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ORIGINAL ARTICLE

Cytotoxicity and genotoxicity of gliotoxin on human lymphocytes *in vitro*

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Abstract The cytotoxic effects on human lymphocytes of two gliotoxin samples (one pure sample produced in the laboratory for this study, and one sample purchased from a standard source) were assessed at four different concentrations (25, 50, 100 and 200 ng/ml) using the methylthiazol tetrazolium (MTT) bioassay. The results showed that growth was inhibited by 21, 39.10, 61.99 and 87.45% for each of the four concentrations of the pure sample, respectively, and by 17.89, 34.92, 58.34 and 85.22% respectively, in the case of the standard purchased sample. Deoxyribonucleic acid (DNA) was extracted from the lymphocytes and analysed by electrophoresis on a 1% agarose gel. Gliotoxin appeared to have the ability to degrade or damage the DNA. The present study showed that both the growth inhibition and DNA damage experienced by the human lymphocytes increased linearly with increasing concentrations of toxin.

1. Introduction

Mycotoxins are secondary fungal metabolites which pose a major risk to the health of humans, animals and crops (Wild and Gong, 2010). Amongst the mycotoxins, gliotoxin is one of the most abundant metabolites produced by *Aspergillus fumigatus* and some other types of fungi. Gliotoxin possesses a wide spectrum of biological activities, which include antibacterial and antiviral activities, inhibiting platelet function and is also a potent immunomodulating agent (Waring and Beaver, 1996) (Fig. 1).

In vitro, gliotoxin has been shown to be able to inhibit the activation of antigen mediated lymphocyte stimulation, cytotoxic-T-cell activation, and gamma interferon production by CD4 lymphocytes (Wichmann et al., 2002). It has also been shown to inhibit mitogenic stimulation of human lymphocytes (Richard et al., 1994). At higher concentrations, gliotoxin induces apoptosis in macrophages and lymphocytes by a mechanism which is distinct from its antiphagocytic effects. This toxicity against the immune system induces the apoptotic cell

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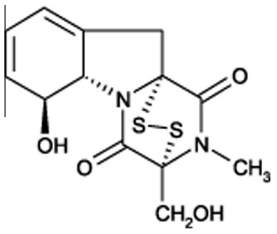

Figure 1 Chemical structure of gliotoxin.

death of thymocytes, peripheral lymphocytes, macrophages, spleen cells and other characteristic changes caused by DNA fragmentation and adduct formation (Golden et al., 1998; Waring and Beaver, 1996). The toxicity of gliotoxin is thought to be mediated in at least two ways: firstly, through conjugation to proteins with susceptible thiol residues, and their subsequent inactivation; secondly, through generation of reactive oxygen species *via* redox cycling (Kwon-Chung and Sugui, 2009).

An important property of gliotoxin, which has been specifically identified in human lymphocyte cells following exposure to gliotoxin, is its ability to go through a redox cycle in the presence of an appropriate reducing agent (Waring and Beaver, 1996). Hydrogen peroxide, as well as other reactive oxygen species (ROS) which are produced by gliotoxin during the redox cycle in a cell-free system is known to damage plasmid directly and cellular DNA (Schnabl et al., 2002).

The objective of the present study is to assess the cytotoxic and genotoxic effects of gliotoxin purified from local *A. fumigatus* isolate and standard gliotoxin (purchased from Sigma–Aldrich for laboratory use) on human lymphocytes *in vitro* using an MTT assay.

2. Materials and methods

2.1. Extraction and purification of gliotoxin

Gliotoxin was produced by *A. fumigatus* under the following conditions: *A. fumigatus* was inoculated on a long grain rice medium for 10 days at 37 °C. The gliotoxin was extracted twice with an electric homogenizer using 50 ml chloroform and filtered through a Whatman No. 1 filter paper. The collected filtrate after extraction with chloroform was pooled and dried by evaporation and stored at 4 °C (Kosalec et al., 2005). The entire dried residue was combined, and dissolved in 2 ml methylene chloride before being filtered through a 0.45 μm Millipore filter. The filtrate was then placed on a silica gel Sep-Pak column and primed with 5 ml of methylene chloride. The Sep-Pak was eluted with 2 ml of hexane, ethyl acetate, chloroform, and methanol and was then evaporated to dryness and dissolved in 1 ml methylene chloride (Richard et al., 1989). After purification, the concentration was determined by Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) and compared with standard gliotoxin, which had been purchased from Sigma–Aldrich PubChem Substance ID 24895384 (molecular weight of 326.39 and produced from *Gliocladium fimbriatum*).

The TLC plates were used for detection of the gliotoxin with a methylene chloride: methanol (97:3 v/v) solvent system as the mobile phase. The plates were air-dried and gliotoxin was visualized with a spray reagent of 5% silver nitrate in ethanol: water (90:5 v/v).

2.2. Preparation of toxin concentration

Both standard gliotoxin and gliotoxin extract were dissolved in methanol at 1 mg/ml and used to prepare concentrations of 25, 50, 100 and 200 ng/ml in complete culture media (RPMI-1640 medium supplemented with 10% fetal calf serum, containing a solution of penicillin 100 units/ml and streptomycin 100 μg/ml) (Freshney, 2012).

2.3. Collection and processing of human lymphocytes

Peripheral venous blood was taken from a healthy 26 year old male donor and lymphocytes were extracted following Rafael and Vaclav (2000). The suspension of cultured human lymphocytes was adjusted until the number of cells was about 1×10^4 cells/ml. 100 μl of the cell suspension was then dispensed into each of the 96 wells of a microtiter plate to give a final cell count of 1000 cells/well. The plates were then incubated at 37 °C in an incubator supplemented with 5% CO_2 for 24 h (Wichmann et al., 2002). After incubation, gliotoxin was added to each well at different concentrations and incubated for 24 h (Rafael and Vaclav, 2000).

2.4. Cytotoxicity assay using the 3-[4,5-dimethylthiazoyl]-2, 5-diphenyltetrazolium bromide (MTT) test

This test was performed by dissolving 3-[4,5-dimethylthiazoyl]-2, 5-diphenyltetrazolium bromide in phosphate buffered saline (PBS) at 2 mg/ml, filtrated by a 0.22 μm millipore filter. 50 μl of the MTT dye was added to each of the microtiter plate wells containing human lymphocytes treated with different concentrations of gliotoxin for 24 h. The MTT-formazan crystals, which are formed only by live cells, were dissolved in 100 μl Dimethyl sulphoxide (DMSO), enabling the optical density of each well to be measured using an ELISA reader at a transmitting wavelength of 620 nm (Freshney, 1994). The inhibitory rate was measured according to Wang et al. (2003) as follows:

$$\text{Growth Inhibition } \% = \frac{\text{O.D. of control} - \text{O.D. of Sample}}{\text{O.D. of control}} \times 100$$

2.5. Genotoxicity assay

After the incubation period, all the treated human lymphocyte content of the wells was transferred from the well of the microtiter plate by micropipette before staining to a sterile Eppendorf tube for use in the genotoxicity assay. DNA was extracted from the cultured cells using a genomic DNA mini kit (Geneaid Company). The samples were analysed by electrophoresis on a 1% agarose gel, following Sambrook et al. (1989). Visual observations of DNA were recorded with UV camera system (Abraham et al., 2008). Statistical analysis was performed on all the concentrations and the control with mean ± standard error and differences between means being performed by One Way Analysis of Variance (ANOVA) according to SAS (2004).