

Deoxynivalenol (DON) is toxic to human colonic, lung and monocytic cell lines, but does not increase the IgE response in a mouse model for allergy

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Abstract

We examined whether the common crop mycotoxin deoxynivalenol (DON) from *Fusarium* species is toxic to human colonic (Caco-2), lung (A549) and monocytic (U937) cell lines. Moreover, since DON reportedly induces increased levels of Th2 cytokines and total IgE, and we have observed that mould extracts adjuvated allergy development in mice, possible adjuvant effect of DON on allergy was studied in a mouse model. For all the cells, exposure to DON for 24 h reduced cellular protein synthesis, proliferation and survival rate dose-dependently. In addition, production of IL-8 in the U937 cell line increased up to eight-fold at levels of DON just lower than the most toxic one, suggesting that IL-8 can be used as an additional index for cytotoxicity in mononuclear phagocytes. However, DON did not increase levels of allergen-specific IgE or IgG1 in the mouse model for allergy. These results suggest that DON, when inhaled or ingested, may have toxic effect on human alveolar macrophages and epithelial cells in lungs and colon, but does not increase the allergic response to allergens.

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1. Introduction

Deoxynivalenol (DON), a.k.a. vomitoxin, is a mycotoxin mainly produced by the fungi *Fusarium culmorum* and *F. graminearum* while growing on crops

(Morooka et al., 1972; Miller et al., 1983). It is a trichothecene with relatively low toxicity. However, DON is of interest because it is one of the most common contaminants of wheat, corn and barley (Rotter et al., 1996 review) and only partly reduced during processing of grain (Young et al., 1984). Thus, DON is found in agricultural products like bread, beer and feed. Moreover, the mycotoxin may represent an environmental problem at work places where cereals are processed, like mills, bakeries and breweries.

Abbreviations: DON, deoxynivalenol; OVA, ovalbumin

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In animals, low ingestion of DON promotes anorexia, whereas higher doses induce vomiting (Rotter et al., 1996 review), hence the name vomitoxin. The cytotoxicity of DON is linked to its potent inhibition of protein, DNA and RNA synthesis (Rotter et al., 1996 review). Leukocytes and the immune system are a primary target for trichothecene mycotoxins like DON (Bondy and Pestka, 2000 review). In vitro, DON and related trichothecenes exhibit immunotoxic effects on B and T cells (Rotter et al., 1996 review) and NK cells (Berek et al., 2001), in addition to toxicity to Chinese hamster lung cells (Rogers and Heroux-Metcalf, 1983) and enterocytes (Kasuga et al., 1998). In vivo DON is found to induce development of autoimmune IgA nephropathy (Rotter et al., 1996 review). Exposure to DON modulates normal regulation of the IgA response in Peyer's patches (PP) by affecting T-cells and macrophages and elevating their cytokine production (Yan et al., 1998; Ouyang et al., 1996; Wong et al., 1998). DON increased IL-2, IL-4 and IL-5 levels in murine T cell cultures (Ouyang et al., 1996), and IL-1 β , IL-6 and TNF α secretion by murine macrophages (Wong et al., 1998). In vivo, DON fed mice express increased IL-1 β , IL-2, IL-6, IL-10, IL-12 and TNF α mRNAs in spleen and PP (Zhou et al., 1998). The result is an increase in IgA positive B-cells in PP and spleen cells and elevated levels of IgA in serum, mainly of the polyform (Bondy and Pestka, 1991; Petska et al., 1989), which is normally found on mucous membranes. DON may induce T cell-mediated switching to both IgA- and IgE-positive cells in PP (Petska and Dong, 1994). Moreover, ingestion of DON in mice is also found to induce increased levels of circulating total IgE (Petska and Dong, 1994).

DON is toxic to immune cells (B and T cells, NK cells, macrophages; Rotter et al., 1996 review; Berek et al., 2001) and immuno-competent cells like epithelial lung cells (Rogers and Heroux-Metcalf, 1983). Hence it is not surprising that DON induces a reduced resistance to *Listeria monocytogenes* infection, a facultative intra-cellular bacteria in macrophages (Tryphonas et al., 1986). These bacteria also most often result in airway infections, which, in the case with DON-affected patients, may be explained by toxicity of inhaled DON on both lung

epithelial cells and alveolar macrophages. Although DON is mostly ingested and toxic to enterocytes (Kasuga et al., 1998), there are no reports on a general increase in alimentary tract infections after DON ingestion. The reason may be a preventive effect of the DON-induced increase in IgA positive B-cells in PP (Bondy and Pestka, 1991). Depressed host resistance does also involve suppression of several other cellular functions (Rotter et al., 1996 review).

Recently, we have found that mould extracts increased the allergic response to the model allergen, ovalbumin (OVA), in mice (Instanes et al., in press). Since DON is a mould product and it also induces Th1 and Th2 type of cytokines as well as increased IgE production, we wanted to investigate possible adjuvant effect of DON on allergy in the very same mouse model that was used in the previous study with mould extracts. Moreover, since DON affects the alimentary tract when ingested and possibly lungs when inhaled, we examined the toxic effect of low doses of DON on a human colonic cell line (Caco-2), as well as on immuno-competent monocytic (U937) and lung (A549) cell lines.

2. Materials and methods

2.1. Cells

Human colorectal adenocarcinoma cell line Caco-2 (ATCC no. HTB-37), human lung adenocarcinoma A549 (ATCC no. CCL-185) and histiocytic lymphoma U937 cell lines (ATCC no. CRL-1593.2) were cultured at 37 °C in air with 5% CO₂, in RPMI 1640 (Gibco-BRL, Paisley, Scotland) with 10% fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 100 U/ml of penicillin (P) and 100 μ g/ml of streptomycin (S) (Gibco).

2.2. Animals

Female Balb/c mice (6–8 weeks old) were obtained from Gl. Bomholt gård Ltd., Ry, Denmark, and rested for 1 week after arrival. The mice were earmarked, housed eight per cage (one group) and given water and feed ad libitum. The experiments were approved by

the local representative for the National Animal Research Committee and were performed in accordance with standards published by the Norwegian Ministry of Agriculture.

2.3. Reagents

Deoxynovalenol (DON) was purchased from Sigma–Aldrich (cat. no. SD0156) and diluted in PBS with 10% (w/v) ethanol (stock) and further diluted in PBS and PBS with 10% Balb/c serum for in vitro and in vivo usage, respectively. Ovalbumin (Sigma–Aldrich; cat. no. A7641) was prepared as described (Ormstad *et al.*, 1998).

2.4. Assay for [^3H]leucine incorporation

Cells of similar age were seeded at 10^5 /well in 96-well cell culture plates (Nunc, Nuncleon, Roskilde, Denmark) in leucine-free RPMI (Sigma) with additives and incubated with or without DON (0–25 $\mu\text{g/ml}$) for 24 h, and then pulsed overnight with 1 $\mu\text{Ci/well}$ of [^3H]leucine (sp. act. 25–50 Ci/mM; Amersham Life Science, Amersham Place, Buckinghamshire, UK). Then the supernatants were removed, the cells lysed with 1 M NaOH, cellular protein trichloroacetic acid (TCA)-precipitated and its content of radioactivity recorded in a β -counter (Tri-Carb, Model 1500, Packard Instruments Co., Inc. Meriden, CT, USA) after addition of scintillation fluid. The results are presented as percentage of [^3H]leucine incorporation of control cells incubated with medium alone. In all in vitro experiments, wells incubated with 0.5% (w/v) of ethanol were used as controls for wells incubated with DON diluted in a similar end concentration of ethanol.

2.5. Assay for [^3H]thymidine incorporation

Cells were cultured in 96-wells plates (Nunc) in RPMI 1640 with additives and with or without DON for 24 h, whereafter 25 μl of [^3H]thymidine (1.25 $\mu\text{Ci/well}$, sp. act. 98 Ci/mg; Amersham) was incubated with each well overnight. A Scatron cell harvester (Scatron A/S, Lier, Norway) transferred the cells onto filter paper, which was washed and dried. The radioactivity on the filter, corresponding to each

culture well, was recorded in a β -counter. The results are presented as percentage of [^3H]thymidine incorporation of control cells incubated with medium alone.

2.6. Assay for survival of cells

Cell cultures were handled similarly as above, except for omitting the isotopes, stained for 15 min at 37 °C with trypan blue, washed and fixed in 2.5% glutaraldehyde. The percentage of live cells counted in a cross-section in each cell culture well, was determined by trypan blue exclusion using an inverse phase-contrast microscope.

2.7. IL-8 measurement

Human IL-8 levels in cell supernatants were determined by a sandwich-type ELISA obtained from R&D Systems (Barton Lane, Abingdon, Oxon, UK). The assay is based on a monoclonal anti-human IL-8 capturing antibody (cat. no. MAB 208) coated onto polystyrene microtiter wells (Immunulon II M 129B, Dynatech Laboratories Ltd., Chantilly, VA, USA) and a biotinylated, polyclonal anti-human IL-8 detection antibody (cat. no. BAF 208). The reaction was developed with Strept-ABComplex (cat. no. K0391; Dako AS, Glostrup, Denmark) and phosphate substrate in 10% diethanolamine buffer (Sigma). The absorbances were read on a Dynatech MR 7000 ELISA (Alexandria, VA).

2.8. Procedure for animal experiments

The procedure for the animal experiments is described in detail elsewhere (Ormstad *et al.*, 2000). In brief, groups of eight female Balb/c mice (7–9 weeks old) were injected with 0.08–10.0 μg of DON plus 10 μg of OVA, DON (2 μg) alone, medium or OVA alone into the right hind footpad. All groups were boosted with OVA on day 21. An additional group contained untreated animals. The experiments were terminated by exsanguination on day 26, and the popliteal lymph node (PLN) was taken out from both hind limbs. The PLN weight and cell numbers were determined (PLN assay, PLNA), as well as levels of serum IgE, IgG1 and IgG2a anti-OVA.

2.9. Assays for PLN and specific serum IgE, IgG1 and IgG2a anti-OVA

The PLN assay and the ELISA used for measurement of OVA-specific IgE have been described in detail previously (Ormstad et al., 1998). PBS with 10% Balb/c serum was used as medium. The IgG1 and IgG2a assays were similar to the one for IgE anti-OVA, except for coating with rat anti-mouse IgG1 (clone LO-MG1-13) and IgG2a antibody (clone LO-MG2a-7; Serotec Ltd., Oxford, UK), respectively, and using OVA-specific standard IgG1 or IgG2a serum (Ormstad et al., 2000). Results are expressed as arbitrary units (AU)/ml.

2.10. Statistical analysis

One-way analysis of variance (ANOVA) on ranks for multiple comparisons and Spearman's rank correlation coefficient ρ were used. Groups were compared with parametric *t*-test when normally distributed, oth-

erwise with nonparametric rank sum test; *P* values < 0.05 were considered significant.

3. Results

3.1. Effect of DON on allergic and PLN responses in mice

Possible adjuvant effect of DON on the production of specific serum IgE antibodies against the model allergen OVA was studied in Balb/c mice injected with OVA $\pm$ DON in the right hind footpad. However, there was no systemic IgE effect of DON (Fig. 1). The negative specific IgE results were confirmed by analysis of serum IgG1 anti-OVA antibodies (data not shown). Moreover, examination of weight and cell number in the harvested PLN, to measure the local cellular response, did not reveal any adjuvant effect of DON on OVA or of DON alone in this local inflammatory response (data not shown).

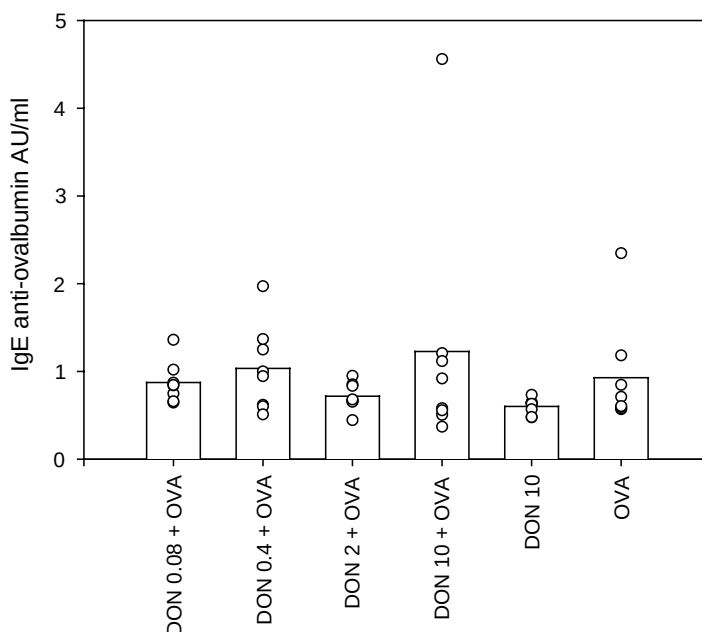


Fig. 1. Levels of OVA-specific IgE measured in mouse serum on day 26 after immunization. Mice were injected with DON + OVA, DON or OVA on day 0. Doses of DON were: 0.08, 0.4, 2 and 10 μ g given together with 10 μ g of OVA. Control mice were given 10 μ g of DON or OVA alone. On day 21 all mice were boosted with 10 μ g of OVA. Values are given in arbitrary units (AU) for each mouse (circle) and mean for groups of eight mice (columns).

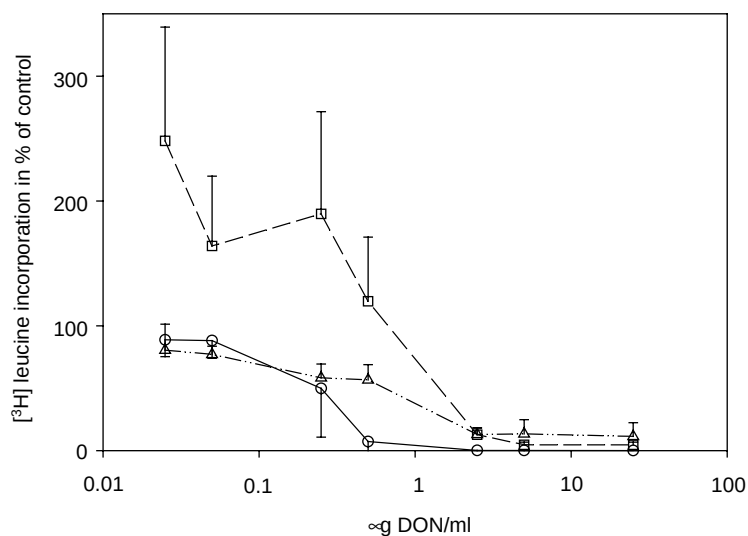


Fig. 2. Levels (% of untreated control) of [^3H]leucine incorporation in U937 (○), A549 (△) and Caco-2 (□) cell cultures incubated with 0.025–25.0 μg of DON for 24 h. Values (mean) for control cells incubated with medium were 438 477, 138 175 and 52 907 cpm for the respective cell lines. The data points represent mean values of three separate experiments each performed in triplicate and the vertical bars represent one standard error.

3.2. Effect of DON on cellular protein synthesis, proliferation and survival

We then examined the toxic effects of DON after 24 h on human A549 and U937, and Caco-2

cell lines, representative for cells from lung and intestines, respectively. For all cell lines, DON inhibited both the incorporation of [^3H]leucine (Fig. 2) and of [^3H]thymidine (Fig. 3) at concentrations as low as 2.5 $\mu\text{g}/\text{ml}$. In U937 cells the incorporation of

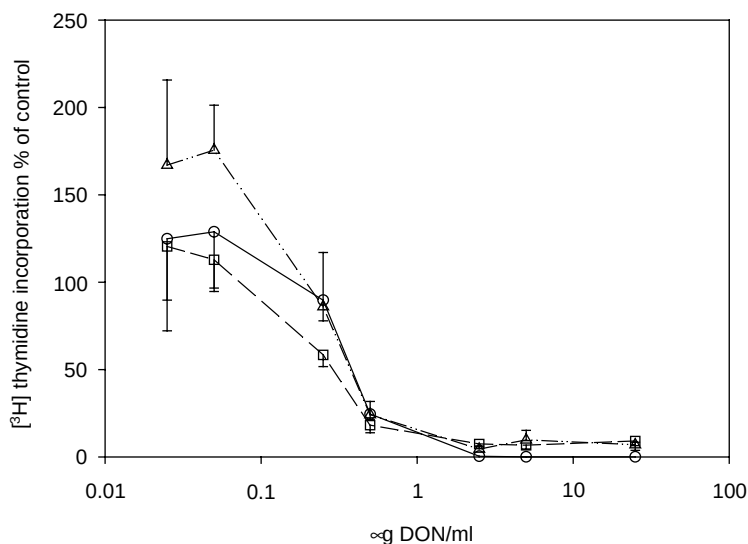


Fig. 3. Levels (percentage of untreated control) of [^3H]thymidine incorporation in U937 (○), A549 (△), and Caco-2 (□) cell cultures incubated with DON for 24 h. Values (mean) for control cells incubated with medium were 441 893, 11 984 and 2 873 cpm for the respective cell lines. The data points represent mean values $\pm$ S.E. (vertical bars) of three separate experiments performed in triplicate.

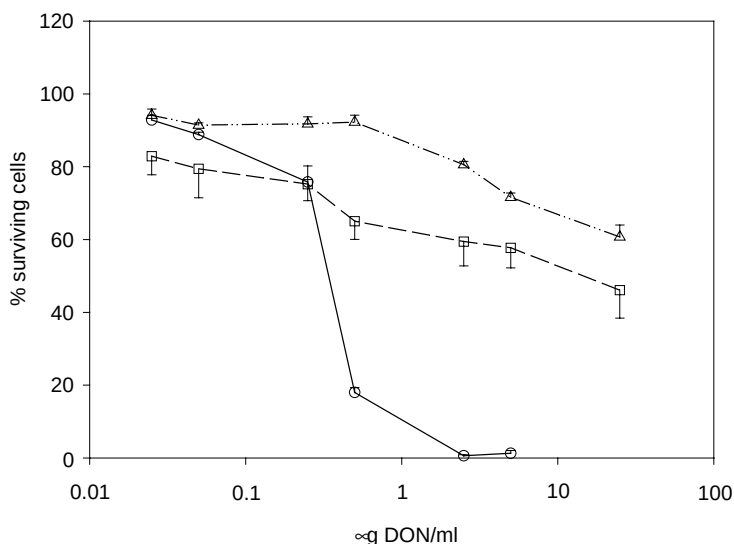


Fig. 4. Survival rate of U937 (○), A549 (△) and Caco-2 (□) cell cultures incubated with DON for 24 h. The values (mean survival rates) for control cells incubated with medium were 90.7, 89.5 and 89.1% for the respective cell lines. The data points represent mean values $\pm$ S.E. (vertical bars) of three separate experiments performed in triplicate.

[^{3} H]leucine was even inhibited at 0.5 μ g/ml (Fig. 2). DON reduced the survival rates of A549 and Caco-2 cells with approximately 30% and 40%, respectively, over a concentration of 25 μ g/ml (Fig. 4), and the toxin killed all U937 cells at 2.5 μ g/ml (Fig. 4).

There was a highly negative correlation between the levels of these three parameters for each of the cell types and the concentrations of DON (Spearman $\rho \leq -0.69$, $P \leq 0.04$). Moreover, except for correlations between proliferation and survival rates in A549 cells ($\rho = 0.51$, $P = 0.12$) and protein synthesis and survival of Caco-2 cells ($\rho = 0.61$, $P = 0.08$), there were highly positive and statistically significant inter-correlations ($\rho > 0.70$, $P \leq 0.03$) between levels of the parameters for each cell line.

3.3. Effect of DON on cellular IL-8 biosynthesis

The IL-8 concentration in cell supernatants was measured by ELISA. Whereas U937 and Caco-2 cells had a mean $\pm$ S.E. baseline IL-8 synthesis after 24 h incubation of 120 ± 21 pg/ml and 227 ± 124 pg/ml, respectively, A549 cells showed a 20- to 30-fold higher ($P \leq 0.03$) baseline IL-8 production (4.0 ± 1.2 μ g/ml). DON induced a peak in the IL-8 production of U937 cells (0.6 ± 0.2 μ g/ml) over a DON concentration of

0.25 μ g/ml (Fig. 5). Although the IL-8 peak values were not statistically significantly increased, they were 1.5- to 8-fold higher than corresponding values from unexposed cells. DON had less effect on the IL-8 production in A549 and Caco-2 cells (data not shown).

4. Discussion

Our results show that the mycotoxin DON is highly toxic to human monocytic cells and epithelial cells from the colon and lung, as demonstrated by strongly reduced cellular protein synthesis, proliferation and survival rate of model cells exposed to increasing doses of DON. In contrast, there was neither an adjuvant effect of DON on the systemic allergic response nor on the local lymph node reaction in the PLNA mouse model. DON (vomitoxin) induces vomiting when ingested, and the currently and previously (Kasuga et al., 1998) reported toxic effect of DON on cells (Caco-2) from the alimentary tract may contribute to nausea. Since DON may be inhaled as well, its potential toxic effects on epithelial cells in the lungs and on alveolar macrophages is yet another concern. It may be relevant for farmers or mill workers during harvest and processing of crops.

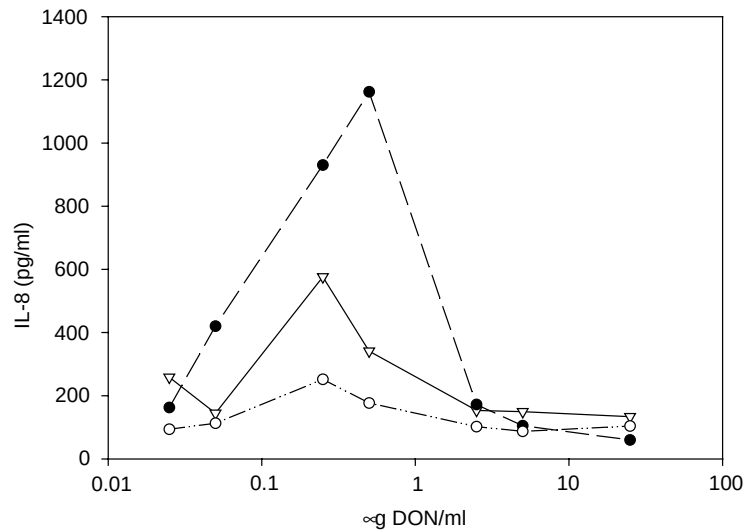


Fig. 5. Levels (pg/ml) of IL-8 in three individual experiments with U937 cells incubated with DON for 24 h. The values for control cells incubated with medium were 159 pg/ml (○), 84 pg/ml (▽) and 116 µg/ml (●). Each experiment was performed in duplicate.

This may also affect the handling of contaminated flour by bakers and brewers.

Judged by data on protein synthesis and cell survival, A549 and Caco-2 cells seemed to be less susceptible to DON compared with U937 cells. The high degree of inter-correlation between levels of the parameters for each cell line indicates that there is a close relationship between protein synthesis, proliferation and survival of these cancer cells. The lower correlation coefficients between survival rate and proliferation in A549 cells, and survival and protein synthesis in Caco-2 cells, are due to large variations in these parameters at lower DON concentrations. The current doses of DON used *in vitro* are similar to those employed previously by Rotter et al. (1996).

In contrast to the activity of DON in PP (Yan et al., 1998), there was no local inflammatory response in the draining PLN after DON injection into the corresponding hind footpad of the mice. In addition, there was no adjuvant effect of DON on the PLN parameters when injected together with OVA, which by itself gives a low inflammatory response in the PLN. Substances injected into the footpad will be taken up by Langerhans cells which phagocytose foreign material in the skin for processing and presentation to T cells in draining lymph nodes (PLN). In addition to Th1- and Th2-type of cytokines, DON is also reported to

increase both the pro-inflammatory $\text{TNF}\alpha$, IL-1 β and IL-8 (Zhou et al., 1998; Sugita-Konishi and Pestka, 2001) and anti-inflammatory (as well as Th2 type) IL-6 and IL-10 (Zhou et al., 1998; Sugita-Konishi and Pestka, 2001) cytokines. Hence, the overall outcome of DON stimulation in a local inflammatory reaction like the one in the PLN, was unpredictable. One could possibly have expected a toxic effect with diminished cell numbers and reduced PLN weight because DON induces apoptosis in lymphocytes (Petska et al., 1994). Hence, low doses of DON were administered to avoid toxic cell death in the footpad. The highest dose given in this experiment was similar to that of adjuvant substances from mould and fungi, which mostly had systemic effects in similar animal experiments (Ormstad et al., 2000). Nevertheless, the lack of systemic adjuvant effect of DON on IgE anti-OVA is surprising because DON has been shown to induce increase in total serum IgE and IL-4 and IL-5 cytokine (Th2-type) levels (Petska and Dong, 1994; Azcona-Olivera et al., 1995). On the other hand, DON has also been found to induce IL-2 and IL-12 (Meky et al., 2001; Zhou et al., 1998) which favor a Th1-response and depress a Th2-response according to the Th1/Th2 paradigm. Furthermore, in contrast with administration by the respiratory route which mainly favors a Th2-dependent response, s.c. administration

of antigen in mice used in the present paper, promotes a Th-1 dependent response (Constant et al., 2000). In order to get a final answer, it is pertinent to examine the adjuvant effect after administration of the mycotoxin via the airways and the alimentary tract. The immunological effect of DON may be confined to mucosal membranes only and it would have been of interest to examine possible adjuvanticity of DON in food allergy.

The monocytic U937 cells were used as a model for human alveolar macrophages. It has been shown that induction of apoptosis in U937 cells increases their IL-8 production (Muhl et al., 1999). Hence, we propose that IL-8 can be used as an additional variable of cytotoxicity. DON is also found to upregulate IL-8 production in human macrophages (Sugita-Konishi and Pestka, 2001). Since DON according to the cellular tests used was cytotoxic, we examined the synthesis of the IL-8 cytokine by the cell lines in response to DON. The IL-8 production in U937 cells peaked at the very same DON concentration (0.25 µg/ml) that inhibited 50% of the cells' protein synthesis. This does not necessarily represent a controversy because a substance may inhibit the general protein synthesis without reducing that of a specific protein like IL-8. Moreover, approximately 80% of the cells were still alive and presumably able to respond to a stimulus. Recently, DON has been shown to induce IL-8 production in differentiated U937 cells in higher concentrations than reported here with unstimulated U937 cells, and the IL-8 production also occurred at cytotoxic concentrations of DON (Sugita-Konishi and Pestka, 2001). Although the current rise in IL-8 was not statistically significant due to a large variance, the peak consists of up to eight-fold increased values and is most likely due to de novo IL-8 synthesis, pointing towards apoptosis as the subsequent mechanism of cell death. The increased production of IL-8 in the U937 cells due to toxins presumably occurs in order to attract PMN to the site for ingestion of injured and dying cells.

In conclusion, we observed a dose-dependent reduction in cellular protein synthesis, proliferation and survival rate when human cancer cell lines isolated from blood, lung and colon were exposed to DON. Moreover, the IL-8 production in U937 cells peaked just prior to the maximal toxic DON level, indicating the applicability of IL-8 as an additional index of cytotoxicity. Our results suggest that DON, when inhaled

or ingested, may have toxic effect on human alveolar macrophages and epithelial cells in lungs and colon. Although DON induces increased total IgE (Pestka and Dong, 1994), it does not seem to affect the allergic response.

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