A study of zearalenone cytotoxicity on human peripheral blood mononuclear cells

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Abstract
The mycotoxin zearalenone (ZEA) is a common contaminant of all major cereal grains worldwide with estrogenic and anabolic activity. We investigated the in vitro cytopathic effects of ZEA on freshly isolated human peripheral blood mononuclear cells (PBMC) in relation to proliferation and cell death patterns of untreated and mitogen-activated cells. The higher concentration of 30 μg/ml ZEA was found to totally inhibit T and B lymphocyte proliferation from the stimulation with phytohemagglutinin and pokeweed mitogen. The inhibitory effects of ZEA were further related to cell necrosis/apoptosis. Flow cytometry analysis showed a distinct necrotic effect on PBMC, irrespective of mitogen stimulation, whereas apoptotic activity was less evident. Necrosis was observed in both the lymphocyte and monocyte/granulocyte gates. Measurements of ZEA-induced intracellular calcium ion (Ca²⁺) mobilization showed an increase of both Ca²⁺ levels and the number of cells with high Ca²⁺ only in the monocyte/granulocyte gated cells. Using phenylmethyl sulfonyl fluoride (PMSF), a serine protease inhibitor, and ammonium chloride (NH₄Cl), a lysosomal inhibitor, both associated with cell necrosis inhibition, we showed that PMSF at 0.05 mM and NH₄Cl at 1 and 10 mM reduced the cytopathic effects induced by 30 μg/ml ZEA, whereas apoptosis was less affected. Exposure of PBMC to 1 μg/ml ZEA did not alter the viability of the cells. Our results suggest that high ZEA concentrations in the blood may well exert cytotoxic effects that merit further investigation.

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1. Introduction
Zearalenone (ZEA) is a non-steroidal estrogenic mycotoxin produced by several Fusarium species, such as Fusarium graminearum, Fusarium culmorum, Fusarium equiseti, and Fusarium crookwellense (Bennett and Klich, 2003). All these species are regular contaminants of cereal crops worldwide (Kuiper-Goodman et al., 1987) destined for human and animal consumption. ZEA concentrations of up to 289 μg/g in human foods have been reported (Kim et al., 1993; Yawai et al., 1994).

The major mycotoxicity of ZEA and its metabolites is attributed to their estrogenic and anabolic activi-
ities (Etienne and Jemmali, 1982) that modulate or disrupt endocrine function in animals and possibly in humans (Ryu et al., 2002). ZEA has been implicated in numerous incidences of mycotoxicosis in farm animals (Kuiper-Goodman et al., 1997; Hussein and Brasel, 2001) especially in pigs (Coulombe, 1993). Human estrogen receptors bind ZEA, although their influence on target tissues seems to be weaker (80–160 less active) as compared to diethylstilbestrol or estradiol (Forsell and Peskka, 1985; Ueno and Tashiro, 1981; Tomaszewski et al., 1998). Although ZEA elicits permanent reproductive tract alterations (Ito and Ohtsubo, 1994), evidence for teratogenic or carcinogenic effects were limited in laboratory animals (Maaroufi et al., 1996; Creppy, 2002).

Several changes of histological, haematological and immunological parameters were induced by high ZEA concentrations (Abdelhamid et al., 1992; Berek et al., 2001; Maaroufi et al., 1996; Forsell et al., 1986; Cooray, 1984). A dose-dependent reduction of phytohemagglutinin (PHA)-induced lymphocyte proliferation was observed using rat or human peripheral blood lymphocytes (Atkinson and Miller, 1984). Inhibitory effects of ZEA were observed in blastogenesis assays with leukoagglutinin, concanavalin A and pokeweed mitogen, indicating that ZEA was capable in inhibiting all B and T cell subsets (Forsell and Peskka, 1985).

The cytotoxic and genotoxic effects of ZEA is not fully understood. It was recently shown that ZEA increased DNA fragmentation in a concentration-dependent manner in three cell lines, Vero, Caco-2 and DOK, after 24 h exposure at concentrations of 10, 20, and 40 μM (Abid-Essefi et al., 2003, 2004). ZEA induced also male germ cell apoptosis in rats in a time-dependent and stage-specific pattern, contributing to germ cell depletion and testicular atrophy (Kim et al., 2003). On the other hand, ZEA is reported to induce sister chromatid exchanges (SCEs), chromosome aberrations (CAs) (Ouanes et al., 2003), and only a slight induction of sister chromatid exchanges (SCEs), chromosome aberrations (CAs) (Ouanes et al., 2003), and only a slight reduction in cell viability to ZEA-treated bovine lymphocytes at a concentration of 4 μM after 24 h exposure, which did not seem to implicate an apoptotic mechanism of action, whereas at higher concentrations and at a longer exposures, necrotic cells were observed (Las et al., 2004).

The inconclusive results on the toxicity of ZEA prompted us to investigate the cytotoxic effects of ZEA on human peripheral blood mononuclear cells (PBMC). We report in this study the effects of ZEA on the proliferation and cell death patterns of untreated and mitogen-activated PBMC.

2. Materials and methods

2.1. Cell isolation

Buffy coats were collected from healthy, HIV-1/hepatitis b sera-negative blood donors obtained from Venizelio Hospital Blood Transfusion Service, Heraklion, Crete. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation with the use of ficoll-paque (Amersham—Pharmacia, Upsala, Sweden) and resuspended in RPMI-1640 medium supplemented with antibiotics and 5% human serum (Zadneprovskaya et al., 1997).

2.2. Proliferation assay to ZEA exposure

PBMC at a concentration of 1 × 10^6 cells/ml were cultured in 96-well plates (Costar, US) in RPMI-1640, at 37°C in 5% CO2-buffered and humidified incubator. For proliferation assays, ZEA (Sigma—Aldrich Chemical, Germany) was added at concentrations of 0.1, 1, 10, 50 μg/ml in cell cultures in triplicate wells. For mitogen stimulation, either 5 μg/ml phytohemagglutinin (PHA; Sigma, Germany) (Vlata et al., 2005) or 5 μg/ml pokeweed mitogen (PWM, Sigma, Germany) selected after an initial titration using 1, 5 and 10 μg/ml PWM, were added at the same time as ZEA. At appropriate time period of 3 days of culture, cells were harvested after a pulse period of 18 h with [3H]thymidine (Amersham, UK). Cell proliferation was evaluated as counts per minute of each sample measured in an LS1701 beta counter (Beckman, USA) (Barzitaki et al., 2002a,b).

2.3. Measurement of apoptotic–necrotic cells by flow cytometry

The effect of ZEA on cell viability was determined by the trypan blue (0.4% solution in PBS, Sigma Chemical Co.) exclusion technique in a kinetic study of 0, 1, 3, and 7 days. PBMC at 1 × 10^6 cells/ml with and without 5 μg/ml PHA or PWM mitogens co cultured with ZEA at selected concentrations of 1 and 30 μg/ml. In order to evaluate cell apoptosis and necrosis (Barzitaki et al., 2002a,b), a parallel kinetic study was performed using flow cytometry: briefly, at the appropriate time period, 1 × 10^6 cells were collected in tubes and washed with 10 mM phosphate buffered saline (PBS), pH 7.4. The cell pellet was resuspended in 100 μl annexin V-fluos labelling solution (10 mM Hepes/NaOH, 140 mM NaCl, and 5 mM CaCl2, in pH 7.4), containing a certain amount of 2 μl annexin V-fluos (Boehringer Mannheim Biochemica, Germany) and 2 μl of 50 μg/ml propidium iodide solution (ICN Biomedicals, US). Cells were incubated at room temperature for 15 min. Finally, incubation buffer (400 μl) was added to each tube and apoptosis–necrosis was analysed immediately by FACS (Calibur, Becton–Dickinson, US) using the CELLQuest programme.
2.4. Measurement of intracellular calcium ion (Ca^{2+}) mobilization

To investigate the influence of ZEA on the Ca^{2+} signalling, we examined the intracellular changes of Ca^{2+} levels loading the cells with the Ca^{2+} Indicator Fluo-3 (Princen et al., 2003). Using the flow cytometric method we measured the Ca^{2+} mobilization in PBMC treated with 1 and 30 \mu g/ml ZEA at 1, 3, and 7 days of exposure: briefly, 1 \times 10^6 cells were centrifuged and resuspended in RPMI-1640 medium supplemented with 2% FBS, and 4\muM Fluo-3/acetoxymethylester (AM) prepared from 2\muM stock solution of Fluo-3/AM (Molecular Probes, Leiden, The Netherlands) in anhydrous dimethyl sulfoxide (DMSO). After incubation for 45 min at room temperature, cells were washed two times at room temperature in Ca^{2+} flux assay buffer (Hank’s balanced salt solution containing 20\muM HEPES and 0.2% bovine serum albumin, pH 7.4) to remove extra cellular dye. After the second wash, cells were incubated for 10 min at room temperature, and resuspended in 500\mu l PBS. Green fluorescence emission of Fluo-3 was detected at 530 nm by FACS (Callibur, Becton–Dickinson, US) using the CELLQuest programme.

2.5. PMSF and NH_4Cl inhibition of ZEA-induced cell killing

The ability of phenylmethyl sulfonyl fluoride (PMSF) to protect ZEA mediated killing was examined. PBMC were cultured for 24h with or without 30 \mu g/ml ZEA in the presence or absence of 0.05\muM PMSF prepared from 100\muM stock PMSF (Sigma) dissolved in methanol. The cells were washed to remove the inhibitor and ZEA and resuspended in RPMI culture medium for further 11 days incubation. At the appropriate time period of 1, 3, 5, 7, 9 and 11 days, flow cytometric analysis for early apoptotic events with annexin V and cell necrosis with propidium iodide exclusion was performed as described previously.

The capacity of ammonium chloride (NH_4Cl) to inhibit necrosis was also examined. Untreated and treated PBMC with 30\mug/ml ZEA in the presence and absence of 1 mM or 10 mM NH_4Cl were cultured. After 4 days of ZEA exposure, the cell necrosis–apoptosis was determined by annexin V–propidium iodide flow cytometry.

3. Results

3.1. Proliferation of human lymphocytes exposed to ZEA

The effects of ZEA on PBMC proliferation rates were investigated using a concentration range of 0.1, 1, 5, 10 and 30 \mu g/ml of ZEA, after stimulation with 5\mug/ml PHA or PWM. Thymidine incorporation after 3 days of ZEA exposure showed a dose-dependent inhibitory effect of ZEA on both PHA (Fig. 1A) and PWM (Fig. 1B)-induced proliferation. The IC50 (concentration of ZEA for 50% inhibition of mitogen stimulation) for PHA was 9.5 \mu g/ml and for PWM 5.0 \mu g/ml. The experiments were carried out on PBMC from five different donors.

3.2. Measurement of cell necrosis–apoptosis

To determine whether the observed decline on mitogen-induced proliferation was associated with cell death, we investigated cell viability in an extended time course (0, 1, 3, and 7 days). Fig. 2 summarizes the percentage of viable cells on unstimulated and stimulated PBMC either with 5 \mu g/ml PHA or PWM, and exposed to a low (1 \mu g/ml) and high (30 \mu g/ml) concentration of ZEA. The above results showed a distinct decrease on cell viability with 30 \mu g/ml ZEA, as measured by both trypan blue exclusion (Fig. 2A) and flow cytometry (cells double negative to propidium iodide and annexin V staining) (Fig. 2C). Exposure to 1 \mu g/ml ZEA did not affect cell viability as shown in Fig. 2B (trypan blue exclusion) and Fig. 2D (flow cytometry), respectively.

We further investigated the nature of the ZEA-induced cell death (Fig. 3). Exposure of unstimulated
Fig. 2. Cell viability in ZEA-treated PBMC unstimulated and stimulated with 5 μg/ml PHA or PWM mitogens. Viable cells were estimated by negative trypan blue staining after exposure to (A) 30 μg/ml ZEA and (B) 1 μg/ml ZEA. Viable cells were estimated by flow cytometry (annexin V/propidium iodide negative cells) after exposure to (C) 30 μg/ml ZEA and (D) 1 μg/ml ZEA. Values represent the mean (± S.D.) activity from two different donors.

Fig. 3. Flow cytometry analysis of ZEA-induced necrosis and apoptosis on unstimulated PBMC: (A) percentage of necrotic cells, positive staining with propidium iodide; (B) percentage of apoptotic cells, positive staining with annexin V. Values represent the mean (± S.D.) activity from two different donors.

PBMC to 30 μg/ml ZEA induced increased necrosis as compared to control cells (Fig. 3A). High ZEA levels (30 μg/ml) also induced apoptosis (Fig. 3B). The corresponding apoptotic profile however, was less distinct. Exposure to low levels of ZEA (1 μg/ml) gave similar results to controls.

In addition, we studied the necrotic and apoptotic patterns induced by 30 μg/ml ZEA after PHA and PWM stimulation (Fig. 4). The necrotic (Fig. 4A) and apoptotic (Fig. 4B) profiles were not altered by either PHA or PWM stimulation. The increasing percentages of necrotic and not the apoptotic PBMC indicated that ZEA induces the same necrotic effect in both resting and stimulated lymphocytes.

3.3. ZEA-induced intracellular Ca²⁺ levels

We studied the intracellular Ca²⁺ mobilization in ZEA-exposed unstimulated PBMC. Using conventional forward and side scatter gating in flow cytometry, we investigated the lymphocytic and monocyte/granulocyte subpopulations. The results showed an increase in Ca²⁺ levels in the monocyte/granulocyte gate that consisted of 40–70% CD14⁺ cells (data not shown), on day 1 of 30 μg/ml ZEA exposure (Fig. 5A), followed by a reduction in the number of cells with high Ca²⁺ on days 3 and 7. High Ca²⁺ levels were observed in the corresponding gate of the control PBMC on day 3. The lymphocyte pop-
Fig. 4. Comparative effect of 30 μg/ml ZEA on unstimulated and stimulated with 5 μg/ml PHA or PWM peripheral blood mononuclear cells (PBMC) measured by flow cytometry: (A) percentage of necrotic cells, positive staining with propidium iodide; (B) percentage of apoptotic cells, positive staining with annexin V. Values represent the mean (±S.D.) activity from two different donors.

3.4. PMSF and NH₄Cl reverse ZEA mediated necrotic cell killing

In order to verify the necrotic effect of ZEA on PBMC, we used the serine protease inhibitor PMSF that is reported to inhibit proteolytic enzymes released from the collapsing lysosomes during necrosis. We treated PBMC for 24 h with 30 μg/ml ZEA in the presence or absence of 0.05 mM PMSF. The cells were then washed and incubated for a further 11 days period. As shown by a kinetic study of annexin V–propidium iodide flow cytometry, PMSF inhibited PBMC from ZEA-induced necrosis that peaked on day 9 (Fig. 6A). In contrast, PMSF did not greatly affect the apoptotic cell events (Fig. 6B).

We further investigated the inhibition of necrosis with the lysosomal inhibitor NH₄Cl 4 days after exposure of PBMC to 30 μg/ml ZEA in the presence and absence of 1 mM or 10 mM NH₄Cl. Our results (Fig. 6C) showed a
4. Discussion

Contamination of cereals intended for human and animal consumption by mycotoxins constitutes a potential health hazard to both humans and animals with agro-economic importance (Hussein and Brasel, 2001). ZEA is considered one of the most frequent mycotoxin contaminants (Berek et al., 2001). Although several studies have reported in vitro and in vivo genotoxic effects of ZEA in some mammalian species (Thust et al., 1983; Hsiang et al., 1988; Grosse et al., 1997), the mechanisms by which ZEA affects immune cells remain unclear (Creppy et al., 2004).

We investigated the in vitro cytopathic effects of ZEA on human PBMC. ZEA exerted a dose-dependent inhibitory effect on the proliferation of both PHA- and PWM-stimulated lymphocytes, in agreement with previous reports (Atkinson and Miller, 1984; Forsell and Pestka, 1985).

By examining the apoptosis and necrosis profiles using flow cytometry, and cell viability, we showed that ZEA at 30 μg/ml exerted a distinct necrotic effect on PBMC, irrespective of mitogen stimulation. Interestingly, Lioi et al. (2004) reported that no apoptotic activity was observed in ZEA-treated bovine lymphocytes after 24 h exposure to 4 μM (=1.2 μg/ml) of ZEA, while at higher concentrations and at longer exposures only necrotic cells were obtained.

We investigated calcium ion (Ca²⁺) levels of PBMC during ZEA exposure. There is strong evidence that a perturbation in intracellular Ca²⁺ influx can cause cytotoxicity and trigger either apoptotic or necrotic cell death (Orrenius et al., 2003) in almost all cell types (Waring, 2005). Our results showed that only activated cells (monocytes and granulocytes) were affected, with an increase of both Ca²⁺ levels and the number of cells with high Ca²⁺ levels. The lymphocytic gate did not show any significant Ca²⁺ changes, although a noticeable reduction in the number of gated cells was observed.

Sawada et al. (2000) applied the serine protease inhibitor PMSF to inhibit l-leucine methyl ester-induced lysosome disruption and necrosis of monocyte-derived THP-1 cells. Using a similar PMSF dosage (0.05 mM), we demonstrated that ZEA-induced cytopathic necrosis was inhibited by PMSF, but not apoptosis. Similar patterns were observed with the lysosomal inhibitor, NH₄Cl (at 1 and 10 mM) that also reduced ZEA-induced necrosis. Apoptosis was not reduced, and indeed NH₄Cl appeared to enhance the apoptotic phenomenon at the higher concentration (10 mM).

Certain mycotoxins appear to affect the human immune system. The trichothecene mycotoxin T-2 was reported to alter the activation-induced cell death mechanism of T lymphocytes (Vlata et al., 2005). Here, we reported that ZEA can induce necrosis in the PBMC populations. We showed that ZEA at a concentration of 30 μg/ml exerts direct cytopathic effects on human PBMC. It has been reported that patients with serum levels of 19–100 μg/ml ZEA exhibited an increasing incidence of early thelarche (JECFA, 2000). The concentration of ZEA in human foods can reach at much higher levels (Kim et al., 1993; Yuwai et al., 1994) with a...
reported frequency of contamination at 69% (Vrabcheva et al., 1996). Moreover, ZEA has been shown to be rapidly absorbed after oral administration in rats, rabbits, pigs and humans (Kuiper-Goodman et al., 1987; Biehl et al., 1993), and also that human intestinal microflora cultured in a continuous flow system were unable to degrade ZEA (JECFA, 2000). All these lines of evidence render ZEA a health hazard and its cytopathic effects on immune cells merits further investigation.

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References


