



Mycotoxins nivalenol and deoxynivalenol differentially modulate cytokine mRNA expression in Jurkat T cells

Lorella Severino ^a, Diomira Luongo ^{a,b}, Paolo Bergamo ^b, Antonia Lucisano ^a,
Mauro Rossi ^{b,*}

^a Department of Pathology and Animal Health, Division of Toxicology, University of Naples “Federico II”, via Delpino 1, 80137 Naples, Italy

^b Istituto di Scienze dell’Alimentazione, CNR, via Roma 52, 83100 Avellino, Italy

Received 21 July 2006; received in revised form 25 October 2006; accepted 2 November 2006

Abstract

Deoxynivalenol (DON) and its hydroxylated form nivalenol (NIV) are *Fusarium* mycotoxins that occur in cereal grains alone or in combination. Several studies have shown that these metabolites affect lymphocyte functions. However, the molecular mechanisms underlying their activities are still partially known. To address this issue, we examined the influence of NIV and DON in modulating IFN γ , IL-2 and IL-8 mRNA levels in Jurkat T cells. In PMA/ionomycin stimulated cells, pre-incubated with increasing concentrations of NIV, transcription was induced in the range 0.06–2 μ M; higher concentrations of NIV were found non-stimulating (4 μ M) or inhibitory (8 μ M) for IFN γ and IL-2 whereas IL-8 was still induced. DON administration elicited a similar profile for IL-8 and IFN γ , whilst IL-2 mRNA was induced in a broader range of concentrations. Combination of NIV and DON at 1:1 and 1:10 ratios essentially restored the cytokine transcriptional pattern observed with NIV alone but the level of transcripts, with the exception of IL-8, peaked at lower concentrations suggesting interactive effects. Moreover both mycotoxins caused inhibition of cell proliferation, mediated by induction of apoptosis, confirming previous results and highlighting the usefulness of Jurkat as a T-cell model to study the effects of mycotoxins on the immune functions in humans.

© 2006 Elsevier Ltd. All rights reserved.

Keywords: Nivalenol; Deoxynivalenol; Jurkat T cells; Immunotoxicity

1. Introduction

Nivalenol (NIV) and deoxynivalenol (DON)¹ are mycotoxins produced by soil fungi of the *Fusarium* genus which are abundant in many cereal crops in the field (wheat, maize, barley, oats and rye) and also in processed grains (malt, beer and bread). A recent data collection on the occurrence of *Fusarium* toxins in food in the European

Union showed a 57% incidence of positive samples for DON and 16% for NIV out of several thousands of samples analysed [1].

Chemically, NIV (12,13-epoxy-3,4,7,15-tetrahydroxy-trichothec-9-en-8-one) and DON (12,13-Epoxy-3,4,15-trihydroxytrichothec-9-en-8-one) belong to the trichothecenes group, very stable compounds both during storage/milling and processing/cooking of food [2].

Different toxic properties have been associated to trichothecenes. In particular a series of studies with experimental animals demonstrated effects on the immune system, including impaired delayed-type hypersensitivity responses, phagocyte activity [3–5] and modulation of host response to enteric infections [6]. In particular, NIV inhibited total and antigen specific IgE production in ovoalbumin specific T-cell receptor $\alpha\beta$ transgenic mice [7]. *In vitro* analyses showed that trichothecenes can both

* Corresponding author. Fax: +39 0825299104.

E-mail address: mrossi@isa.cnr.it (M. Rossi).

¹ Abbreviations used: DON, deoxynivalenol; NIV, nivalenol; 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; NP-40, Nonidet P-40.

suppress and stimulate immune functions [8]. NIV inhibits blastogenesis in cultured human lymphocytes producing 50% inhibition of thymidine incorporation in mitogen-stimulated human peripheral lymphocytes [9]. Thuvander et al. [10] found that NIV inhibited proliferation of human male and female mitogen-stimulated lymphocytes. DON instead induced inhibition of human lymphocyte proliferation and did not have significant effects on cytokine profiles, in particular there was a marked increase in IL-2 production, a modest increase in both IL-4 and IFN γ and, in contrast, an inhibition of IL-6 production [10]. In another study, DON was shown to induce IL-2, IL-4 and IL-5 production [11]. Some other studies indicated that trichothecenes induce apoptosis in cells [3,12,13]. More in particular, Shifrin et al. [14] found that NIV and DON were, respectively, intermediate and strong inducers of apoptosis in Jurkat cells.

Among biochemical effects ascribed to NIV and DON, inhibition of protein synthesis was reported [15] mediated by specific blocking of peptidyl transferase activity [16]. Initiation of a 'ribotoxic stress response' by protein synthesis inhibitors has been postulated to be a signal for MAPKs activation [17]; interestingly, DON was shown to induce MAPK phosphorylation in Jurkat cells paralleled by induction of apoptosis and cytokine production [18].

Several mycotoxins are commonly present in food. However there is relatively little information about the interaction between concomitantly occurring trichothecenes and the consequences for their immunotoxicity. Some recent studies focused on the combined administration of trichothecenes in *in vitro* test systems showing that co-occurrence of toxins can exert interactive effects [19]. The purpose of this study was to further elucidate the activity of NIV and DON, alone and in combination, on cytokine mRNA expression in Jurkat cells, a model of human T lymphocyte, to gain more information about the immune effects exerted by these toxins and, more in particular, to define the minimal doses able to modulate the cell system.

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). Deoxynivalenol (D-0156), Nivalenol (N-7769) 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% CO₂/95% air in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 1% (w/v) non-essential amino acids. Cells were kept in the exponential growth phase by passages at 2–3 days intervals.

2.3. Measurement of cytokine mRNA levels

The expression of cytokine mRNAs in the Jurkat cell line was assessed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Briefly, cells were cultured in 24-well plate (2×10^6 /well) and incubated for 24 h with different concentrations of DON and NIV, in the presence/absence of 1 μ g/L PMA and 0.5 μ M ionomycin [20]. 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 μ g 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 28–35 cycles of 1 min denaturation (94°), 1 min annealing at 60 °C (IL-8), 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 oligonucleotides used for amplification and the size of the PCR products are described in Table 1. In preliminary experiments we found that L-32 gene [21] expressed transcript levels more comparable to the cytokine mRNAs content than of other commonly used housekeeping genes (not

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'	
IL-8	Sense	5'-ATGACTTCCAAGCTGGCCGTGGCT-3'	289 bp
	Anti-sense	5'-TCTCAGCCCTCTTCAAAACTTCTC-3'	

shown). The primers were designed in our laboratory by using the software Lasergene Primerselect (DNASTAR Inc., Madison, Wisconsin, USA) so that amplified cDNA fragments sizes could be readily distinguished from those obtained from genomic DNA, if present. Negative control was performed by omitting RNA from the cDNA synthesis and specific PCR amplification. PCR products were analysed 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). Results were expressed as cytokine/L-32 mRNA ratio.

2.4. 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 DON (0.0625–80 μM) and NIV (0.0625–8 μM). Eighteen hours prior to harvesting cells were pulsed with 1 μCi /well [^3H]-thymidine. Cultures were harvested on filters using a semiautomatic cell harvester (Filtermate, Packard, Danvers, MA). [^3H]-thymidine incorporation was assessed by a microplate liquid scintillator (Top Count NXTTM, Packard, Danvers, MA). Results were expressed as cpm (counts per minute).

2.5. Apoptosis 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 DON and NIV for 24 h in 48-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 (10,000g, 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 nmoles of AMC/mg protein. Cells treated with 10 μM actinomycin D were used as positive control.

2.6. Cytotoxicity assay

Lactate dehydrogenase (LDH) was used as *in vitro* marker for cellular toxicity [22]. Cells were incubated at 2×10^5 /well in 48-well plates with different doses of DON and NIV for 24 h. After 20 min at 4 °C the cell suspensions were centrifuged (4000g, 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)

Nonidet P-40 (NP-40), 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) NP-40 and calculated as follows: [(experimental value – blank value)/(total lysis – blank value) \times 100].

2.7. Statistical analysis

The results were expressed as means \pm 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 $P < 0.05$ for all studies.

3. Results

3.1. Assessment of NIV and DON treatments on cytokine mRNA expression

ELISA experiments indicated that DON, but not NIV, induced IL-2 and IL-8 protein secretion in Jurkat cells [18]. Starting from this point we investigated the minimal concentration of NIV and DON affecting the mRNA profile of these cytokines and of IFN γ , as we reported that in Jurkat this pro-inflammatory molecule can be modulated by mycotoxins [23]. Transcript levels were evaluated by semi-quantitative RT-PCR, after normalization to the mRNA content of the housekeeping gene L-32. As expected, cytokine mRNA production could be detected only following cell stimulation with PMA/ionomycin, whereas L-32 levels were similar in unstimulated and stimulated cells (Fig. 1). On the other hand, incubation of mycotoxins in the full range 0.06–8 μM with unstimulated cells did not evidence any transcript (data not shown). In stimulated cells, pre-incubated with increasing concentrations of NIV, the levels of both IFN γ , IL-2 and IL-8 mRNA progressively increased and peaked at 1–2 μM whereas higher concentrations of NIV were found less stimulating (4 μM) or inhibitory (8 μM) for the examined Th1-type cytokines; on the contrary, IL-8 was induced in almost all the concentration range (Fig. 1). Similarly, DON induced the expression of IL-8 and IFN γ mRNAs; nevertheless, the latter appeared to peak later and with a lower increase than that reported for NIV (Fig. 1). Very interestingly, this mycotoxin appeared to differently affect IL-2 mRNA levels; in fact, raising concentrations of DON were paralleled by a constant increase of IL-2 transcripts, with no evidence of a dose-dependent differential activity. The analysis of RT-PCR data pooled from different experiments more accurately revealed that IFN γ mRNA peaked at 1 μM NIV (fourfold increase) and 4 μM DON (threefold; Fig. 2); similarly IL-2 transcripts peaked at 1 μM NIV (threefold

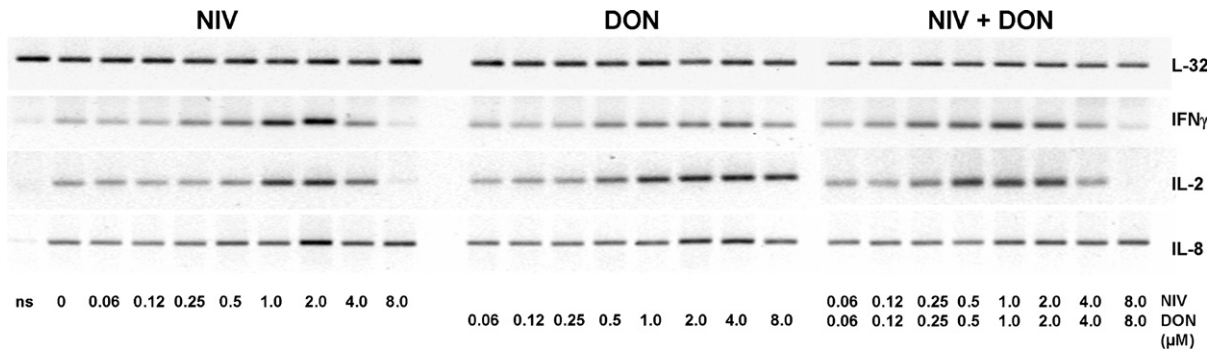


Fig. 1. Assessment of NIV and DON treatment on cytokine mRNA expression. Jurkat T cells, stimulated with PMA/ionomycin, were treated with different doses of NIV and DON, alone or in combination (1:1 ratio), for 24 h. mRNA levels of IL-2, $\text{INF}\gamma$, IL-8 and the housekeeping gene L-32 were assessed by a semiquantitative RT-PCR; the products were then analysed on a 2.0% agarose gel.

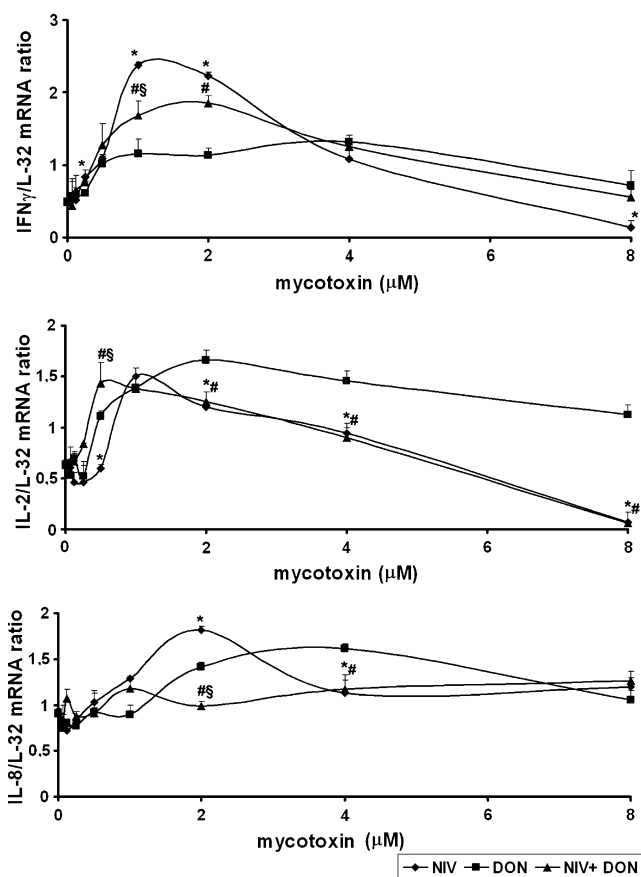


Fig. 2. Densitometric analysis of cytokine mRNAs. Scanning of fluorescent bands was carried out and each cytokine value, normalized to the relative content of L-32, was expressed as cytokine/L-32 mRNA ratio. Results represent mean \pm SD of three independent experiments. *Different from DON (NIV); #different from DON (mixture); §different from NIV (mixture); $P < 0.05$.

increase), whereas they reached a plateau at 2 μM DON with a fourfold increase. For IL-8 mRNA a twofold increase was registered for both mycotoxins in the range 2–8 μM (Fig. 2).

The effects of NIV and DON co-incubation, with increasing concentrations of the two mycotoxins, were then

examined. Combination of NIV and DON (1:1 ratio) essentially restored the IL-2 mRNA profile observed with NIV alone, but the level of transcript peaked at lower concentrations (Fig. 1), suggesting an interactive effect exerted by the two mycotoxins. Very interestingly, at higher concentration the inhibitory effect of NIV prevailed over the inductive activity of DON. In the same way, the $\text{INF}\gamma$ profile following co-administration of NIV and DON still reflected that observed with NIV alone while IL-8 mRNA expression was normally induced. The densitometric assessment showed that IL-2 mRNA peaked at 0.5 μM NIV plus 0.5 μM DON (threefold increase; Fig. 2); for $\text{INF}\gamma$ the induction profile was similar to that observed in the presence of NIV alone but the mRNA levels were found lower; IL-8 levels essentially reflected those registered for the single mycotoxins (Fig. 2). Considering that in field samples the concentration of DON is often found higher than NIV [24] we also analysed the combined effect of the two mycotoxins in the presence of DON overload (1:10; NIV:DON ratio). As reported in Fig. 3, higher concentrations of DON still induced a 2- to 3-fold increase in the expression of IL-8 and $\text{INF}\gamma$ mRNAs; on the other hand DON was found inhibitory for the transcription of IL-2 starting from 40 μM . Co-incubation at the 1:10 ratio essentially confirmed the results previously reported for the 1:1 ratio; in particular, inhibition of $\text{INF}\gamma$ and a trend to further decrease IL-2 mRNA levels were registered starting from the 2:20 μM combination. Interestingly, full induction of IL-8 was observed at all the examined concentrations.

3.2. The effects of NIV and DON on cell vitality

Jurkat cells, incubated with different concentrations of NIV and DON, ranging from 0.0625 to 8 μM , showed a decrease in thymidine incorporation for both mycotoxins (Fig. 4A); such a decrease became significant and dose-dependent at 0.5 and 1 μM for NIV and DON. Co-incubation caused a further dose-dependent decrease of cell proliferation both at the 1:1 (Fig. 4A) and 1:10 ratio (not shown).

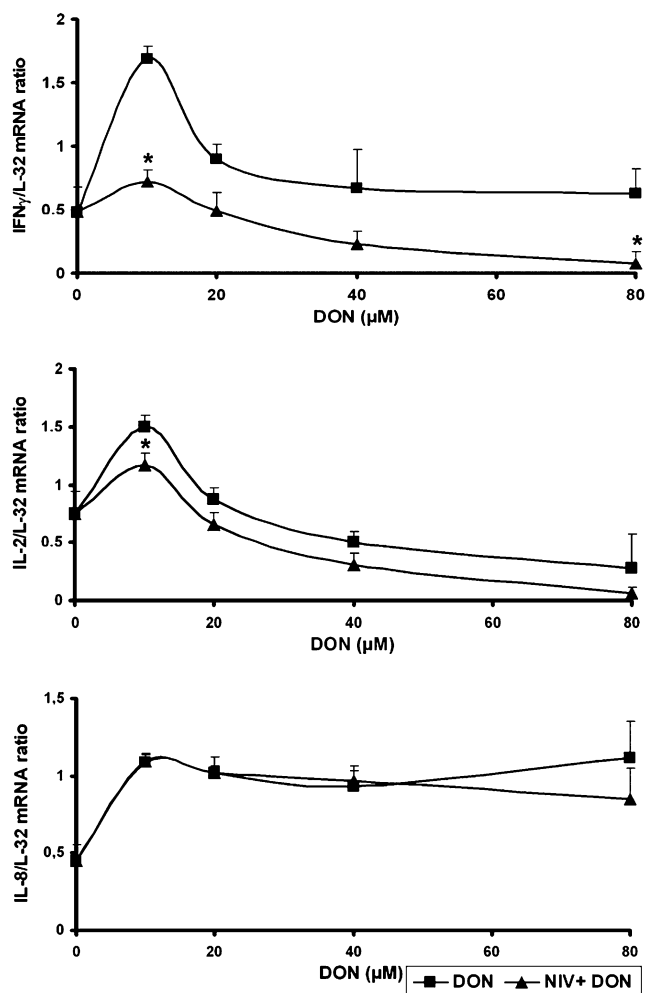


Fig. 3. Cytokine mRNAs in Jurkat following co-incubation with NIV and DON (1:10 ratio). Stimulated Jurkat T cells were treated with different doses of DON in the range from 10 to 80 μ M, alone or in combination with NIV at 1:10 ratio for 24 h. mRNA levels of IL-2, IFN γ , IL-8 and IL-32 were assessed by RT-PCR. Fluorescent bands were analysed as in Fig. 3. Results represent mean \pm SD of three independent experiments. *different from DON alone; $P < 0.05$.

To discern whether mycotoxin-induced inhibition of proliferation was associated with apoptosis induction at all the various examined doses, the activity of caspase 3 was determined. Exposure of cells to increasing concentrations of both mycotoxins resulted in a dose-dependent raise of caspase 3 activity, compared to untreated cells (Fig. 4B). At 8 μ M we were not able to estimate the enzyme specific activity due to the very low cell recovery. Similar levels of activity were still observed following co-incubation of NIV and DON at 1:1 ratio. Next we evaluated the effects of mycotoxins on cell integrity by measuring the LDH release in the spent medium. Results showed that NIV and DON treatment caused a slightly and non-statistically significant increase of LDH activity (Fig. 4C). Again, co-administration of mycotoxins did not significantly modify the levels of the released enzyme both at the 1:1 (Fig. 4 C) and 1:10 ratio (not shown).

4. Discussion

In the present study we provided new insight into how mycotoxins NIV and DON, frequently found in food, might modulate human T-cell functions. To address this issue the Jurkat T-cell line was used as a lymphocyte model. Jurkat cells have extensively been used for different immunological studies. They have proved helpful, particularly for the dissection of the IL-2 and Ag-receptor structures, as well as molecules of signal transduction. The use of Jurkat cell culture offers several advantages over other methods for the assessment of immunotoxicity; for instance, present analytical systems, mainly based on physical-chemical evaluations, do not take into account for a given mycotoxin interaction with other mycotoxins. On the other hand, it is known that the capacity of different transduction pathways to react to external stimuli is altered in Jurkat in comparison with normal T cells [25]; consequently, results gained in Jurkat needs careful interpretation before to extrapolate them to the normal T-cell biology. In particular our data were in line with previous results on normal T lymphocytes [9–11,13], so confirming the validity of this *in vitro* cell model for studying mycotoxins.

It must be emphasised that the actual doses required for human effects represent a major concern in studies on mycotoxins. Thus, our work was initially focussed on the identification of the minimal concentrations of NIV and DON sufficient for modulating cytokine production. To date there are no studies which have investigated the activities of NIV and DON at the mRNA cytokine level in human lymphocytes. Interestingly, we found that these mycotoxins differentially influenced the examined Th1-type cytokine transcripts. In particular NIV appeared to exert a dual effect, inductive at low doses (0.06–2 μ M) and suppressive at higher concentration (8 μ M). The inductive effect may be explained by assuming that, in analogy with other trichothecenes [18,26], NIV can increase the binding activity of the transcription nuclear factors NF- κ B and AP-1 via MAPK activation. We speculated that the reduced levels of IFN γ and IL-2 transcripts at 8 μ M are instead due to activation of specific inhibitory pathways. In fact, inhibition of the overall protein synthesis by NIV cannot be claimed to explain this result, as transcriptional up-regulation of IL-8 was reported at the very same NIV concentration. Experiments are in progress in our lab to better elucidate this aspect. Apparently, our results are in contrast with ELISA data describing no variation of IL-2 and IL-8 protein content in the supernatants of NIV-treated Jurkat cell cultures in comparison with that of untreated cells, at NIV concentration we reported to be inductive [18]. This divergence may well found explanation assuming differences in assay sensitivity (RT-PCR vs ELISA), as well as the consequence of a still undefined post-transcriptional NIV activity.

Conversely our data on the modulation activity of DON on IL-2 and IL-8 transcripts were in agreement with

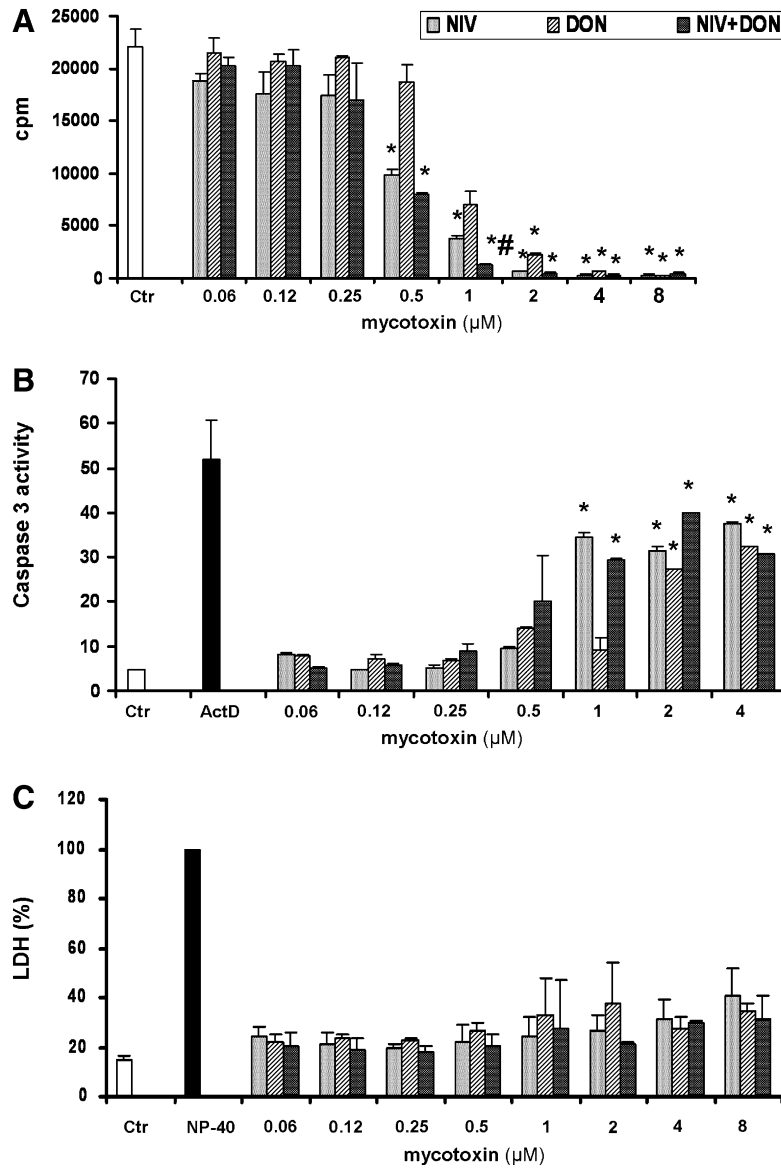


Fig. 4. Effects of NIV and DON on Jurkat cell vitality. Jurkat T cells were cultured for 24 h in the presence of increasing concentrations of NIV and DON, tested alone and in combination (1:1 ratio), at concentrations ranging from 0.06 to 8 μM. (A) DNA synthesis was measured by [³H]-thymidine incorporation and expressed as cpm. (B) For assessment of apoptosis induction, caspase-3 activity was analysed and values calculated as nmoles AMC/mg protein; cells treated with 10 μM actinomycin D were used as positive control. (C) Cytotoxicity of mycotoxins 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% NP-40). Each value represents the mean ± SD of three independent experiments. Ctr, control, cells incubated in the absence of mycotoxins. *different from non-treated cells. #Different from NIV- and DON-treated cells; $P < 0.05$.

ELISA and published results describing increased protein levels of the two cytokines in Jurkat cells [18]. Accordingly, we also found DON-mediated induction of IFN γ , but lower than that reported for NIV. Interestingly, DON and NIV induced a completely different effect on IFN γ and IL-2 transcripts at high concentration. In particular, we found that NIV was inhibitory while DON was inductive at 8 μM. The inductive effect was lost at concentration higher than 40 μM. If we consider that the two trichothecenes have a similar sesquiterpenoid structure, differing only for one more OH- group hold in position 7 by NIV, together our data indicate that this chemical difference plays a

role at 8 μM concentration, driving a well defined fine-tuning of the lymphocyte immune functions. In particular, the ability of DON to up-regulate IL-2 mRNA is in line with other results in murine T cells [26,27] and represent a very important clue, considering the central role of this cytokine in modulating T-cell development in physiological and inflammatory conditions. Moreover, DON-induced IL-2 overproduction may change the dynamics of proliferating lymphocytes with implications for development of autoimmune diseases [28,29]. As underlined above T-cell cytokine synthesis can be also modulated by post-transcriptional mechanisms such as stabilisation of cytokine mRNA

through activation of various kinase pathways [30]. This possibility has been suggested to explain superinduction of IL-2 gene expression by DON in murine EL-4 thymoma cells [27]. Accordingly the influence of NIV and DON on cytokine mRNA stability in Jurkat remains another point to be addressed.

An important concern to human health is represented by the consumption of plant products usually containing concomitantly different mycotoxins. Among the several combinations that frequently occur, NIV and DON are often mentioned [31]. So we used our *in vitro* system to analyse the effect of mycotoxin co-incubation on the cytokine transcription at 1:1 and 1:10 (NIV:DON) ratios. At lower concentration (0.06–4 μ M) our data suggested an interactive effect for IFN γ and IL-2, in agreement with our proliferation studies. Interestingly, at 8 μ M the effect of NIV prevailed, suggesting that NIV can be considered a competitive inhibitor of DON, acting on the same pathway for modulating the IFN γ and IL-2 mRNA expressions. This property of NIV was also maintained at a 1:10 ratio. Studies are in progress in our lab to identify this biochemical route. For IL-8 the mRNA profile following co-administration of NIV and DON at both examined ratios confirmed the inductive effect of these mycotoxins with no further enhancement as a consequence of co-administration.

Cell proliferation studies showed inhibition exerted by both mycotoxins. This finding is in close agreement with other studies [10,13] that identify trichothecenes as potent inhibitors of proliferation. This effect can be related to the capacity of the compounds to bind ribosomes and inhibit protein synthesis [15,32]. We evidenced that this inhibition can be exerted *in vitro* at very low concentrations (0.5 and 1 μ M for NIV and DON, respectively) consistent with the levels that can be found in the tissues of treated mice [33]. Interestingly, combination of NIV and DON produced effects in our lymphocyte proliferation test, suggesting interaction between the two mycotoxins, as co-administration further lowered cell growth, still in accordance with other studies [10,19]. We confirmed that inhibition of cell proliferation is essentially mediated by apoptosis, in agreement with previous studies of several trichothecenes [18,33,34] and suggestive of a cellular stress derived by the interaction with these mycotoxins. In particular, a prolonged activation of p38 was reported for DON [33] and it is known that longer activation of MAPK can lead to expression of genes critical for apoptosis induction [35].

In conclusion, the findings from this study highlight the potential immunomodulatory effects of NIV and DON at different concentrations on a model of human lymphocyte. The assessment of mRNA transcription showed different effects of NIV and DON in modulating Th1-type cytokines, depending on the dose whereas trichothecenes were found to interact and inhibit lymphocyte proliferation through apoptosis. Future work will be addressed to dissect the intracellular pathways involved to gain further information about the immunotoxicity of trichothecenes,

in the perspective to more accurately state safety levels for humans.

Acknowledgement

The work was supported by a grant from the Consiglio Nazionale delle Ricerche. Ph.D. fellowship of Dr. Diomira Luongo was supported by the University “Federico II” of Naples.

References

- [1] Schothorst RC, van Egmond HP. Report from SCOOP task 3.2.10 “Collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU member states”. Subtask: trichothecenes. *Toxicol Lett* 2004;153:133–43.
- [2] Grove JF. Non-macrocytic trichothecenes. *Nat Prod Rep* 1988;5: 187–209.
- [3] Pestka JJ, Yan D, King LE. Flow cytometric analysis of the effects of *in vitro* exposure to vomitoxin (deoxynivalenol) on apoptosis in murine T, B and IgA⁺ cells. *Food Chem Toxicol* 1994;32:1125–36.
- [4] Kidd MT, Hagler Jr WM, Qureshi MA. Trichothecene mycotoxins depress the mononuclear-phagocytic system of young turkeys. *Immunopharmacol Immunotoxicol* 1995;17:385–98.
- [5] Rotter BA, Prelusky DB, Pestka JJ. Toxicology of deoxynivalenol (vomitoxin). *J Toxicol Environ Health* 1996;48:1–34.
- [6] Li M, Cuff CF, Pestka J. Modulation of murine host response to enteric reovirus infection by the trichothecene deoxynivalenol. *Toxicol Sci* 2005;87:134–45.
- [7] Choi CY, Nakajima-Adachi H, Kaminogawa S, Sugita-Konishi Y. Nivalenol inhibits total and antigen-specific IgE production in mice. *Toxicol Appl Pharmacol* 2000;165:94–8.
- [8] Bondy GS, Pestka JJ. Immunomodulation by fungal toxins. *J Toxicol Environ Health B Crit Rev* 2000;3:109–43.
- [9] Forsell JH, Pestka JJ. Relation of 8-ketotrichothecene and zearalenone analog structure to inhibition of mitogen-induced human lymphocyte blastogenesis. *Appl Environ Microbiol* 1985;50:1304–7.
- [10] Thuvander A, Wikman C, Gadhasson I. *In vitro* exposure of human lymphocytes to trichothecenes: individual variation in sensitivity and effects of combined exposure on lymphocyte function. *Food Chem Toxicol* 1999;37:639–48.
- [11] Meko FA, Hardie LJ, Evans SW, Wild CP. Deoxynivalenol-induced immunomodulation of human lymphocyte proliferation and cytokine production. *Food Chem Toxicol* 2001;39:827–36.
- [12] Ouyang YL, Li S, Pestka JJ. Effects of vomitoxin (deoxynivalenol) on transcription factor NF-kappa B/Rel binding activity in murine EL-4 thymoma and primary CD4⁺ T cells. *Toxicol Appl Pharmacol* 1996;140:328–36.
- [13] Rocha O, Ansari K, Doohan FM. Effects of trichothecene mycotoxins on eukaryotic cells: a review. *Food Addit Contam* 2005;22: 369–78.
- [14] Shifrin VI, Anderson P. Trichothecene mycotoxins trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 mitogen-activated protein kinase and induces apoptosis. *J Biol Chem* 1999;274:13985–92.
- [15] Ueno Y. Toxicological features of T-2 toxin and related trichothecenes. *Fund Appl Toxicol* 1984;4:S124–32.
- [16] Corrier DE. Mycotoxicosis: mechanisms of immunosuppression. *Vet Immunol Immunopathol* 1991;30:73–87.
- [17] Iordanov MS, Pribnow D, Magun JL, Dinh TH, Pearson JA, Chen SL, et al. Ribotoxic stress response: activation of the stress-activated protein kinase JNK1 by inhibitors of the peptidyl transferase reaction and by sequence-specific RNA damage to the alpha-sarcin/ricin loop in the 28S rRNA. *Mol Cell Biol* 1997;17:3373–81.
- [18] Pestka JJ, Uzarski RL, Islam Z. Induction of apoptosis and cytokine production in the Jurkat human T cells by deoxynivalenol: role of

- mitogen-activated protein kinases and comparison to other 8-ketotrichothecenes. *Toxicology* 2005;206:207–19.
- [19] Tajima O, Schoen ED, Feron VJ, Groten JP. Statistically designed experiments in a tiered approach to screen mixtures of *Fusarium* mycotoxins for possible interactions. *Food Chem Toxicol* 2002;40:685–95.
- [20] Luongo D, Bergamo P, Rossi M. Effects of conjugated linoleic acid on growth and cytokine expression in Jurkat T cells. *Immunol Lett* 2003;90:195–201.
- [21] Hamalainen HK, Tubman JC, Vikman S, Kyrola T, Ylikoski E, Warrington JA, et al. Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. *Anal Biochem* 2001;299:63–70.
- [22] Tipton DA, Lyle B, Babich H, Dabbous MK. In vitro cytotoxic and anti-inflammatory effects of myrrh oil on human gingival fibroblasts and epithelial cells. *Toxicol in Vitro* 2003;17:301–10.
- [23] Luongo D, Severino L, Bergamo P, De Luna R, Lucisano A, Rossi M. Interactive effects of fumonisin B1 and α -zearalenol on proliferation and cytokine expression in Jurkat T cells. *Toxicology in vitro* 2006;20:1403–10.
- [24] Lepschy-v Gleissenthal J, Dietrich R, Martlbauer E, Schuster M, Suss A, Terplan G. A survey on the occurrence of *Fusarium* mycotoxins in Bavarian cereals from the 1987 harvest. *Z Lebensm Unters Forsch* 1989;188:521–6.
- [25] Astoul E, Edmunds C, Cantrell DA, Ward SG. PI 3-K and T-cell activation: limitations of T-leukemic cell lines as signaling models. *Trends Immunol* 2001;22:490–6.
- [26] Li S, Ouyang Y, Yang GH, Pestka JJ. Modulation of transcription factor AP-1 activity in murine EL-4 thymoma cells by vomitoxin (deoxynivalenol). *Toxicol Appl Pharmacol* 2000;163:17–25.
- [27] Li S, Ouyang YL, Dong W, Pestka JJ. Superinduction of IL-2 gene expression by vomitoxin (deoxynivalenol) involves increased mRNA stability. *Toxicol Appl Pharmacol* 1997;147:331–42.
- [28] Massarotti EM, Liu NY, Mier J, Atkins MB. Chronic inflammatory arthritis after treatment with high-dose interleukin-2 for malignancy. *Am J Med* 1992;92:693–7.
- [29] Richards JM, Mehta N, Ramming K, Skosey P. Sequential chemimmunotherapy in the treatment of metastatic melanoma. *J Clin Oncol* 1992;10:1338–43.
- [30] Chen CY, Del Gatto-Konczak F, Wu Z, Karin M. Stabilization of interleukin-2 mRNA by the c-Jun NH2-terminal kinase pathway. *Science* 1998;280:1945–9.
- [31] Eskola M, Parikka P, Rizzo A. Trichothecenes, ochratoxin A and zearalenone contamination and fusarium infection in Finnish cereal samples in 1998. *Food Addit Contam* 2001;18:707–18.
- [32] Azcona-Olivera JI, Ouyang YL, Warner RL, Linz JE, Pestka JJ. Effects of vomitoxin (deoxynivalenol) and cycloheximide on IL-2, 4, 5 and 6 secretion and mRNA levels in murine CD4+ cells. *Food Chem Toxicol* 1995;33:433–41.
- [33] Yang GH, Jarvis BB, Chung YJ, Pestka JJ. Apoptosis induction by the satratoxins and other trichothecene mycotoxins: relationship to ERK, p38 MAPK, and SAPK/JNK activation. *Toxicol Appl Pharmacol* 2000;164:149–60.
- [34] Nagase M, Alam MM, Tsushima A, Yoshizawa T, Sakato N. Apoptosis induction by T-2 toxin: activation of caspase-9, caspase-3, and DFF-40/CAD through cytosolic release of cytochrome c in HL-60 cells. *Biosci Biotechnol Biochem* 2001;65:1741–7.
- [35] Nasri T, Bosch RR, Voorde ST, Fink-Gremmels J. Differential induction of apoptosis by type A and B trichothecenes in Jurkat T-lymphocytes. *Toxicol In Vitro* 2006;20:832–40.