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Original article

Gliotoxin from *Aspergillus fumigatus* affects phagocytosis and the organization of the actin cytoskeleton by distinct signalling pathways in human neutrophils

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Abstract

Gliotoxin is a mycotoxin having a considerable number of immuno-suppressive actions and is produced by several moulds such as *Aspergillus fumigatus*. In this study, we investigated its toxic effects on human neutrophils at concentrations corresponding to those found in the blood of patients with invasive aspergillosis. Incubation of the cells for 10 min with 30–100 ng/ml of gliotoxin inhibited phagocytosis of either zymosan or serum-opsonized zymosan without affecting superoxide production or the exocytosis of specific and azurophil granules. Gliotoxin also induced a significant re-organization of the actin cytoskeleton which collapsed around the nucleus leading to cell shrinkage and the disappearance of filopodia. This gliotoxin-induced actin phenotype was reversed by the cAMP antagonist Rp-cAMP and mimicked by pCPT-cAMP indicating that it probably resulted from the deregulation of intracellular cAMP homeostasis as previously described for gliotoxin-induced apoptosis. By contrast, gliotoxin-induced inhibition of phagocytosis was not reversed by Rp-cAMP but by arachidonic acid, another member of a known signalling pathway affected by the toxin. This suggests that gliotoxin can affect circulating neutrophils and favour the dissemination of *A. fumigatus* by inhibiting phagocytosis and the consequent killing of conidia.

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1. Introduction

*Aspergillus fumigatus* (*A. fumigatus*) is a ubiquitous mould whose airborne conidia are continuously inhaled from the environment both indoors and outdoors and it is the most common cause of aspergillosis in animals and humans. Various veterinary pathologies have been described in poultry and cattle, such as mastitis or placentitis as well as different kinds of pulmonary infections, notably in poultry. In humans, the most serious form of the infection is invasive pulmonary aspergillosis (IPA) which occurs in immunocompromized patients as a result of conidia germination and mycelial development in
the lung parenchyma. *A. fumigatus* represents the most prevalent airborne fungal pathogen because of the increasing number of immuno-suppressive therapies and immuno-suppressive diseases with poor prognosis. It remains a main cause of death in this patient population with mortality ranging from 30% to 40%, partly due to a lack of diagnostic tools and the relatively poor efficacy of anti-fungal therapy.

*A. fumigatus* produces several mycotoxins whose role in aspergillosis remains unknown. Gliotoxin is among the most abundant. It belongs to the family of epipolythiodioxopiperazines that are characterized by a disulfide bridge across a piperazine ring which is essential for their toxicity [1]. Gliotoxin is suspected to be a significant actor in aspergillosis pathology for three main reasons. Firstly, it has multiple cellular activities that are mainly immuno-suppressive such as induction of leukocyte apoptosis, inhibition of cytotoxic T-cell response, phagocytosis and respiratory burst [2–11]. Secondly, it has been shown to be produced in situ during animal aspergillosis and also during human IPA, where its concentration in serum is in the order of 100 ng/ml [12,13]. Thirdly, it has been shown that gliotoxin could exacerbate experimental aspergillosis in mice [14].

Gliotoxin-induced apoptosis is the result of an increase in the cellular concentration of cAMP, leading to protein kinase A stimulation [7] and stabilization of IκB in the cytoplasm with the consequent inhibition of NFκB [6]. Gliotoxin inhibits O₂⁻ production in phagocytes by impairing the assembly of the NADPH-oxidase subunits in the plasma membrane [11].

Gliotoxin could positively exacerbate aspergillosis by inducing leukocyte apoptosis and inhibiting the respiratory burst during mycelial development, but also by inhibiting phagocytosis either at the initial stage of the infection during spore germination or at a later stage by facilitating conidia dissemination. However, the mechanism by which gliotoxin inhibits the phagocytic process remains unknown. In the present study, we have attempted to explore the effect of gliotoxin on the phagocytosis of serum-opsonized and non-opsonized yeast wall particles by human neutrophils. We report that low gliotoxin concentrations specifically affected phagocytosis and the organization of the actin cytoskeleton through distinct signalling pathways.

2. Materials and methods

2.1. Isolation of neutrophils and drug treatment

Neutrophils were isolated from blood collected from healthy donors after dextran T-500 sedimentation and centrifugation through a Ficoll separating solution as previously described [15]. They were incubated at 37 °C for 20 min in HEPES-buffered minimal essential medium at pH 7.4 (MEM/HEPES) and for the next 10 min in the presence of different concentrations of gliotoxin. In some experiments, neutrophils were pre-incubated for 30 min in the presence of 100 μM of the cAMP antagonist Rp-cAMP (adenosine 3',5'-cyclic monophosphothioate Rp-isomer triethylammonium salt, Sigma) or the cAMP agonist pCPT-cAMP (8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate sodium salt, Sigma) prior to gliotoxin addition. When arachidonic acid was used, it was added at 30 μM to the medium concomitantly with zymosan particles.

2.2. Apoptosis and necrosis

Neutrophils at 5 × 10⁶ cells/ml were incubated in MEM/HEPES for 5 h at 37 °C in the presence of 10, 30, 60, 100, 200, 300, 400 or 500 ng/ml of gliotoxin (Sigma, Saint-Quentin Fallavier, France). The cells were sedimented by centrifugation for 10 min at 200 × g at 4 °C in two aliquots. Cells from the first centrifugation were spread onto glass coverslips, fixed for 10 min at 4 °C in 3.7% formaldehyde and permeabilized for 5 min in PBS containing 0.25% Triton X-100. The cell nuclei were stained for 15 min at room temperature with 0.1 μg/ml final concentration of 4',6-diamidino-2-phenylindole (Sigma) in PBS plus 0.25% Triton X-100. Finally, the coverslips were washed in PBS and mounted in Mowiol (Calbiochem/VWR International, Fontenay sous Bois, France). Apoptotic cells showing condensed and fragmented nuclei were counted using fluorescence microscopy (at least 150 cells per condition). The second sample of sedimented cells was used to measure cell viability by a spectrophotometric measurement of lactate dehydrogenase activity in the extracellular and intracellular compartments [16].

2.3. Superoxide production

Zymosan (Sigma) was opsonized by incubation with human serum at 37 °C as described [16]. NADPH-oxidase of neutrophils (10⁶ cells/ml) was stimulated by the addition of 25 particles/cell of opsonized zymosan (OZ) and incubated for 1 h at 37 °C. The generation of superoxide by intact neutrophils was determined by the superoxide dismutase-inhibitable cytochrome c reduction assay as described previously [17] for discontinuous measurement, using a double-beam spectrophotometer Uvikon 930 (Kontron, Plaisir, France).

2.4. Measurement of granule exocytosis

Neutrophil (5 × 10⁶ cells/ml) degranulation was triggered by the addition of 25 particles of OZ/cell for 1 h. The reaction was stopped by placing the cells on ice and they were centrifuged at 200 × g for 10 min at 4 °C. The cells were lysed in 0.1 M Na₂HPO₄/KH₂PO₄, pH 7.2, 0.5 M NaCl, 3 mM KCl and 1% Triton X-100, and passed several times through a 25-gauge needle. Enzyme-linked immuno-absorbant detection of lactoferrin was performed on the extracellular and intracellular compartments as previously described [15]. The enzymatic activity of β-glucuronidase was measured spectrophotometrically in these compartments according to the method described in Ref. [17] using 4-nitrophenyl-β-d-glucopyranosiduronic acid (Merck/VWR international) as substrate.

2.5. Phagocytosis

Neutrophils (7 × 10⁵/ml) adhering to glass coverslips, as previously described [18], were exposed to 30 or 100 ng/ml
gliotoxin for 10 min. Z or OZ was then added at 25 particles/cell and incubated at 37°C for 30 or 10 min, respectively. At these time points, the phagocytic process was maximal and the ingested particles were present in phagolysosomes as attested by the presence of Hck at the phagosomal membrane [18]. Cells were then fixed and permeabilized in methanol at −20°C, washed in PBS with 0.1% Tween-20 and exposed to a rabbit polyclonal antibody anti-Hck (Santa Cruz Biotechnologies, Santa Cruz, CA) and FITC-conjugated secondary antibody (Sigma). Hck-positive phagosomes were examined by fluorescence microscopy and quantification was performed by counting the percentage of neutrophils that had ingested at least one particle. For each condition, at least 150 cells were counted. Phagocytosis was also observed using F-actin staining of neutrophils incubated for 1 or 5 min in the presence of OZ, rapidly fixed in acetone and permeabilized at −20°C for 5 min. Cells were then washed in PBS 0.1% Tween-20 and incubated for 20 min at room temperature in the presence of 0.8 μM of rhodamine-coupled phallolidin, as previously described [19]. This permitted to study actin re-organization during phagocytosis.

2.6. Detection of F-actin

Neutrophils (7 × 10^5/ml) adhering to glass coverslips were incubated at 37°C for 20 min in MEM/HEPES and then exposed to the drugs and gliotoxin. In some experiments, neutrophils were incubated for 1 or 5 min in the presence of OZ, washed in PBS, fixed and permeabilized in acetone at −20°C for 5 min. Cells were then washed in PBS 0.1% Tween-20 and incubated for 20 min at room temperature in the presence of 0.8 μM of rhodamine-coupled phallolidin.

2.7. Quantification of actin polymerisation in whole cells

Neutrophils incubated at 37°C in MEM-HEPES at 2 × 10^7 cells/ml were stimulated for 30 s by 1 μM n-formyl-Met-Leu-Phе (n-FMLP). Cells were fixed by 1% glutaraldehyde for 20 min at 37°C and permeabilized in the presence of 1% nonidet P40 for 20 min at 37°C. F-actin was stained with rhodamine-phallolidin (210 nM final concentration) and the cytoskeletal proteins were sedimented by centrifugation at 280 × g for 9 min. The detergent insoluble pellet was dissolved in methanol for 1 h and the fluorescence was read in 1 ml quartz cuvettes in a fluorescence spectrophotometer (ex 540, slit 3 nm, em 564 nm, slit 3 nm) as previously described [20].

3. Results

3.1. Gliotoxin inhibits both opsonized and non-opsonized phagocytosis

The effect of gliotoxin on phagocytosis by neutrophils was studied using zymosan (Z) or serum-opsonized zymosan (OZ) which involves distinct signalling pathways. Z is recognized by the lectin site of CR3, while OZ contained on its surface iC3b that is recognized by the complement site of CR3 and IgG that is recognized by Fc receptors. Receptors for opsonins very actively induced particle ingestion as attested by the presence of OZ-containing phagosomes in approximately 90% of the cells after 10 min. To obtain a similar percentage of phagocytic cells in the Z experiments, co-incubation of Z with neutrophils was carried out for 30 min before cell fixation. Gliotoxin has been shown to exert several of its effects within the first few minutes after its addition to neutrophil suspensions (i.e. inhibition of cell adhesion and NADPH-oxidase activity) [2,10]. Therefore neutrophils were pre-incubated for 10 min in the presence of gliotoxin before the addition of Z or OZ. As shown in Fig. 1, gliotoxin inhibited the phagocytosis of both Z and OZ in a dose-dependent manner. At 30 ng/ml gliotoxin, the difference between the inhibition of Z and OZ phagocytosis (88.3 ± 2.5% versus 51.1 ± 11%, respectively) might be due to the different incubation times used to carry out these experiments (40 min for Z and 20 min for OZ). In fact, when gliotoxin was pre-incubated at 30 ng/ml for 30 min and OZ co-incubated with neutrophils for the next 10 min, the inhibition was greater than 95% (n = 2). As a control, two experiments were performed using 100 ng/ml of dimethylthio-gliotoxin, the inactive reduced form of gliotoxin. No inhibition of the phagocytic activity was observed confirming the necessity of the disulfide ring for the action of gliotoxin.

The effect of gliotoxin on phagocytosis was probably not the result of neutrophil apoptosis since after 2 h of incubation with gliotoxin up to 10 μg/ml, no apoptosis was detected [9]. To verify that the gliotoxin batches used in our experiments were able to affect previously well-characterized cell responses, we examined whether they triggered apoptosis in human neutrophils as described [9]. Neutrophils were incubated for 5 h with different gliotoxin concentrations. Apoptosis was...
apparent in 30% of the cells when incubated with 100 ng/ml gliotoxin and in 40% at 500 ng/ml gliotoxin whereas apoptosis in control cells was under 4%. In these experiments, we did not detect any variation in the release of LDH in the absence or presence of gliotoxin up to 500 ng/ml, indicating that the toxin was not cytotoxic.

3.2. Gliotoxin had no effect on neutrophil degranulation and NADPH-oxidase activity

The inhibitory effect of gliotoxin on NADPH-oxidase is one of the anti-microbial activities observed at micromolar concentrations [11]. At the concentrations used above (from 10 to 100 ng/ml), gliotoxin did not inhibit the production of superoxides triggered by OZ and did not significantly affect the exocytosis of specific and azurophil granules as shown by the release of $\beta$-glucuronidase and lactoferrin, respectively (Fig. 2). Thus gliotoxin strongly inhibited phagocytosis without affecting the other anti-microbial responses of neutrophils indicating that neutrophils were fully activated by OZ and that gliotoxin, at the concentrations tested, specifically inhibited phagocytosis.

3.3. Gliotoxin affects the organization of the actin cytoskeleton

Gliotoxin has been reported to induce cell detachment and to perturb the microfilament structure of cultured liver cells [21]. Microfilaments were stained with rhodamine-coupled phalloidin and the neutrophils were then examined by direct fluorescence microscopy. Control neutrophils showed a characteristic spread shape with filipodia at the cell periphery (Fig. 3A). In the presence of 100 ng/ml gliotoxin, a pronounced change in the neutrophil morphology was observed for more than 50% of the cells (Fig. 3A and B). Considerable cell shrinkage was observed with collapsed F-actin around the nucleus showing as large patches.

The effect of gliotoxin on the F-actin content of cells was measured in resting or activated neutrophils. The F-actin content was identical in control or gliotoxin (30 or 100 ng/ml)-treated neutrophils. When neutrophils were stimulated by 1 $\mu$M n-FMLP, the F-actin content was increased by 1.56 $\pm$ 0.13-fold ($n = 2$ duplicates) in the absence or presence of 100 ng/ml gliotoxin, respectively, as compared to unstimulated cells. These data indicate that gliotoxin-induced F-actin re-organization without modifying actin polymerisation.

3.4. The anti-phagocytic property of gliotoxin is AA-dependent and cAMP-independent

The ability of neutrophils to polymerize actin in the presence of gliotoxin was confirmed by focusing the examination on the only cells able to ingest OZ (Fig. 4A). Like in control cells, we observed that F-actin-positive phagocytic cups (1 min) and phagosomes (1 and 5 min) are formed in the presence of gliotoxin. Interestingly at 5 min, these gliotoxin-treated cells have ingested a large number of particles like control cells (G and C panels 5 min). After 1 min of contact with OZ, visualization of OZ under UV (panels OZ) and F-actin (panels Act), for the same cells (either control (C) or gliotoxin-treated (G)), showed F-actin-rich areas situated around bounded OZ particles attesting to local actin polymerisation during OZ ingestion. So, in the presence of gliotoxin, the process of actin polymerisation occurring...
During phagocytosis takes place in the few cells which internalize OZ.

Among its various cellular effects, gliotoxin has been shown to increase the intracellular cAMP concentration [7] and the production of arachidonic acid [22]. Therefore we examined whether the action of gliotoxin on phagocytosis involves these second messengers. The inhibitory effect of 100 ng/ml gliotoxin on phagocytosis was not modified in the presence of 100 μM Rp-cAMP which inhibits cAMP signalization (Fig. 4B). On the contrary, in the presence of 30 μM AA added concomitantly to OZ, phagocytosis was restored in gliotoxin-treated neutrophils (Fig. 4C). These results suggest that inhibition of AA production by gliotoxin could be responsible for phagocytosis inhibition.

3.5. The gliotoxin-induced F-actin re-organization is cAMP-dependent and arachidonic acid-independent

Next, we examined the effect of cAMP and AA on the F-actin re-organization triggered by gliotoxin. In contrast to its action on phagocytosis, the treatment of neutrophils with 100 μM pCPT-cAMP, a cAMP agonist, induced a phenotype similar to that observed with gliotoxin with the cells showing a shrunken morphology associated with a reduction of

![Image]

Fig. 3. The effect of gliotoxin on the cell morphology is reversed by the cAMP antagonist Rp-cAMP but not by AA. Neutrophils were treated or not (Ctrl) for 30 min with 100 μM pCPT-cAMP (pCPT), or 100 μM Rp-cAMP (Rp); then, gliotoxin (100 ng/ml) was added or not for 10 min. AA (30 μM) was added 10 min after gliotoxin and further incubated for 10 min. Cells were fixed, permeabilized and incubated with rhodamine-coupled phalloidin to stain F-actin. (A) Cells were examined by fluorescence microscopy and a representative experiment out of four (for pCPT-cAMP and Rp-cAMP) or out of three (for AA) is shown. (B) Quantification of these experiments is shown (Ctrl); Gliotoxin (G), pCTP-cAMP (pCPT), Rp-cAMP (Rp), Rp-cAMP + gliotoxin (Rp + G), AA (AA) or AA + gliotoxin (AA + G). Results are expressed as mean ± SD of three or four experiments performed in duplicates (Bar = 10 μM). Statistical differences were calculated for treated cells versus control cells using a Student’s t-test, **P < 0.01.
filopodia and the condensation of actin microfilaments around the nucleus (Fig. 3A). This phenotype was observed for more than 50% of gliotoxin-treated cells and more than 30% of pCPT-cAMP, respectively, as quantified in Fig. 3B. On the contrary, neutrophils incubated for 30 min in the presence of 100 μM Rp-cAMP retained a characteristic spread shape with the numerous filopodia, similar to the control neutrophils (Fig. 3A and B). Moreover, in the presence of Rp-cAMP, the...
effect of gliotoxin on the cell morphology and the actin cytoskeleton was markedly diminished (Fig. 3A and B). When neutrophils were incubated with AA, no significant actin condensation and change in cell shape were observed and the shrunken cell morphology induced by gliotoxin was not reversed (Fig. 3A and B). Thus, cAMP but not AA mimicked the effect of gliotoxin on F-actin re-organization.

4. Discussion

Gliotoxin shows a number of broad toxic and inhibitory effects directed towards immune cells. Several targets of the toxin-action have been identified such as the induction of apoptosis or the inhibition of NADPH-oxidase. Here we report that gliotoxin inhibited phagocytosis by human neutrophils at concentrations found in the serum of IPA patients and that this inhibitory effect was reversed by AA. A strong re-arrangement of the actin cytoskeleton was also observed and reversed by cAMP antagonists. Thus gliotoxin has a large panel of effects that contribute to the depression of immune responses and thereby favour the survival of A. fumigatus.

Gliotoxin inhibited the phagocytosis of Z and OZ in a dose-dependent manner. The effect was rapid, being observed after only 10 min incubation in the presence of the toxin, and was effective at very low concentrations (below 100 ng/ml). Since Z and OZ are taken up via distinct receptors and distinct molecular mechanisms [23], it suggests that gliotoxin blocks a common step involved in these two types of phagocytic processes.

By contrast, gliotoxin up to 100 ng/ml did not inhibit the respiratory burst in OZ-stimulated neutrophils but rather slightly increased it. In fact, the inhibition of NADPH-oxidase by gliotoxin that has been reported was at higher concentrations, that is above 1 μg/ml [11]. Our study also showed that at 100 ng/ml, gliotoxin had a minor effect on the exocytosis of granules by neutrophils. The absence of a major action of gliotoxin up to 100 ng/ml on these two anti-microbial activities showed that neutrophils remained able to be fully activated by OZ even if phagocytosis was strongly inhibited by the toxin. Moreover, at this concentration and incubation time, no apoptosis was detected in gliotoxin-treated cells. Thus phagocytosis appears to be the most sensitive process affected by gliotoxin after a short incubation period at low concentrations. This is very interesting in the light of the recent data obtained in vivo showing that in mice or human IPA [13], the level of circulating gliotoxin is in the order of 100 ng/ml.

Therefore during the dissemination of conidia, the phagocytic activity of phagocytes is probably altered. In studies with animal models, several micrograms of the toxin have been measured in A. fumigatus-infected tissues [12]. Thus in infected tissues, the toxin concentration is sufficient to abolish bactericidal activities of neutrophils (phagocytosis, O$_2^-$ production) and to induce apoptosis. Gliotoxin is probably playing a key role in aspergillosis pathology both in blood and in tissues.

To study further the role of gliotoxin on the phagocytic process, we examined whether the actin cytoskeleton could be affected. The phagocytosis of both opsonized and non-opsonized substrates requires re-organization of F-actin even though distinct signalling pathways are involved [23]. In the presence of gliotoxin, neutrophils shrank, most of the F-actin collapsed around the nucleus and filopodia disappeared. This re-organization of F-actin distribution occurred without affecting the polymerisation process as the F-actin content was identical in gliotoxin-treated and control cells.

cAMP has been shown to modulate adhesion-associated events such as the dynamics of the actin cytoskeletal, cell migration and the formation of filopodia or microspikes [24]. In addition, the pro-apoptotic action of gliotoxin is mediated by cAMP in a protein kinase A-dependent manner [7]. Since the shrunken cell phenotype induced by gliotoxin was reversed by Rp-cAMP, a cAMP antagonist and treatment of neutrophils with the cAMP analog pCPT-AMP mimicked the gliotoxin-induced F-actin collapse, it is likely that gliotoxin modifies the cAMP steady-state which induces a rapid effect on F-actin and, later on, triggers apoptosis. So, the previously reported inhibitory effect of gliotoxin on cell adhesion and migration could be due to its action on F-actin organization [3,8]. By contrast, the inhibitory effect of gliotoxin on phagocytosis which is also an actin-dependent process was not reversed by modulating cAMP levels. In addition, AA is able to restore phagocytosis without restoring a spread phenotype. So, we propose that the gliotoxin effect on phagocytosis involves an inhibition of the release of AA in an F-actin independent manner. Actually, gliotoxin has been described to inhibit the release of AA mediated by PLA$_2$ [22]. PLA$_2$ activation is a necessary early event in the phagocytosis of IgG opsonized particles [25–28]. Two types of PLA$_2$ have been proposed to regulate phagocytosis: the calcium independent iPLA$_2$ and secreted sPLA$_2$ as inhibitors of these PLA$_2$s inhibit phagocytosis in an AA restorable manner [26–28]. In addition, a recent study performed in mouse peritoneal macrophages has shown that sPLA$_2$ V translocates to phagosomes and positively regulates phagocytosis of zymosan [29]. Inhibition of iPLA$_2$ leads to an accumulation of small vesicles underneath the phagocytic cup suggesting that PLA$_2$(S) are controlling the fusion of vesicles with the plasma membrane to provide membrane for the forming phagosomes [28]. Interestingly, in our system, addition of exogenous AA partially restored the OZ phagocytosis that was inhibited by gliotoxin. This suggests that part of the signalling pathway involved in the anti-phagocytic action of gliotoxin leads to a decrease in AA release. A supply of membrane to nascent phagosomes is required for phagocytosis irrespective of the receptors involved. If PLA$_2$ is a (direct or indirect) target of gliotoxin it could explain therefore why both the Z and OZ phagocytic pathways were inhibited.

F-actin re-organization observed in gliotoxin-treated cells appeared from our results to be clearly dissociated from its anti-phagocytic properties. We observed that when 90% of the cells are unable to internalize OZ or Z in the presence of 100 ng/ml of gliotoxin, only 55% of them exhibit a shrunken phenotype with F-actin collapse. The action of gliotoxin on
the actin cytoskeleton was shown to be cAMP-dependent but AA-independent. Conversely, the gliotoxin-induced inhibition of phagocytosis was reversed by AA but not Rp-cAMP. Our results are in accordance with previous results showing that the cAMP-dependent effect of gliotoxin on macrophage apoptosis was unrelated to its anti-phagocytic properties [4].

Neutrophils are essential for the eradication of pathogens in the acute stage of most fungal infections [30]. Gliotoxin is more deleterious for neutrophils than for the other immune cells [8] and we report here that it is very efficient at inhibiting phagocytosis and F-actin organization. Gliotoxin therefore plays a key role in the pathogenicity of Aspergillus fumigatus by enabling the fungus to avoid ingestion and killing by neutrophils.

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