Stimulator Cells. The data in Fig. 2 suggest that early events in the induction of alloreactivity are affected by gliotoxin. The possibility that gliotoxin specifically inhibits the generation or release of one or more of the necessary lymphokines required for cytotoxic T-cell induction was tested by the addition of 3% Con A-activated

Table 1. Gliotoxin-induced inhibition of BALB/c anti-C57BL/10 alloreactive cytotoxic T-cell generation *in vitro*

Stimulator cells*	Gliotoxin, ng/ml	% specific lysis of ^{51}Cr C57BL/10 TGM†
Spleen	0	39.9 (1.9)
Spleen	1000	−1.3 (1.3)
Spleen	100	19.3 (1.7)
TGM	0	37.7 (3.6)
TGM	1000	−3.9 (1.2)
TGM	100	−3.4 (1.1)

*Spleen cells were irradiated with 2000 R from a ^{60}Co source. Spleen and TGM cells were treated with gliotoxin for 1 hr and then added to cultures 2×10^6 and 1×10^6 cells per well, respectively.

†Mean percent specific ^{51}Cr release over a 4-hr period, with SEM of three replicate samples given in parentheses. Spontaneous release was 24%. The values given are from titration curves at 1/90 fraction of culture.

Table 2. Inhibition of induction of CBA anti-BALB/c alloreactive cytotoxic T cells *in vitro*

Treatment of stimulator cells*	% specific lysis of ^{51}Cr P815†
None	55.3 (1.3)
Gliotoxin (1000 ng/ml)	−0.4 (0.3)
Gliotoxin (100 ng/ml)	4.1 (0.4)
Gliotoxin (1000 ng/ml) and CS	0.1 (0.5)
Gliotoxin (100 ng/ml) and CS	58.8 (1.5)
UV irradiation	3.3 (0.6)
UV irradiation and CS	60.5 (4.1)

CS, Con A-activated lymphocyte supernatant.

*BALB/c spleen cells were given 2000 R from a ^{60}Co source and then treated with gliotoxin for 1 hr at a concentration of 1×10^6 cells per ml, or they were inactivated by irradiation from a UV source at 960 μW/cm^2 for 4 min. Stimulator cells (2×10^6) were cocultured with 1×10^7 CBA responder cells.

†Mean percent specific ^{51}Cr release over a 4-hr period. Spontaneous release was 16.0%. The values given are from titration curves 1/30 fraction of culture. SEM of three replicate samples is given in parentheses.

Table 3. Effect of gliotoxin on target-cell lysis in antibody and complement and cytotoxic T-cell assays

	Gliotoxin, ng/ml	% specific ^{51}Cr release*			
		CBA-TGM	L929	BW	Con A blast
Anti-H-2^k antibody and complement	0	64 (16)	92 (10)	85 (8)	87 (7)
	10	50 (15)	80 (11)	84 (8)	88 (7)
	100	55 (29)	91 (12)	86 (8)	90 (8)
	1000	69 (20)	81 (17)	76 (8)	99 (9)
Anti-H-2^k cytotoxic T cell	0	56 (28)	74 (22)	80 (17)	69 (23)
	10	55 (31)	81 (24)	67 (16)	76 (23)
	100	16 (65)	84 (25)	69 (16)	78 (27)
	1000	6 (54)	47 (49)	32 (29)	44 (27)

*Mean percent specific ^{51}Cr release over a 3-hr period for antibody and complement-mediated lysis and over a 6-hr period for cytotoxic T-cell mediated lysis. Spontaneous release is given in parentheses. All values given are from titration curves at 1:200 dilution of antisera or 1/90 fraction of cytotoxic T-cell culture.

lymphocyte supernatant to CBA anti-BALB/c MLCs in which BALB/c spleen cells were treated with gliotoxin (Table 2). Treatment of stimulator cells with gliotoxin at concentrations of 1000 ng/ml and 100 ng/ml totally abrogated their ability to induce alloreactive cytotoxic T cells; a similar effect was obtained by UV irradiation of stimulator cells. Addition of Con A-activated lymphocyte supernatant had no effect when stimulator cells were treated with gliotoxin at 1000 ng/ml. However, full alloreactivity was obtained when Con A-activated lymphocyte supernatant was added to gliotoxin-treated (100 ng/ml) and UV-irradiated stimulator cells.

Gliotoxin Treatment of Target Cells. To investigate the effect of gliotoxin on target susceptibility to lysis by antibody and complement and by alloreactive cytotoxic T cells, a panel of target cells (CBA-TGM, L929 and BW tumor cells, and CBA-Con A blast targets) were labeled with ^{51}Cr and then incubated for 1 hr with various concentrations of gliotoxin. Susceptibility to lysis of these targets was tested by (*i*) anti-H-2^k antibody and complement-mediated lysis over a 3-hr period and (*ii*) lysis by alloreactive BALB/c anti-CBA immune cytotoxic T cells (Table 3). No difference in target-cell susceptibility was observed in antibody plus complement-mediated lysis over a 3-hr period. Similarly, no changes in spontaneous release were observed over this period of time in all four targets tested. However, in the cytotoxic T-cell assay over a 6-hr period, target-cell lysis of all four targets was impaired with gliotoxin treatment at 1000 ng/ml. This was accompanied by an increase in spontaneous release of ^{51}Cr. CBA-TGM targets were also affected by gliotoxin concentrations of 100 ng/ml, again showing increased spontaneous release.

DISCUSSION

We have shown here that gliotoxin, a metabolite of *A. fumigatus* and related fungi, is a potent immunosuppressive agent, as tested by the induction of alloreactive cytotoxic T cells *in vitro*. Gliotoxin also inhibits phagocytosis in TGM at concentrations of 20–50 ng/ml. In contrast, gliotoxin is produced in fungal cultures at concentrations of 20–80 μg/ml (5). Loss of phagocytic ability is rapid after gliotoxin contact (5–10 min) and is not reversible for at least 48 hr. To what extent, however, this loss of phagocytic activity is responsible for loss of stimulatory activity is not known. Spleen cells and TGM, when treated with gliotoxin, lose their ability to induce alloreactive cytotoxic T cells. The block occurs early in induction of cytotoxic T cells, and gliotoxin does not seem to prevent cell proliferation unless it is present in the culture within the first 24 hr. The presence of gliotoxin at 48 hr or later has no effect. Similarly, macromolecular synthesis in T- and B-cell lymphoblasts appears unaffected by gliotoxin concentrations sufficient to inhibit macrophage phagocytosis and induction of T-cell alloreactivity (unpublished observation). TGM and spleen allogeneic stimulator cells when treated with gliotoxin at 100 ng/ml can induce alloreactivity when exogenous lymphokines are added. The block of induction, however, cannot be overridden by exogenous Con A-activated lymphocyte supernatant when gliotoxin concentrations of >300 ng/ml are used. This suggests that gliotoxin at low concentrations interferes with interleukin-related processes and does not impair antigen presentation. At higher concentrations, however, antigen presentation also fails.

The results obtained with antibody and complement and cytotoxic T-cell-mediated lysis of gliotoxin-treated target cells (Table 3) further supports the idea that gliotoxin does not modify cell-surface antigenic structures *per se*. However, the increased spontaneous release of ^{51}Cr from TGM targets in the cytotoxic T-cell assay at gliotoxin concentrations of 100 ng/ml and 1000 ng/ml suggests that TGM membrane changes eventually take place after exposure to gliotoxin. Thus, gliotoxin appears to be a potent immunosuppressive agent *in vitro* with action on macrophages and antigen-presenting cells.

The role gliotoxin may play in the etiology of aspergillosis and other immune-deficiency syndromes is a matter of speculation. What is known is that there are a number of toxic fungal metabolites which, like gliotoxin, possess an epidithiodioxopiperazine ring (5); these substances are generally produced by closely related fungi. It is worth noting that recently a fungus (*Thermoascus crustaceus*), which is closely related to *A. fumigatus*, was isolated from monocytes of patients with acquired-immunodeficiency syndrome (AIDS) (8). In either disease, the *in vivo* production of such immunosuppressive fungal metabolites could compromise host defense mechanisms and thus lead to exacerbation of the disease state.

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