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Interactive effects of fumonisin B1 and α-zearalenol on proliferation and cytokine expression in Jurkat T cells

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Abstract

Mycotoxins are secondary metabolites of fungi that grow on various food and feed. These compounds elicit a wide spectrum of toxicological effects, including the capacity to alter normal immune function. Feed commodities are usually contaminated with more than one mycotoxin; however, extensive information on the interaction between concomitantly occurring mycotoxins and the consequence for their toxicity is lacking. In the present study, we examined the effects *in vitro* of fumonisin B1 (FB1) and α-zearalenol (α-ZEA), alone or in combination, on the immune function in the human lymphoblastoid Jurkat T cell line. Treatment of cells with increasing concentrations of FB1 resulted in a dose-dependent induction of proliferation. In contrast, α-ZEA showed a marked inhibitory effect on cell proliferation, even at very low doses, essentially mediated by apoptosis. In stimulated cells pre-incubated with FB1, the levels of IL-2 and IFN γ mRNAs were similar to control whereas a reduction of cytokine transcripts was reported following α-ZEA treatment. Interestingly, co-administration of mycotoxins resulted in further inhibition of both proliferation and IFN γ mRNA expression when compared with α-ZEA alone. In conclusion, FB1 and α-ZEA showed different immunomodulation abilities when individually administered. Combination of mycotoxins resulted instead in interactive effects.

Keywords: Fumonisin B1; α-zearalenol; Jurkat T cells; Immunotoxicity

1. Introduction

Mycotoxins are secondary metabolites of fungi that grow on various food. Their global occurrence is considered to be a major risk factor affecting human and animal health. It is also of interest the capacity of some mycotoxins to alter the normal immune function even if present in food at levels below observable overt toxicity (Oswald et al., 2005). In particular, mycotoxins-mediated immune effects have been confirmed both *in vivo* (Bondy and Pestka, 2000) and *in vitro* (Berek et al., 2001).

The most important mycotoxins that may potentially affect human and animal health and productivity are *Fusarium* toxins, particularly fumonisin B1, zearalenone and deoxynivalenol. The *Fusarium* fungi are commonly found on cereals grown in the temperate areas of America, Europe and Asia (Creppy, 2002).

Fumonisin B1 (FB1) is a potent and naturally occurring mycotoxin, produced by the pathogen fungi belonging to *Fusarium* spp. (*Fusarium verticillioides* and *F. proliferatum*) commonly infesting corn and other cereals. It is linked to the etiology of several species-specific toxicoses in domestic and laboratory animals (Colvin and Harrison, 1992; Gelderblom et al., 1991; Kuiper-Goodman, 1998) and it has been

Abbreviations: FB1, fumonisin B1; α-ZEA, α-zearalenol; IL-2, interleukin-2; IFN γ, interferon γ; FBS, foetal bovine serum; PCR, polymerase chain reaction; cpm, counts per minute; PMA, phorbol 12-myristate 13-acetate; AMC, 7-amino-4-methylcoumarin; Ac-DEVD-AMC, acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin; LDH, lactate dehydrogenase; NAD, nicotinamide adenine dinucleotide

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correlated to the incidence of human oesophageal cancer (Marasas, 1995; Abnet et al., 2001). The pathophysiological effects of FB1 are attributable to disruption of the sphingolipids metabolism since this mycotoxin is an inhibitor of ceramide synthase (Ramasamy et al., 1995; Yoo et al., 1996). Ceramide and glycosphingolipids are important signalling molecules and recognition sites in the cellular immune response and attachment sites for many infectious agents and microbial toxins. FB1 affects a variety of cell signalling molecules including protein kinase C (PKC), involved in a number of signal transduction pathways that include cytokine induction, carcinogenesis and apoptosis (Merrill and Murphy, 1997; Gopee and Sharma, 2004). The effects of fumonisin B1 on the immune system remain controversial: FB1 causes immune-suppression in poultry (Li et al., 2000), swine (Harvey et al., 1995), bovine (Osweiler et al., 2003) and immune-stimulation in rodent species (Dombrink-Kurtzman et al., 2000; Dresden-Osborne and Noblet, 2002; Sharma et al., 2004).

Zearalenone (ZEA), regularly found in corn and corn derived products, is synthesised by *Fusarium graminearum* and other *Fusarium* species. ZEA is a macrocyclic lactone with high binding affinity to estrogen receptors. Zearalenone causes estrogenic effects in pigs (Diekman and Green, 1992) and it has been suggested as a causative agent of infertility and reduced milk production in cattle (D'Mello and Mac Donald, 1997). Alcohol metabolites of ZEA, α-zearalenol (α-ZEA) and β-zearalenol, as well as its reduced form, are also estrogenic (Cheeke, 1998).

It is known that the immune system is a potential target for estrogenic endocrine disruptors because estrogen receptors are also present in the various cells on the immune system (Igarashi et al., 2001). In spite of that, only few studies have been carried out on the immune effects of zearalenone and its metabolites. In particular, studies in geriatric mice showed that α-zearalanol decreases IFN γ (Calemine et al., 2003) whereas zearalenone caused *in vitro* a slight reduction of both mitotic index and cell survival of bovine lymphocytes (Lioi et al., 2004).

Feed commodities are usually contaminated with more than one mycotoxin, often produced by the same mold species but there is little information on the interaction between concomitantly occurring mycotoxins and the consequence for their toxicity (Speijers and Speijers, 2004). In *in vitro* studies an interactive (synergistic) effect of citrinin and ochratoxin in renal cells was described (Heussner et al., 2006). Regarding *Fusarium* toxins, co-occurrence can exert additive and synergistic effects, as demonstrated for various trichotecenes (Tajima et al., 2002), but may also act as antagonists (Koshinsky and Khachatourians, 1992). More in particular, a very limited number of studies have been carried out to assess the immunotoxicity of mycotoxins co-occurrence. In an *in vivo* study in rats, Theumer et al. (2003) showed different immunobiological effects produced by a mixture of aflatoxin B1 and fumonisin B1, in comparison to the individual action of the same toxins, but the observed changes were not an addition of the effects produced individually by AFB1 and FB1. Another work on *Penicillium* mycotoxins showed that the majority of examined mixtures produced *in vitro* less-than-additive effects (Bernhoft et al., 2004).

The objective of the present study was to analyse two fusarium mycotoxins, FB1 and α-ZEA, using the human acute T cell leukaemia Jurkat cell line, a model of T lymphocyte. In particular, we compared the activity of the two mycotoxins, alone and in mixture, to gain more information about their immunomodulatory effects. We provide evidence that FB1 and α-ZEA affected proliferation of Jurkat cells and cytokine expression differently when individually tested. Instead, administration of a mixture elicited a synergistic effect on α-ZEA activity.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium, L-glutamine, streptomycin, penicillin, foetal bovine serum (FBS) and non-essential aminoacids were purchased from Cambrex Bioproducts Europe (Verviers, Belgium). Fumonisin B1 (F 1147), α-Zearalenol (Z 0166) and reagents for LDH assay were from Sigma (St. Louis, MO, USA). Caspase 3 substrate, acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC), and caspase 3 standard, 7-amino-4-methylcoumarin (AMC) were purchased from Calbiochem (San Diego, CA, USA). TRIZOL and all other reagents used for reverse transcription, PCR amplification and gel electrophoresis were from Invitrogen Ltd (Paisley, UK).

2.2. Cell culture

Jurkat T cells (ATCC, Manassas, VA) were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2/95% air in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% (w/v) non-essential amino acids. Cells were maintained in the exponential growth phase by passages at 2–3 days intervals.

2.3. Cell proliferation assay

Cells were cultured in flat bottom 96-well plates (2×10^4/well) for 24 h in the presence of different concentrations of FB1 (20–150 μM) and α-ZEA (10–80 μM). The concentrations ranges of mycotoxins were chosen on the basis of preliminary dose–response experiments (data not shown) and literature data (Creppy et al., 2004; Kouadio et al., 2005; Abid-Essefi et al., 2004). Eighteen hours prior to harvesting cells were pulsed with 1 μCi/well [^{3}H]-thymidine. Cultures were harvested on filters using a semiautomatic cell harvester (Filtermate, Packard, Danvers, MA). [^{3}H]-thymidine incorporation was assessed by a microplate liquid scintilla-

Table 1
Primer sequences

L-32	Sense	5′-CCT CAG ACC CCT TGT GAA GC-3′	145 bp
	Anti-sense	5′-GCC CTT GAA TCT TCT ACG AAC C-3′	
IL-2	Sense	5′-AAC TCA CCA GGA TGC TCA CAT TTA-3′	148 bp
	Anti-sense	5′-TTC CTG GGT CTT AAG TGA AAG TTT-3′	
IFN γ	Sense	5′-TCA GCT CTG CAT CGT TTT GG-3′	120 bp
	Anti-sense	5′-GTT CCA TTA TCC GCT ACA TCT GAA-3′	

tor (top count NXT^{TM}, Packard, Danvers, MA). Results were expressed as cpm (counts per minute).

2.4. Measurement of cytokine mRNA levels

The expression of cytokine mRNA in Jurkat cell line was assessed by semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR). Briefly, cells were cultured in 24-well plates (2×10^6/well) and incubated for 24 h with different concentrations of FB1 and α-ZEA, in the presence/absence of 1 μg/l PMA and 0.5 μM ionomycin (Luongo et al., 2003). At the end of the incubation, total RNA was isolated from cells using TRIZOL reagent according to the manufacturer's protocol. Reverse transcription of 1 μg RNA was primed using oligo-$(dT)_{12–18}$ and different aliquots of cDNA were then used for PCR using 1 U of Taq DNA polymerase and 20 pmoles of each primer. The thermo-amplification program consisted of an initial denaturation (5 min at 94 °C), followed by 28 (L-32 and IFN γ) or 35 cycles (IL-2) of 1 min denaturation (94°), 1 min annealing at 54 °C (L-32) or 52 °C (IL-2 and IFN γ) and 30 s elongation at 72 °C with a final 5 min extension period at 72 °C. The primer sequences and their respective PCR fragment lengths are shown in Table 1. The primers were designed using the Lasergene software (DNASTAR Inc. Madison, Wisconsin). Negative control was performed by omitting RNA from the cDNA synthesis and specific PCR amplification. PCR products were separated on a 2% (w/v) agarose gel stained with VISTRA Green (Amersham International plc, Buckinghamshire, UK). Fluorescence scanning and quantitative analysis of detected bands were carried out on STORM 860 system by IMAGEQUANT software (Molecular Dynamics, Inc, Sunnyvale, CA). The housekeeping L-32 gene was used as reference and results were expressed as cytokine/L-32 mRNA ratio.

2.5. Caspase assay

Caspase-3 activity was measured by determining the release of 7-amino-4-methylcoumarin (AMC), following enzyme hydrolysis of acetyl Asp-Glu-Val-Asp 7-amido-4-methylcoumarin (Ac-DEVD-AMC). Cells (6×10^5/well) were incubated with different concentrations of α-ZEA for 24 h in 96-well plates, then washed with PBS and resuspended in 100 μl lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM DTT) for 20 min at 4 °C. Lysates were centrifuged (10,000*g*, for 5 min at 4 °C) and supernatant aliquots incubated with reaction buffer (lysis buffer supplemented with 2 mM EDTA and 50 μM Ac-DEVD-AMC) for 60 min at 37 °C. Fluorescence data ($360_{EX}/460_{EM}$) were recorded and used to calculate the concentration of released AMC by means of calibration curve with pure standard. The caspase-3 activity was finally expressed as nmols of AMC/mg protein/hour. Cells treated with 10 μM actinomycin D were used as positive control.

2.6. Cytotoxicity assay

Lactate dehydrogenase (LDH) release was measured as *in vitro* marker for cellular toxicity (Tipton et al., 2003). Cells were incubated at 2×10^5/well in 48-well plates with different doses of α-ZEA for 24 h. After 20 min at 4 °C the cell suspensions were centrifuged (4000*g*, 5 min, 4 °C). The supernatants were recovered, whereas the cell pellets were lysed in 100 μl of 0.2 M Tris/HCl pH 8.0, containing 1% (w/v) Triton X-100, for 30 min at 4 °C. Lysates and supernatant aliquots (100 μl) were then incubated with 100 μl reaction buffer (0.7 mM *p*-iodonitrotetrazolium violet, 50 mM L-lactic acid, 0.3 mM phenazine methosulphate, 0.4 mM NAD, 0.2 M Tris/HCl pH 8.0) for 30 min at 37 °C. Absorbance was read at 490 nm; results were expressed as percentage of total LDH release from control cultures treated with 1% (w/v) Triton X-100 and calculated as follows: [(experimental value – blank value)/(total lysis – blank value) × 100].

2.7. Statistical analysis

The results of caspase 3 activity and LDH release were expressed as mean ± SD. Differences among the various treatment groups were determined by one-way analysis of variance (ANOVA). Multiple comparison of treatment means were made using the Tukey test. The criterion for significance was $P < 0.05$.

2.8. Isobole method analysis

The type of interaction between FB1 and α-ZEA was determined by evaluating the effect of mycotoxin combinations on cell proliferation at two doses of FB1 (20 and 40 μM). Results were expressed as percentage of the proliferation in untreated cells. The concentrations of α-ZEA producing significant inhibition of cell proliferation at a 50% level, were estimated by extrapolating results from the dose-response curves and analysed for interactive effects according to Berenbaum (Berenbaum, 1989). Student's *t*-test

was used for statistical analysis of the estimated combined effects. The criterion for significance was $P < 0.05$.

3. Results

3.1. Influence of mycotoxins on Jurkat cell proliferation

To investigate whether FB1 and α-ZEA affect cell proliferation, we used [^{3}H]-thymidine incorporation as an indicator of DNA synthesis. Initially, time-dependent response experiments were performed and, consequently, a 24 h time period was chosen as optimal incubation time for both mycotoxins (data not shown). Treatment of Jurkat cells with increasing concentrations of FB1 showed a consistent dose-dependent increase in cpm at low doses, that reached a plateau at 80 μM FB1 (Fig. 1A). In contrast, when cells were incubated in the presence of increasing concentrations of α-ZEA, we observed a marked inhibitory effect on cell proliferation even at very low doses (Fig. 1B).

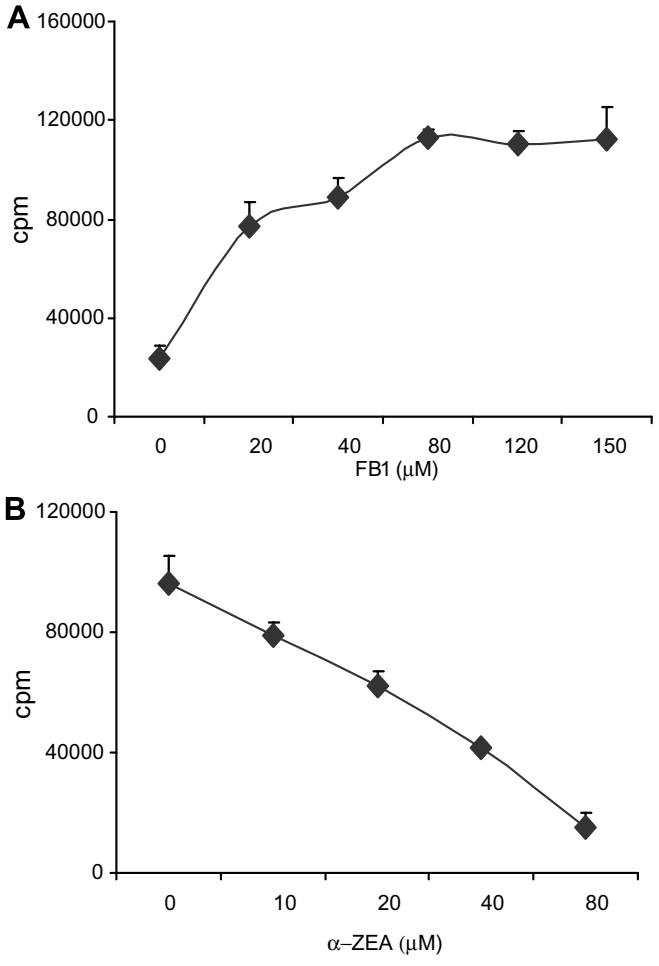

Fig. 1. Effects of FB1 and α-ZEA on Jurkat cell proliferation. Jurkat T cells (2×10^4 cells/ml) were cultured in 96 well-plates, in the presence of increasing concentrations of FB1 (A) and α-ZEA (B) for 24 h. DNA synthesis was measured by [^{3}H]-thymidine incorporation and expressed as cpm. Each value represents the mean ± SD of three independent experiments.

3.2. Effect of α-ZEA on apoptosis and necrosis in Jurkat cells

To discern whether α-ZEA-induced inhibition of proliferation was associated with apoptosis induction, the activity of the final executioner of apoptosis, caspase 3, was determined. Exposure of cells to 0–80 μM α-ZEA resulted in a dose-dependent increase of caspase 3 activity compared to untreated control (Fig. 2A).

Next, we evaluated the effect of α-ZEA on cell integrity and viability, measuring LDH release in the spent medium. Results showed that α-ZEA treatment at various doses caused a relatively consistent increase of LDH activity at 80 μM (Fig. 2B), indicating that it is cytotoxic only at the highest examined concentration. Taken together, data suggested that inhibition of proliferation by α-ZEA was essentially mediated by apoptosis, whereas necrosis can occur concomitantly following exposure to a high dosage of α-ZEA.

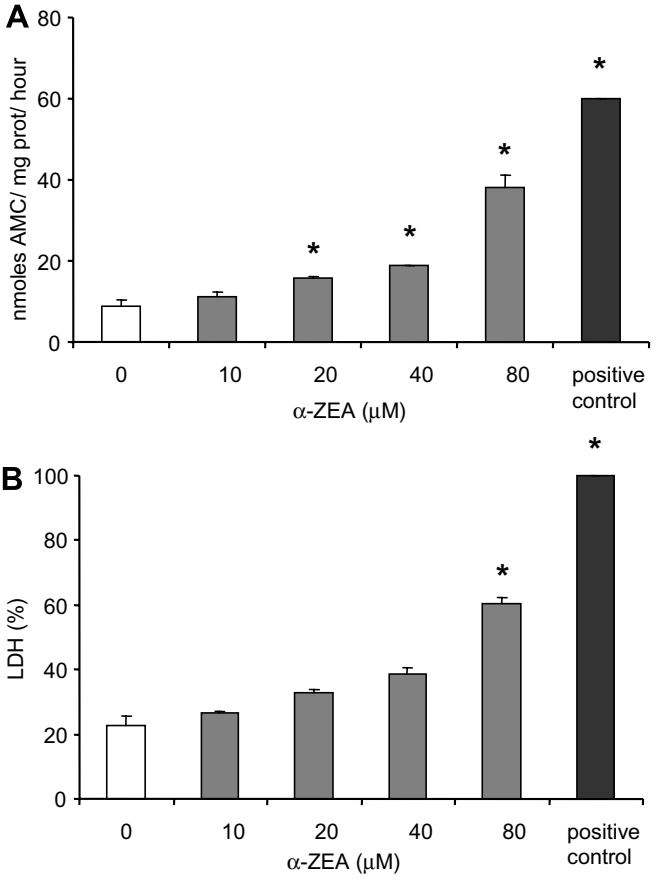

Fig. 2. Effect of α-ZEA administration on cell death. Jurkat T cells (2×10^5 cells/well) were plated in 48 well-plates, and incubated in complete medium with 0–80 μM α-ZEA for 24 h. (A) For assessment of apoptosis induction, caspase-3 activity was analysed and values calculated as nmoles AMC/mg protein/hour; results represent the mean ± SD of triplicate determinations from three separate experiments; cells treated with 10 μM actinomycin D were used as positive control. (B) Cytotoxicity of α-ZEA was assessed by determining the activity of LDH released into the medium; data were expressed as percentage of LDH released from the positive control (cells treated with 1% Triton X-100). Results represent one of three independent experiments. *Significantly different from 0 μM α-ZEA-treated cells.

3.3. Assessment of FB1 and α-ZEA treatments on cytokine mRNA expression

The effects of the two fusariotoxins on the cytokine expression were then evaluated on residual living cells. As expected, cytokine mRNA production could be detected only following cell stimulation with PMA/ionomycin (Fig. 3). In stimulated cells, pre-incubated with FB1 at the highest doses (40 and 80 μM), the levels of IL-2 mRNA were not different from the control (Fig. 3A). Similarly, no effect on the Th1-like cytokine IFN γ was observed (Fig. 3A). Fig. 3B shows the effect of α-ZEA in the range 20–80 μM on the IL-2 and IFN γ transcripts expression. When α-ZEA-treated cells were subsequently stimulated, reduction of IL-2 transcript levels both at 40 and 80 μM concentrations was reported. Accordingly, α-ZEA treatment caused a consistent dose-dependent inhibition of IFN γ mRNA expression.

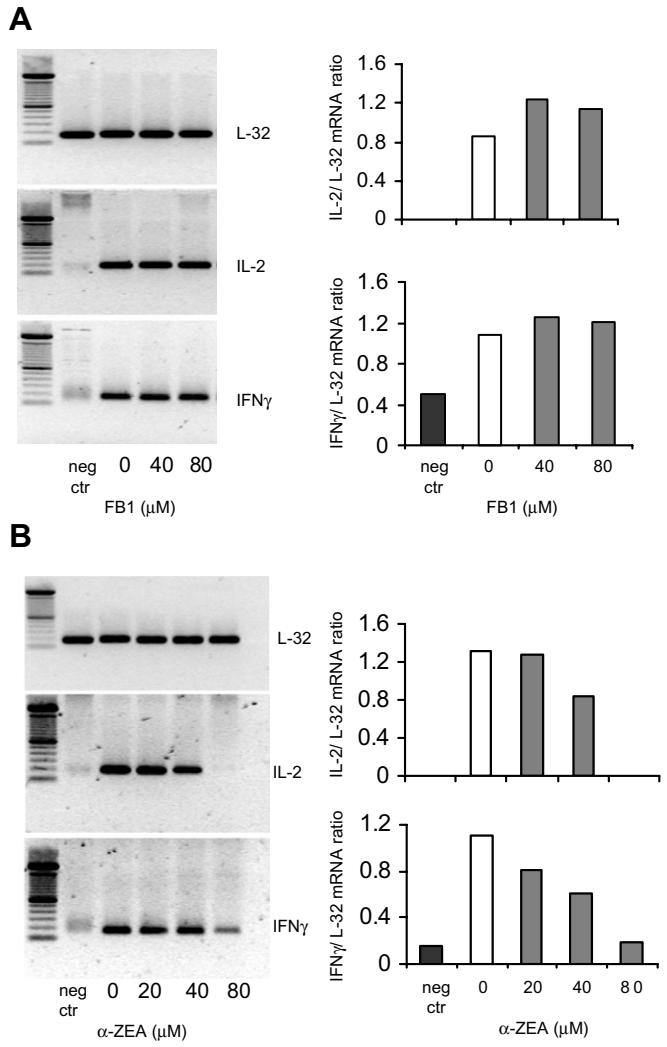

Fig. 3. Assessment of FB1 and α-ZEA treatment on cytokine mRNA expression. Jurkat T cells, stimulated with PMA/ionomycin, were treated with different doses of FB1 (A) and α-ZEA (B) for 24 h before stimulation with PMA/ionomycin. mRNA levels of IL-2, IFN γ and the housekeeping gene L-32 were assessed by a semiquantitative RT-PCR; the products were then analysed on a 2.0% agarose gel. After electrophoresis, a densitometric scanning was carried out and results were expressed as cytokine/L-32 mRNA ratio. Results represent one of three independent experiments. The negative control (neg ctr) is unstimulated cells.

3.4. Effects of FB1 and α-ZEA co-administration on Jurkat cell proliferation

Next, the effects of co-administration of FB1 and α-ZEA on the Jurkat cell proliferation were examined by varying the amount of α-ZEA in the presence of fixed doses of FB1 (20 or 40 μM). Results reported in Fig. 4 showed inhibition of proliferation when both toxins were administered, indicating that the α-ZEA-dependent inhibition prevailed on the stimulatory effect of FB1 (Figs. 1 and 4). To define the type of interaction between the two mycotoxins we applied the isobole method (Berenbaum, 1989) by considering that one of the agent (FB1) did not in any dose produce the effect of the mixture, that is inhibition (heterergic combination; Loewe, 1953). The concentrations of α-ZEA, alone and in the mixtures, producing 50% inhibition of cell proliferation were reported in Table 2. Data showed that the

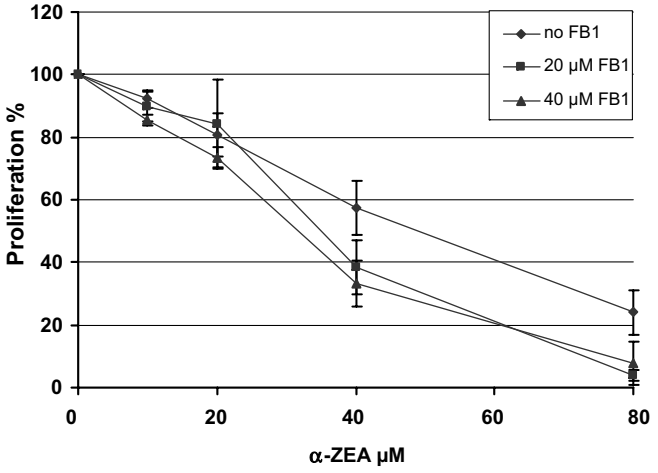

Fig. 4. Influence of FB1 and α-ZEA co-administration on cell proliferation. Jurkat T cells were cultured in the presence of increasing concentrations of α-ZEA alone or a mixture with FB1 (20 or 40 μM) for 24 h. Values are expressed as percentage of proliferation of untreated cells and represent the mean ± SD of three independent experiments.

Table 2
Interactive effect of α-ZEA and FB1

d(a)/D(a) = 1*	Additive
d(a)/D(a) < 1	Synergistic
d(a)/D(a) > 1	Antagonist

Treatment	d(a) (Mean ± SD)	D(a) (Mean ± SD)	*t*-Test ($P < 0.05$)
FB1 + α-ZEA 20 μM	40.37 ± 3.47	51.75 ± 2.99	0.012
FB1 + α-ZEA 40 μM	38.64 ± 3.83	51.75 ± 2.99	0.009

d(a)/D(a) = 1, equation for the zero interaction line of heterergic combinations with two agents (Berenbaum, 1989); d(a), concentration of α-ZEA in the mixture; D(a) concentration of α-ZEA alone isoeffective with the combination. Values were chosen for residual proliferation of 50%.

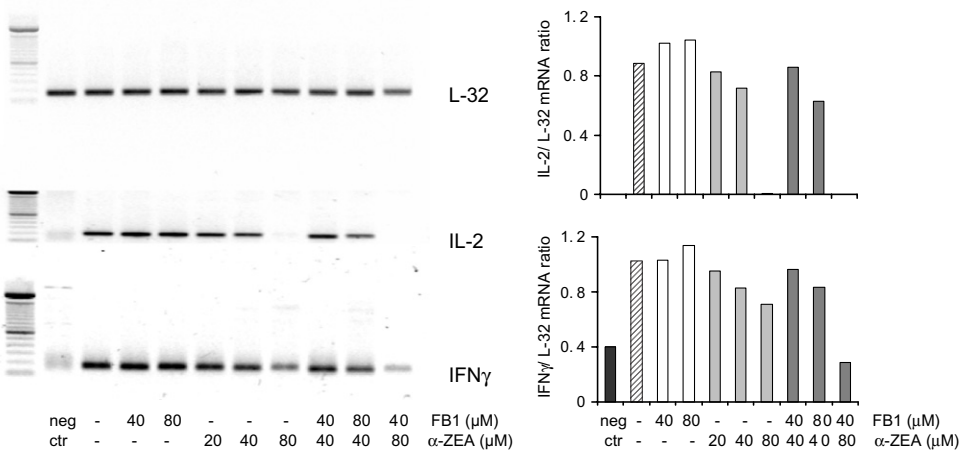

Fig. 5. Effect of FB1 and α-ZEA co-administration on IL-2 and IFN γ mRNA expression. Cells were treated with different doses of FB1 (40–80 μM) and α-ZEA (20–40–80 μM) for 24 h before stimulation with PMA/ionomycin. Analysis was performed as described in Fig. 3. Reported results are representative of three independent experiments. The negative control (neg ctr) is unstimulated cells.

interaction indices both at 20 and 40 μM FB1 in the mixture were significantly less than 1, indicating synergy.

3.5. Effects of FB1 and α-ZEA co-administration on cytokine expression

In order to investigate the effects of FB1 and α-ZEA co-incubation on the expression of IL-2 and IFN γ mRNAs, we treated Jurkat cells with increasing concentrations of the two mycotoxins (Fig. 5). Results confirmed the previously reported effects of α-ZEA and FB1 when individually tested. Interestingly, the α-ZEA-dependent inhibition of cytokine levels was confirmed also in the presence of FB1 at the various examined doses; in particular, we registered that IFN γ mRNA expression, detected following exposure to the mixture at the 80:40 (α-ZEA:FB1) ratio, was lower than the corresponding transcript levels assessed in the presence of 80 μM α-ZEA alone.

4. Discussion

There is major concern over the potential for mycotoxins to negatively influence human health. Moreover, the risk assessment for humans potentially exposed to multi-mycotoxins suffers from the lack of adequate bioassays. In particular, present analytical systems, mainly based on physicochemical evaluations, do not take into account for a given mycotoxin synergism and antagonism with other mycotoxins naturally occurring in the same foodstuffs. From this point of view, the use of cell cultures offers several advantages over other methods, particularly in terms of quantification of toxic effects and for defining organ specificity related to a preferential action on a particular cell type. In the present study, we addressed the issue by evaluating the effects of two mycotoxins, Fumonisin B1 (FB1) and α-zearalenol (α-ZEA), on the immune function by means of *in vitro* assessment of their activities on the human lymphoblastoid Jurkat T cell line. We showed that FB1 and α-ZEA have contrasting effects on specific cell functions when examined separately whereas co-administration resulted in potentiation of α-ZEA overall activity.

The Jurkat T cell line has long served as an useful human T cell model for different immunological studies. They have proved valuable, particularly for the characterisation of the IL-2 and Ag-receptor structures, as well as molecules of signal transduction. However, in comparison with normal T cells, the ability of some transduction pathway to respond to external stimuli is altered (Astoul et al., 2001); so, data obtained in Jurkat needs careful interpretation before to extrapolate it to the normal T cell biology.

FB1 is a mycotoxin produced by *F. verticilloides*, causing inhibition of ceramide synthesis leading to accumulation of free sphingoid bases. Moreover, the ability of FB1 to modulate signal transduction pathways also plays a role in its toxicity (Gopee and Sharma, 2004). The effects of *Fusarium* mycotoxins on immune functions are also well known (Berek et al., 2001; Calemine et al., 2003; Pestka et al., 2005). Very recently, the effect of FB1 on the modulation of the cytokine expression was investigated on pig peripheral blood mononuclear cells (PBMC) indicating that FB1 decreased IL-4 and increased IFN γ synthesis (Taranu et al., 2005). Interestingly, in mice FB1 treatment caused a selective increased expression in pro-inflammatory Th1-cytokines (IL-12, IFN γ and TNFα) with no alteration of Th2-cytokines (IL-4, IL-6 and IL-10) with consequent modification of the cytokine profile (Bhandari et al., 2002). Among cytokines TNFα seems to plays an important role in FB1 toxicity *in vivo*, as the expression of TNFα mRNA in liver and kidney is increased following FB1 exposure in mice (He et al., 2001). Studies aimed at investigating whether sphingoid bases accumulation and TNFα induction are dependent events on each other showed that in LLC–PK1 cells, a pig kidney epithelial cell line, exposure to sphinganine or sphingosine did not significantly alter the expression of TNFα (He et al., 2001). Moreover, inhibition of sphingoid base biosynthesis efficiently blocked the

accumulation of free sphingoid bases in response to FB1, but it did not prevent the induction of TNFα expression, indicating that FB1-induced increase of TNFα expression is independent of sphingoid base accumulation-induced by ceramide synthase inhibition (He et al., 2001). However, we found that FB1 left unaltered the profile of Th1 cytokine expression that characterises Jurkat T cells following stimulation. Instead, FB1 at low doses significantly increased cell proliferation in our system; this was in agreement with FB1-stimulation of DNA synthesis in Swiss 3T3 fibroblasts, related to the accumulation of sphinganine and sphingosine (Schroeder et al., 1994). On the contrary, assessment of FB1 on IPEC-1,a porcine intestinal epithelial cell line, and LLC-PK1 cells showed a significant decrease of cellgrowth at FB1 concentrations determined to be non-cytotoxic for both cell lines (Bouhet et al., 2004). Contrasting effects on FB1-mediated proliferation have also been described in an *in vivo* model, as FB1 administration in rats caused a predominantly zone 3 'toxic' liver injury, with hepatocyte death, but also a concomitant proliferation of hepatic stellate cells (Lemmer et al., 1999).

Taken together, the discrepancies between some reported data and our findings may found explanation in the different nature and histological type of examined cells, in the different concentrations of toxins that were analysed, as well as assuming the existence of interspecies differences in toxin sensitivity. In particular, our data suggest that in Jurkat FB1 positively influences cell proliferation.

α-ZEA instead caused inhibition of cell proliferation in our system, essentially mediated by apoptotic mechanisms of cell death. As the chemical structure of ZEA enables its binding to the estrogenic receptors, our data is in line with previous results indicating that Jurkat cells are sensitive to estrogen treatment (McMurray et al., 2001). Accordingly, our observations of inhibition of IL-2 and IFN γ cytokines mRNA expression by α-ZEA still find explanation assuming an estrogen-like activity (McMurray et al., 2001). Since T-lymphocytes proliferation is essentially mediated by IL-2, the observed inhibition could be considered a primary mechanism of α-ZEA-induced immune-suppression acting also *in vivo*.

Interestingly, the combination of both mycotoxins, that is commonly observed in foodstuff, produced growth inhibition as overall interactive effect. In particular, our data showed that the combination with two different doses of FB1 elicited a statistically significant synergistic effect. Our finding is also in line with previously reported *in vitro* data (Tajima et al., 2002). This is of particular relevance if we consider the regular dietary co-occurrence of α-ZEA with other mycotoxins, such as FB1 and trichothecenes. It is noteworthy that a change from antagonistic to synergistic effect has been also described for the tricothecene mycotoxins T-2 and HT-2 (Koshinsky and Khachatourians, 1992).

We also found that FB1 was unable to revert the inhibitory effect exerted by α-ZEA on the cytokine expression. In particular, incubation with 40 μM FB1 plus 80 μM α-ZEA caused a more marked inhibition of IFN γ transcript levels than that observed with 80 μM α-ZEA alone. We speculated that a threshold of accumulation of free sphinganine and sphingosine inside cells exists: at the examined low doses of FB1, changes in the sphingolipid metabolism do not apparently influence the estrogen receptor-mediated inhibition of proliferation and cytokine transcription. By increasing the FB1 doses, the consequent higher cell content of sphingoid bases may positively influence the α-ZEA activity. Further studies are required to better address this particular issue.

In conclusion, our results confirmed the immunomodulatory abilities exerted by α-ZEA and FB1 alone or in combination. In terms of risk assessment in foodstuff, those mycotoxins which showed interactive effects are of more concern.

Acknowledgements

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