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EVIDENCE THAT GLIOTOXIN ENHANCES LYMPHOCYTE ACTIVATION AND INDUCES APOPTOSIS BY EFFECTS ON CYCLIC AMP LEVELS

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Abstract—Gliotoxin is a secondary metabolite produced by several pathogenic fungi. It has potential clinical applications as an immunosuppressive agent in preventing allograft rejection. At low doses (<30 nM) gliotoxin displays co-mitogenic activity, but at higher doses induces apoptosis in cells. Here we demonstrate that gliotoxin, although not mitogenic in its own right, enhances activation in preactivated splenocytes by a calcium-independent mechanism. The enhancement in activation correlates with a decrease in intracellular cyclic AMP levels. This property is inhibited by dibutyryl-cAMP. Increasing the concentration of gliotoxin to levels that caused apoptosis produced a dose-related increase in cAMP levels. Thus, the effects of gliotoxin on cell activation and the induction of apoptosis may both be mediated by changed levels of cAMP.

Key words: cyclic AMP; apoptosis; proliferation; gliotoxin; fungal metabolite

Gliotoxin, a secondary fungal metabolite produced by several fungi, including *Aspergillus, Gliocladium, Trichoderma,* and *Penicillium* species [1], is a member of the epipolythiodioxopiperazine family of compounds. Some members of this family of compounds have attracted attention because of their biological properties and role in disease. Sporidesmin is responsible for producing facial eczema in sheep [1], and gliotoxin has been linked to the pathogenesis of aspergillosis [2].

The known biological properties of gliotoxin include the capability to undergo redox cycling to generate oxygen free radicals that cause oxidative damage to isolated DNA *in vitro* [3]; immunosuppressive activity in *in vitro* [4] and *in vivo* [5] models, and the induction of apoptotic cell death occurred in both cultured cells [6] and in lymphoid organs of mice injected with gliotoxin [5]. Gliotoxin at low concentrations causes an increase in the incorporation of radiolabelled thymidine in bone marrow cells stimulated with GM-CSF‡ [7], reflecting its comitogenic activity at low doses.

The immunosuppressive properties of gliotoxin have considerable therapeutic potential, as has been shown in several transplantation models [8–10]. These applications depend on the observation that different cell types have variable susceptibilities to gliotoxin, and cells of the immune system are particularly sensitive. For the full potential of gliotoxin to be realised, it is important that its mechanism(s) of action are determined, and any effect of gliotoxin on cellular function could give clues as to these mechanisms. Here we show that the effects of gliotoxin on cell proliferation and cell death are related to cAMP levels.

MATERIALS AND METHODS

Splenocyte proliferation assay

Splenocytes from CBA/H mice (bred pathogen-free in the John Curtin School of Medical Research Animal Unit) were red cell-depleted by hypotonic lysis, and the remaining white cells counted using a haemocytometer. Cells (10^6/mL) were pretreated with varying doses of gliotoxin in F15 culture medium (Multicel, TRACE Biosciences) containing 2% (v/v) foetal calf serum (FCS from Commonwealth Serum Laboratory, Parkville, Australia) for one hour at 37°C and 5% CO_2. To measure proliferation, splenocytes (10^6/mL) were cultured in 96 well plates in 200 μL of culture medium + 2% FCS, with or without reagents. All reagents were obtained from Sigma. After one day in a humidified incubator at 37°C, each well was pulsed for 6 hours with 1 μCi of tritiated thymidine (Amersham), and the counts measured in a 1205 betaplate Pharmacia scintillation counter.

Measurement of intracellular calcium

Red cell-depleted splenocytes were resuspended at 2×10^7 cells/mL in F15 + 2% FCS. For measurement of $[Ca^{2+}]_i$ using flow cytometry, a method modified from Merrit *et al.* [11] was used. Prior to each run, 400 μL of cell suspension was incubated for 10 min at 37°C with 2.4 μL fluo-3 (Molecular Probes) at 1 μg/μL in DMSO. Immediately, 100 μL of the stained cells was mixed with 1.9 mL F15 + 2% FCS and loaded onto a Becton Dickinson FACStar Plus. After 2 min, the toxin or control medium was added to the cell suspension, and data acquisition was continued. The fluo-3 was excited with an argon ion laser of 200 mW, and its fluorescence collected on FL1 with a 530-nm filter. Data was analysed

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‡ Abbreviations: cAMP, adenosine 3′5′-cyclic monophosphate; c-GMP, guanosine 3′5′-cyclic monophosphate; dbc-AMP, N,2′-O-dibutyryladenosine 3′5′-cyclic monophosphate; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; FCS, fetal calf serum; FACS, fluorescence activated cell sorting; DMSO, dimethyl sulphoxide; GM-CSF, granulocyte macrophage colony stimulating factor; $[Ca^{2+}]_i$, intracellular calcium increase; PBS, phosphate buffered saline.

using a WinMDI analysis program kindly provided by Joseph Trotter at the Salk Institute, La Jolla, CSA, U.S.A.

Analysis of cyclic AMP levels

Intracellular cyclic AMP levels were measured using an Enzyme Immunoassay Kit (Cayman Chemical Co., Ann Arbor, MI). After appropriate treatment, 2×10^7 cells in 1 mL of 50 mM phosphate buffer with 5% (w/v) trichloroacetic acid (TCA) were frozen, thawed, and then sonicated to disrupt the cells. Samples were extracted with diethyl ether and acetylated with acetic anhydride as described in the kit. This increases the sensitivity of detection. Levels of cAMP were assayed by inhibiting the binding of enzyme-linked cAMP to plate bound cAMP-specific antisera. Amounts of cAMP were quantitated by comparison with a cAMP standard curve. All results show cAMP in pmolar resulting from lysis of 2×10^7 cells in 1 mL volume.

Measurement of apoptosis

Apoptosis was estimated by estimating the subdiploid population of propidium iodide stained cells using FACS [12]. Cells were treated at 10^6/mL in complete medium with various concentrations of gliotoxin for 6 hr. After treatment, cells were suspended in PBS (1 mL) and made 70% in ethanol with cold absolute ethanol. Cells were fixed for at least 1 hr, washed twice in PBS, and stained with 1 mL of PBS containing propidium iodide (4 μg/mL) and RNAase (200 μg/mL) for 1 hr. Cells were analysed on a Becton Dickinson FACStar Plus and DNA analysed using VERITY ModFit software. The results were in agreement with DNA fragmentation measured using agarose gel electrophoresis (data not shown). Occurrence of apoptosis was also confirmed using electron microscopy. Cells were pelleted after treatment, washed once in phosphate buffered saline, and fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 hr. The preparations were post fixed in osmium tetroxide for 1.5 hr and en bloc stained in 2% uranyl acetate for 1 hr. They were then dehydrated in alcohol and embeded in Spurr's resin. Sections were cut and examined on a Philips 301 instrument. Statistics were carried out using the Statview data analysis program running on a Macintosh computer.

RESULTS

Enhancement of the mitogenic activation of splenocytes by gliotoxin

Red cell depleted splenocytes were pretreated with low doses of gliotoxin for one hour, before incubating for one day in medium containing Phorbol-12-Myristate-13-Acetate (PMA) and/or the calcium ionophore A23187. When splenocytes were incubated with concentrations of either A23187 (0.1 μM) or PMA (5 ng/mL) individually, gliotoxin had little or no effect on thymidine incorporation. Use of A23187 or PMA at these concentrations alone also produced no effect on cell proliferation. However, if concentrations of 0.5 μM A23187 or 50 ng/mL PMA were used, gliotoxin pretreatment produced a substantial increase in proliferation (Fig. 1a,b). These concentrations of A23187 and PMA alone

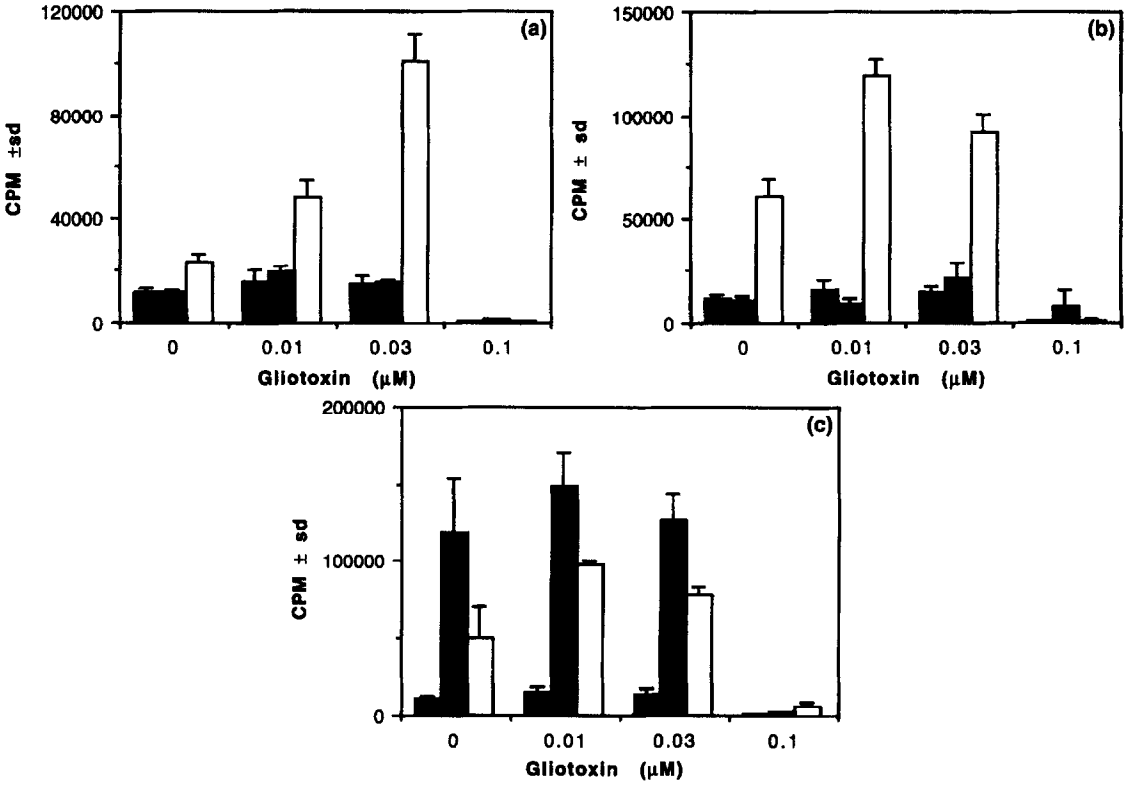

Fig. 1. Co-mitogenic activity of gliotoxin in the presence of PMA and calcium ionophore. (a) Splenocytes pretreated with gliotoxin prior to stimulation with no treatment (■), PMA at 5 ng/mL (▨), and PMA at 50 ng/mL (□). (b) Splenocytes pretreated with gliotoxin prior to stimulation with no treatment (■), A23187 at 0.1 μM (▨), and A23187 at 0.5 μM (□). (c) Splenocytes pretreated with gliotoxin prior to stimulation with no treatment (■), PMA at 50 ng/mL and A23187 at 0.1 μM (▨), and PMA at 50 ng/mL and A23187 at 0.5 μM (□). Proliferation was measured by pulsing with tritiated thymidine for 6 hr after a one-day incubation.

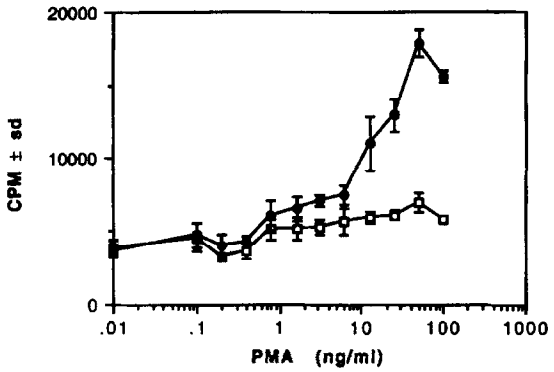

Fig. 2. Effect of gliotoxin upon splenocytes treated with serially diluted PMA. Splenocytes were pretreated with (—●—) or without (—□—) gliotoxin at 0.03 μM, prior to pulsing with tritiated thymidine for 6 hours after a one-day incubation.

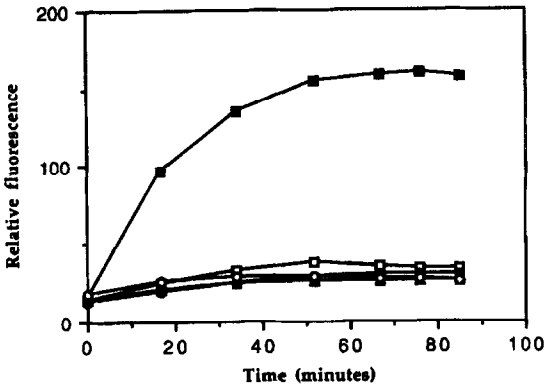

Fig. 3. Mitogenic activity of gliotoxin and PMA on splenocytes is calcium independent. Relative changes to intracellular calcium in untreated cells (—□—) and cells treated with 10 nM thapsigargin (—■—), 0.01 μM gliotoxin (—●—), PMA at 50 ng/mL (—▲—), and gliotoxin and PMA together (—○—). Similar results were obtained using gliotoxin at 30 nM.

are sufficient to produce some proliferation. The mitogenic doses of A23187 (0.5 μM) and PMA (50 ng/mL) used were saturating, as increasing their concentration produced no further increase in proliferation with either PMA (Fig. 2) or A23187 (data not shown). When splenocytes were treated with both PMA and A23187, we still observed some increase in thymidine incorporation after gliotoxin treatment (Fig. 1c).

Treating cells with an increasing concentration of PMA produced a small steady rise in thymidine incorporation, which peaked at 50 ng/mL (Fig. 2). Splenocytes pretreated with 0.03 μM gliotoxin had a greatly increased rate of proliferation, which was more than double the maximum proliferation seen with PMA alone, and peaked at the same concentration (i.e. 50 ng/mL). All subsequent experiments investigated the effect of treating splenocytes with PMA (50 ng/mL) and/or gliotoxin (0.03 μM), as these concentrations exerted maximal effect upon cell proliferation.

Effect of gliotoxin on intracellular calcium levels

Many pathways leading to cellular stimulation require an elevation in intracellular calcium levels ($[Ca^{2+}]_i$). To determine whether gliotoxin caused a similar elevation, splenocytes were treated with gliotoxin and/or PMA and levels of $[Ca^{2+}]_i$ measured by flow cytometry (Fig. 3). Treatment of splenocytes with gliotoxin and PMA at mitogenic concentrations had no effect on $[Ca^{2+}]_i$ levels, compared with untreated cells. In contrast, thapsigargin, which was used as a positive control due to its known property of raising $[Ca^{2+}]_i$ levels, gave a greater than three-fold increase in relative intracellular calcium levels. This indicates that gliotoxin's co-mitogenic activity is calcium independent.

Effect of gliotoxin and PMA on cyclic AMP levels

The lack of effect on $[Ca^{2+}]_i$ indicated that gliotoxin must be acting either via another pathway or downstream from the calcium signal. Cyclic AMP is known to modulate cell activation, and we measured the levels of cAMP within cells stimulated with gliotoxin and PMA. We observed that mitogenic concentrations of PMA alone produced a small decrease in the levels of intracellular cAMP within 30 minutes of treatment (Fig. 4a, $P < 0.01$). There was a significant difference between PMA-treated and PMA/gliotoxin-treated cells, $P < 0.01$. Submitogenic concentrations had no effect upon cAMP levels (not shown). If the cells were also treated with gliotoxin, then the decrease in cAMP levels was much

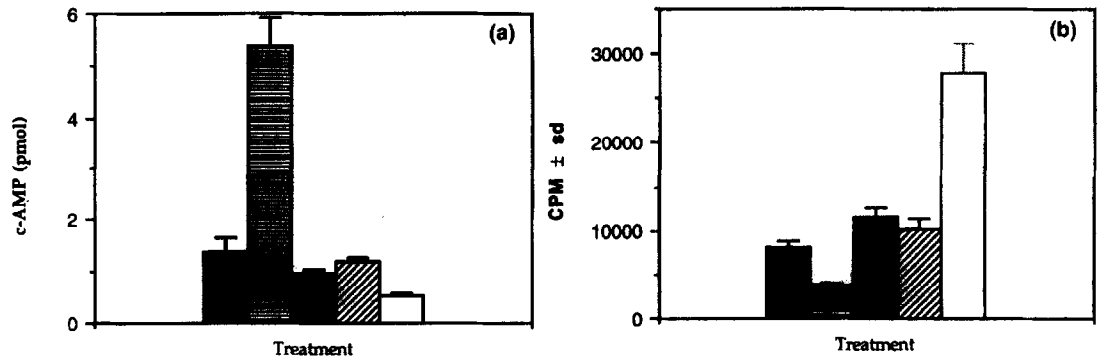

Fig. 4. Effect of gliotoxin and PMA on intracellular cyclic AMP levels and proliferation in splenocytes. (a) Cells were treated as described. Control (■), cholera toxin at 10 ng/mL (▤), PMA at 50 ng/mL (■), 0.03 μM gliotoxin (▨), and PMA and gliotoxin together (□). Measurements were made at 50 min. No effect was seen before 15 min. Typical of six separate experiments. (b) Cells treated simultaneously with PMA and gliotoxin were aliquoted into 96 well plates, and one day later pulsed with tritiated thymidine. Legend is the same as 4a.

Table 1. Time course of cAMP levels in lymphocytes treated with gliotoxin and PMA

Time (hr)	cAMP (pmolar) Untreated	Treated
1	0.56 ± 0.05	0.28 ± 0.07
4	0.30 ± 0.07	0.22 ± 0.03
8	0.18 ± 0.01	0.31 ± 0.12
22	0.27 ± 0.01	0.29 ± 0.08

Table 2. Effect of theophylline on gliotoxin/PMA induced DNA synthesis in lymphocytes

Treatment	CPM
Control	310 ± 35
GT/PMA	2570 ± 430
GT/PMA/1 mM Theo.	570 ± 60
GT/PMA/5 mM Theo.	170 ± 40

greater and correlated with increased proliferation (Fig. 4b). Both PMA and gliotoxin had slight, significant effects ($P < 0.05$), but there was a highly significant difference between thymidine incorporation in cells treated with PMA alone and cells treated with PMA and gliotoxin ($P < 0.01$). Table 1 shows that the decrease in cAMP occurs mainly in the first hour of treatment, and that cAMP levels are the same by 22 hrs. This suggests that it is the initial drop in cAMP relative to control cells that triggers enhanced proliferation. Thus, the increased proliferation seen with gliotoxin and PMA-treated splenocytes could be mediated by a decrease in cAMP levels. If this was true, it would be expected that increasing intracellular levels of cAMP would inhibit stimulation by these reagents. To test this, proliferation experiments were repeated in the presence of N,2′-O-dibutyryladenosine 3′5′-cyclic monophosphate (dbcAMP), a cell-soluble form of cAMP. The addition of dbcAMP to proliferation assays did indeed inhibit the proliferative effect of PMA and gliotoxin at 15 μM, and abolished the effect at 125 uM. The cGMP analogue had little or no effect confirming that the decrease is specifically due to intracellular formation of cAMP (Fig. 5). The phosphodiesterase inhibitor theophylline also inhibits proliferation induced by gliotoxin/PMA (Table 2). Inhibition of endogenous cAMP metabolism by theophylline counters the decrease caused by gliotoxin/PMA and inhibits proliferation.

Gliotoxin-induced apoptosis and cAMP increases

Gliotoxin at concentrations greater than 0.03 μM induces apoptosis in thymocytes. We asked whether gliotoxin could induce apoptosis by a mechanism involving cAMP. Splenocytes were treated with a range of doses of gliotoxin, and the levels of cAMP and apoptosis in these cells measured. Pretreatment with gliotoxin alone up to 0.3 μM produced a dose-related increase in cAMP that correlated with the percentage of cells undergoing apoptosis (Fig. 6). Electron microscopy confirmed the classic morphology of apoptosis in lymphocytes treated with gliotoxin (Fig. 7).

DISCUSSION

Treating splenocytes with both PMA, which activates protein kinase C (PKC), and calcium ionophore A23187, which produces an increase in $[Ca^{2+}]_i$, generates cell activation. At low concentrations, neither signal alone is sufficient to produce this effect, but combined they produce significant cellular proliferation. This provided a useful model for investigating gliotoxin's apparent comitogenic activity.

Pretreating red cell-depleted splenocytes with low doses of gliotoxin enhanced the activation signal provided by mitogenic concentrations of both PMA and A23187 individually, but did not induce activation when submitogenic doses were used. Increasing mitogen concentration did not increase proliferation, indicating that the levels used were saturating. Thus, gliotoxin enhanced the stimulating effects of these mitogens by a mechanism distinct from that of either PMA or A23187. Gliotoxin only significantly increased proliferation if the splenocytes received an activation signal from another source. It had no effect when submitogenic concentrations of reagents were used. Thus, the observed activity of gliotoxin was mediated via a mechanism that is inac-

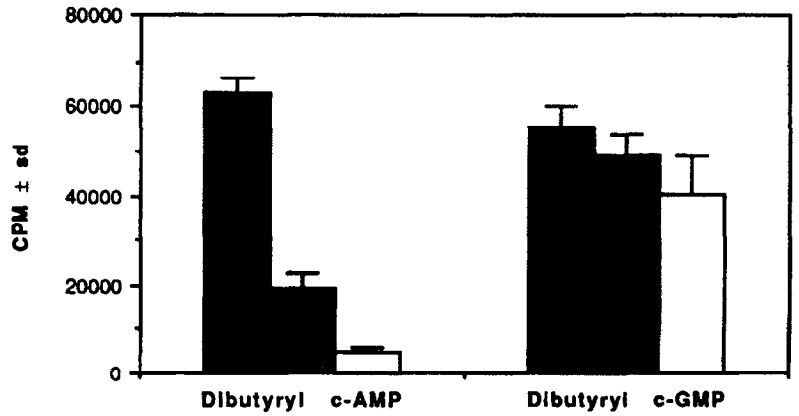

Fig. 5. Inhibition of PMA and gliotoxin mitogenicity by dbcAMP. Red cell-depleted splenocytes were incubated with PMA (50 ng/mL), gliotoxin (0.03 μM), and dbcAMP at 15 μM (▨), 125 μM (□), and untreated with dbcAMP (■) for one day and pulsed for six hours with tritiated thymidine. Corresponding results using dbcGMP from a separate experiment are also shown.

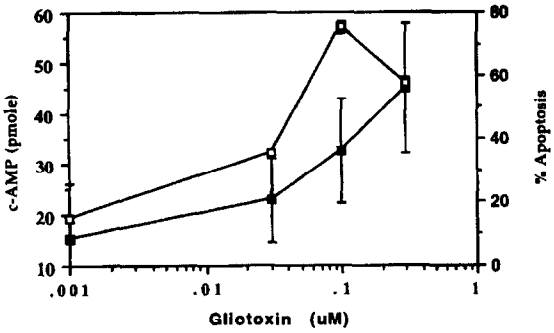

Fig. 6. Elevation of cAMP by high doses of gliotoxin correlates with induction of apoptosis in splenocytes. Apoptosis in splenocytes at 6 hr (—□—). cAMP levels in splenocytes measured at 50 min (—■—).

tive in a quiescent cell but is revealed following treatment with a second mitogen.

Analysis of intracellular elements involved in second messenger signalling demonstrated that gliotoxin acted via a mechanism independent of intracellular calcium. The treatment of splenocytes with PMA and gliotoxin at mitogenic concentrations, however, produced a decrease in cAMP levels within 30 minutes, which inversely correlated with the degree of proliferation measured 24 hours later. The time scale for this decrease in proliferation corresponded with the time required to increase cAMP levels directly with cholera toxin (via GTP-binding proteins, associated with adenylate cyclase at the plasma membrane). That gliotoxin influenced cell proliferation via an effect on cAMP levels was supported by the addition of dbcAMP to proliferation assays, which inhibited the increase in stimulation and the countering effects of theophylline. The effect of reduced cAMP on proliferation occurs in the first few hours of treatment.

The effect of cAMP upon lymphocyte activation has been a matter of controversy over past years, with evidence that cAMP can both increase [13] and inhibit [14] lymphocyte activation. However, the consensus of opinion now appears to be that cAMP provides an inhibitory signal [15, 16]. Tamir and Isakov demonstrated that cAMP inhibited mitogenic signals in T lymphocytes via phosphatidylinositol pathways, increased the binding of the transcription regulator AP-1 to an oligonucleotide containing a TPA-response element (located in the promotor region of many early genes involved in T cell mitogenesis), and altered the composition of *jun* proteins in AP-1 [15]. This led to the down-regulation of many cellular functions. Elevation of cAMP has been demonstrated to inhibit the expression of interleukin-2 (IL2) receptors [17, 18], down-regulate the production of IL1 and IL2 [18], and cause cells to accumulate in early G1 in the cell cycle [19]. Therefore, the observation that gliotoxin further decreases cAMP levels in preactivated lymphocytes could explain the observed effect on cellular proliferation in these cells.

The mechanism by which gliotoxin decreases cAMP levels must be distinct from that of PMA, as the dose of PMA used was saturating and decreased cAMP levels in quiescent cells, whereas gliotoxin could not. PMA provides a signal by activating PKC, which phosphorylates several proteins involved in cell proliferation and leads to activation of the transcription factor AP-1 (*fos*/*jun* heterodimers). In contrast, cAMP acts via PKA, which when activated also phosphorylates transcription factors, leading to activation of cAMP responsive elements (CREs). CRE binding proteins, involved in regulation of the cAMP-dependent activation pathway, are structurally similar to *fos* and *jun* [20]. These two pathways are thus thought to overlap at the nuclear level, and it has been demonstrated that CREs can inhibit activation by AP-1 [21]. This explains the inhibitory action of cAMP in cell activation and how gliotoxin enhances cell proliferation in preactivated cells by further decreasing cAMP levels. The function of cAMP within a cell is extremely complex, as CRE-binding factors can act as activators or repressors (reviewed in ref. [22]).

The toxic activity of gliotoxin is related to its ability to induce cells to undergo apoptosis. Several workers have reported that cAMP is involved in apoptosis, although it is uncertain whether it plays a positive or negative role. The activation of T cell hybridomas through their T cell receptor by immobilised anti-CD3 antibody produced activation-induced cell death, which was inhibited by the elevation of cAMP levels [23]. However, it has been shown that elevation of cAMP can induce apoptosis in thymocytes [24]. Our data are consistent with the latter report.

Here we have shown that low-dose gliotoxin can influence cell division via a reduction in cAMP levels, and that the toxic properties of high doses of gliotoxin may be due to elevation of cAMP, resulting in apoptosis.

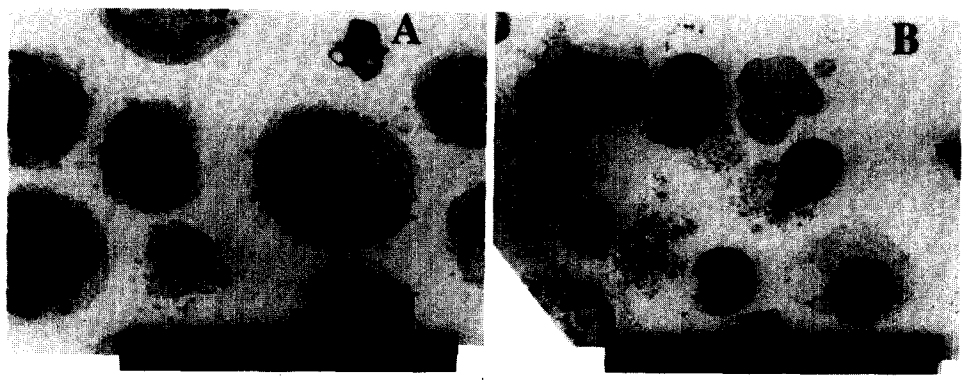

Fig. 7. Morphological evidence of apoptosis in lymphocytes treated with gliotoxin for 6 hr. (A) normal lymphoctytes. (B) lymphocytes treated with 0.3 μM gliotoxin for 6 hr. Note the typical condensed chromatin of the nucleus.

How gliotoxin differentially effects cAMP levels is unknown, and is currently under study.

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