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Methodical aspects of *in vitro* proliferation of porcine blood lymphocytes when exposed to deoxynivalenol (DON)

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Abstract

Trichothecene mycotoxins such as deoxynivalenol (DON) are known to modulate the immune function, but to this date only few studies have been conducted with farm animals, especially pigs, which are regarded as most susceptible to this mycotoxin.

Mitogen-stimulated proliferation of porcine blood lymphocytes was measured using two different assays, the MTT cleavage (metabolic activity) and the BrdU incorporation (DNA-synthesis). Furthermore, immunoglobulin (IgA, IgG and IgM) concentrations of culture supernatants were determined using an indirect ELISA. Various conditions were investigated before establishment of the appropriate method: the grade of stimulation of the T-cell mitogens concanavalin A (ConA) and phythaemagglutinin (PHA) were compared to the B-cell mitogen lipopolysaccharide (LPS) from the cell wall of *E. coli*. Furthermore, the effect of input of 1 x 10^5, 2 x 10^5, and 4 x 10^5 lymphocytes per well was studied in the view of sensitivity to the mycotoxin DON. In addition, the effects of DON dose (0, 70, 140, 280, and 560 ng/ml) were tested in lymphocytes of one particular pig, which were either frozen or freshly processed.

ConA appeared to be the most potent mitogen tested and was applied at 2.5 µg/ml in lymphocyte cultures. 1 x 10^5 cells/well seemed to be more sensitive to DON compared to 2 x 10^5 and 4 x 10^5 cells/well, as the inhibiting concentrations of 50 % (IC_{50}) increased with the cell number from 249, 325 to 337 ng DON/ml, respectively. Addition of 70 to 560 ng DON/ml to ConA-stimulated lymphocytes dose-dependently inhibited the metabolic activity (MTT assay) and DNA-synthesis (BrdU assay). No obvious difference in the toxic action of DON was observed when lymphocytes were frozen or freshly processed since the IC_{50} values were comparable (286 versus 309 ng DON/ml for the MTT and 201 versus 200 ng DON/ml for the BrdU assay). Supernatant Ig concentrations also showed a dose-dependent reduction with IC_{50} values of 108 or 121 ng DON/ml for IgA, 84 or 86 ng DON/ml for IgM, and none or 72 ng DON/ml for IgG when lymphocytes were frozen or freshly processed, respectively.

Keywords: deoxynivalenol, immunoglobulin, in vitro, lymphocyte proliferation, pig

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Zusammenfassung

Methodische Aspekte der *in vitro*-Proliferation von porcinen Blut-Lymphozyten bei Deoxynivalenol (DON)-Exposition

Von den Trichothecene-Schimmelpilzgiften wie Deoxynivalenol (DON) ist bekannt, dass sie die Funktion des Immunsystems modulieren, aber bis heute wurden nur wenige Studien an landwirtschaftlichen Nutztieren, insbesondere Schweinen, die als höchst empfindlich gegenüber diesem Mykotoxin gelten, durchgeführt.

Die Mitogen-stimulierte Proliferation von porcinen Blut-Lymphozyten wurde mittels zweier unterschiedlicher Assays, dem MTT-Abbau (metabolische Aktivität) und dem BrdU-Einbau (DNA-Synthese), gemessen. Weiterhin wurden die Immunglobulin-Konzentrationen im Kultur-Überstand mittels eines indirekten ELISA´s bestimmt. Unterschiedliche Bedingungen wurden zur Etablierung einer geeigneten Methode geprüft: Der Grad der Stimulierung durch die T-Zell-Mitogene Concanavalin A (ConA) und Phythämagglutinin (PHA) wurde mit dem des B-Zell-Mitogens Lipopolysaccharide (LPS) aus der Zellwand von *E. coli* verglichen. Außerdem wurde die Aus-wirkung der Einsaat von 1 x 10^5, 2 x 10^5 und 4 x 10^5 Lymphozyten pro well im Hinblick auf die Sensitivität für das Mykotoxin DON untersucht. Zusätzlich wurde der Effekt einer DON-Applikation (0, 70, 140, 280 und 560 ng/ml) auf die Lymphozyten eines Schweins, die entweder frisch oder eingefroren eingesetzt wurden, getestet.

ConA erschien als das potenteste der untersuchten Mitogene und wurde zu 2.5 µg/ml in die Lymphozyten-Kultur gegeben. Im Vergleich zu 2 x 10^5 und 4 x 10^5 Zellen/well schienen 1 x 10^5 Zellen/well sensitiver gegenüber DON zu sein, da die inhibitorische Konzentration von 50 % (IC_{50}) mit steigender Zellzahl von 249, 325 zu 337 ng DON/ml anstieg. Eine Zugabe von 70 bis 560 ng DON/ml zu ConA-stimulierten Lymphozyten hemmten die metabolische Aktivität (MTT assay) und DNA-Synthese (BrdU assay) dosisabhängig. Dabei gab es keinen sichtbaren Unterschied in der toxischen Wirkung von DON, wenn die Lymphozyten eingefroren oder frisch eingesetzt wurden, da die IC_{50}-Werte vergleichbar waren (286 versus 309 ng DON/ml im MTT und 201 versus 200 ng DON/ml im BrdU assay). Die Ig-Konzentrationen im Überstand waren ebenso dosisabhängig reduziert, mit IC_{50}-Werten von 108 oder 121 ng DON/ml für IgA, 84 oder 86 ng DON/ml für IgM und keinem oder 72 ng DON/ml für IgG, wenn die Lymphozyten eingefroren oder frisch eingesetzt wurden.

Schlüsselworte: Deoxynivalenol, Immunglobulin, in vitro, Lymphozyten-Proliferation, Schwein

1 Introduction

In northern temperate regions, the *Fusarium* toxin deoxynivalenol (DON) is a frequent contaminant of cereal grains occurring in toxicologically relevant concentrations (Bottalico et al., 2002; Logrieco et al., 2002; Placinta et al., 1999). Trichothecenes, such as DON, interfere with protein synthesis at the cellular level, and will therefore predominantly damage quickly proliferating cells as found in the immune system (Rotter et al., 1996). The mitogen-stimulated proliferation of lymphocytes is a common technique to assess immunotoxicity of toxic agents (Rotter et al., 1996). However, the adverse effects of DON on primary lymphocyte cultures of pigs, as the most sensitive species, have only been rarely examined (Goyarts et al., 2006). Furthermore, mycotoxin effects on various cell cultures were determined using different assays, measuring the DNA-synthesis ([^{3}H]-thymidine or BrdU incorporation), metabolic activity (MTT cleavage), integrity of cell membranes (LDH release) and cell death (trypan blue uptake) (Charoenpornsook et al., 1998; Widestrand et al., 1999). On account of this, the applicability and sensitivity of the MTT and BrdU assay were compared from the perspective of DON effects on porcine blood lymphocytes. The yellow tetrazolium salt (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) is metabolized by NAD-dependent dehydrogenase (in active mitochondria) to form a dark blue formazan product (metabolic activity, Mosmann 1983), while BrdU (5-Bromo-2`-deoxyuridine) is incorporated into the DNA of proliferating cells instead of thymidine (DNA-synthesis, Widestrand et al, 1999). In addition, immunoglobulin (IgA, IgG and IgM) concentrations of the culture supernatant were determined as a further parameter to measure the activity of immune cells (Goyarts et al, 2006). Moreover, DON was shown to alter Ig concentrations *in vitro* and *in vivo* (Bondy et al, 2000; Rotter et al, 1996; Pestka 2003).

The aim of this study was to establish the optimum test conditions for a proliferative response of porcine peripheral blood lymphocytes (PBL) in order to investigate the effects of the mycotoxin DON on immune cells *in vitro*.

2 Material and Methods

2.1 Lymphocyte culture

If not further noted, all chemicals were purchased from Sigma (Deisenhofen, Germany).

The preparation of peripheral blood lymphocytes (PBL) was described by Goyarts et al (2006). In brief, 20 ml of diluted, heparinized blood were layered onto 10 ml of a Ficoll (F-4375) density gradient and centrifuged at 400 x g for 15 min to separate PBL. The “buffy coat”, containing more than 95 % lymphocytes, was washed two times (centrifugation 250 x g for 8 min) in RPMI 1640 medium (Sigma R-8758) supplemented with 1 M HEPES buffer (H-3784), 2 mM L-glutamine (G-6392), 5 mM mercaptoethanol (M-7522), 100 U/ml Penicillin G, 0.1 mg/ml Streptomycin, 0.25 µg/ml Amphotericin B (ABAM, A-7292) and 5 % heat inactivated foetal calf serum (FCS, Biochrom AG seromed®, Berlin, Germany).

Cells were counted using an improved Neubauer counting chamber (Roth, Karlsruhe, Germany) and Türk´s solution (VWR, Darmstadt, Germany), while cell viability was evaluated by the trypan blue (Sigma) exclusion technique and was always found to be greater than 95 %. Isolated lymphocytes were adjusted to a final concentration of 1 x 10^6 viable cells/ml and 100 µl of cell suspension was pipetted in quadruplicate into 96-well micro titer plates (MTP, Nunc A/S, Roskilde, Denmark, Cat.No. 167008). PBL of pigs fed a DON free diet were used for the *in vitro* studies.

A final solution volume of 200 µl per well was achieved after adding mitogens and toxins in 50 µl each or the remaining medium. Cell cultures were incubated at 37 °C in a humidified incubator at 5 % CO_2 for 72 h. After centrifugation and collection of 100 µl supernatant for immunoglobulin determination, 10 µl of BrdU or MTT was added and incubated for another 4 h. A BrdU proliferation kit (Roche Diagnostic GmbH, Mannheim, Germany, Cat. No. 1647229) was used according to the manufacturer’s instructions and read with a microplate photometer (Powerwave, Bio-Tek Instruments GmbH, Bad Friedrichshall, Germany) at a test wavelength of 450 nm and a reference wavelength of 690 nm. The optical density of MTT assay was measured by an ELISA reader at 570 nm after dissolving the crystalline formazan product with 100 µl of 0.01 N HCl/SDS-solution overnight at room temperature.

2.2 Mitogens

To find a mitogen most suitable to stimulate porcine PBL, the T-cell mitogens concanavalin A (ConA: 1.25, 2.5, 5, 10 µg/ml) (C-5275) and phythaemagglutinin (PHA: 1, 2, 4, 8 and 20 µg/ml) (L-9132) were compared with the B-cell mitogen lipopolysaccharide (LPS from *E. coli* O26:B6, L-2654: 5, 10, 20, 40 µg/ml) in the MTT assay.

Furthermore, the extent of proliferation stimulation was studied with 1.25, 2.5, 5, 10 and 20 µg/ml of the T-cell-mitogen ConA in the presence or absence of 200 ng/ml (0.68 µM) DON (D-0156) with the MTT (metabolic activity) and BrdU (DNA-synthesis) assay, respectively. In addition, supernatant immunoglobulin concentrations (IgA, IgG and IgM) were determined using an indirect ELISA as described by Goyarts et al. (2006) and Tiemann et al. (2006).

2.3 Cell number

Various numbers of lymphocytes (1 x 10^5, 2 x 10^5, and 4 x 10^5 cells/well) were tested with different concentrations of DON (70, 140, 280, 560 ng/ml [0.24, 0.47, 0.94, 1.88 µM]) to evaluate the proliferation rate after ConA-stimulation by the MTT assay.

2.4 DON exposure and cell status

For technical reasons, e.g., for repeated measurements at different time points with the identical PBL, it was of interest whether porcine lymphocytes are also suitable for the proliferation assay if they are frozen once. Therefore, lymphocytes of one particular control pig were used immediately after separation (fresh) or after freezing and defrosting (frozen). In brief, lymphocytes, supposed to be frozen, were separated, as mentioned above, and the pellet was dissolved in 2 ml ice-cold FCS. One ml of this cell suspension was given into a cryotube containing 800 µl FCS, and 200 µl DMSO (D-2438) were added. Shortly thereafter, the cryotubes were wrapped in cotton wool and frozen in a styrofoam box at -80 °C. On the day of the proliferation test, one cryotube was defrosted at 37 °C in a water bath until only an ice nucleus was seen and the content was transferred in 10 ml supplemented RPMI medium (37 °C) and centrifuged at 250 x g for 8 min. Thereafter the pellet was dissolved in 10 ml supplemented medium for cell counting and a trypan blue exclusion test and treated like the fresh lymphocytes.

The effect of 0, 70, 140, 280 and 560 ng/ml DON was examined in lymphocytes isolated fresh or after the freezing procedure and stimulated with 2.5 µg/ml ConA, which was found to be best suited for stimulation. The proliferation was measured by the MTT and BrdU assay. Additionally, the supernatant IgA, IgG and IgM concentrations of those treated lymphocyte cultures were determined by an indirect ELISA according to Goyarts et al (2006) and Tiemann et al. (2006).

2.5 Calculations and statistics

Optical density of blank wells (medium without cells) was subtracted from the measured extinction values of the cell cultures and mean values and standard deviation of quadruplicates were compared with the corresponding controls.

The stimulation index (SI) was calculated: SI = absorbance of mitogen-stimulated lymphocytes/absorbance of non-stimulated lymphocytes.

The dose-response curves were fitted to a non-linear regression equation and inhibiting concentrations of 50 % (IC_{50}) were calculated according to Mercer et al. (1987, modified):

$$(1) \qquad \%\ \text{of inhibition} = \frac{R_{max} \cdot DON^x}{(K_{0.5})^x + DON^x}$$

$$(2) \qquad IC_{50} = \left(\frac{50 \cdot (K_{0.5})^x}{(R_{max} - 50)} \right)^{\left(\frac{1}{x}\right)}$$

where DON = concentration of DON, R_{max} = maximum theoretical inhibition, $K_{0.5}$ = time at 0.5 · R_{max}, x = apparent kinetic order. The inhibiting concentration of 50 % (IC_{50}) was derived from (2). The non-linear curve fitting module of the Statistica for the Windows™ operating system (StatSoft Inc. 1994) was used to fit the data to equation (1).

Significant mean values differences were evaluated by the Tukey test ($P < 0.05$). All statistics were carried out using the Statistica for Windows™ operating system (StatSoft Inc. 1994).

3 Results

3.1 Mitogens

In Figure 1 the absorbance of lymphocyte culture in the MTT assay was compared to the non-stimulated control (SI = 1). ConA was most potent in stimulating porcine PBL, followed by higher LPS (20 - 40 µg/ml) concentrations, whereas PHA addition even showed a decline of absorbance (Figure 1).

Testing of various ConA concentrations (1.25, 2.5, 5, 10, and 20 µg/ml) in the MTT and BrdU assay revealed that highest stimulation of porcine PBL occurred within a range of 1.25 to 5.0 µg ConA/ml, whereas higher ConA

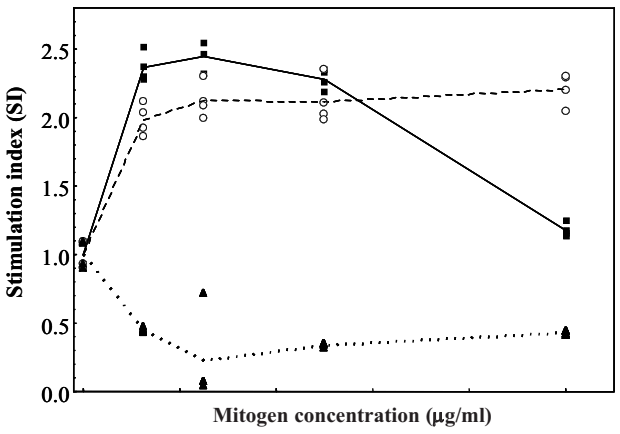

Figure 1:
Dose-dependent effects of the mitogens concanavalin A(■ ConA: 1.25, 2.5, 5, and 10 µg/ml), phythaemagglutinin (▲ PHA: 1, 2, 4, and 8 µg/ml) and lipopolysaccharide (○ LPS: 5, 10, 20, and 40 µg/ml) on the stimulation index (SI) of *in vitro* proliferation of porcine blood lymphocytes in the MTT assay (570 nm); n = 4 for each mitogen concentration.

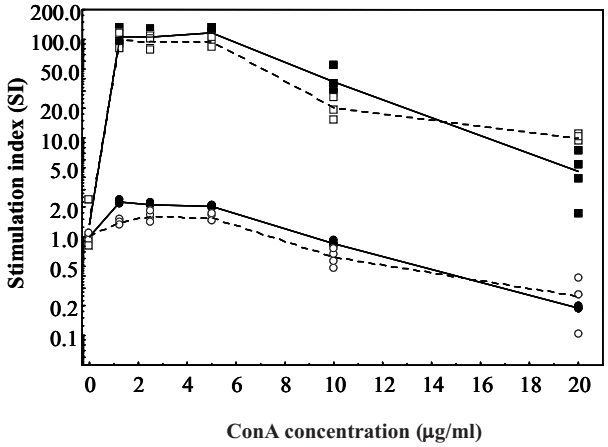

Figure 2:
Effects of increasing ConA concentrations (0, 1.25, 2.5, 5, 10, 20 µg/ml) in the absence (● MTT, ■ BrdU assay) or presence (○ MTT, □ BrdU assay) of 200 ng DON/ml on the stimulation index (SI) of *in vitro* proliferation of porcine blood lymphocytes in the MTT (570 nm) and BrdU (450-690 nm) assay; n = 4 for each ConA concentration.

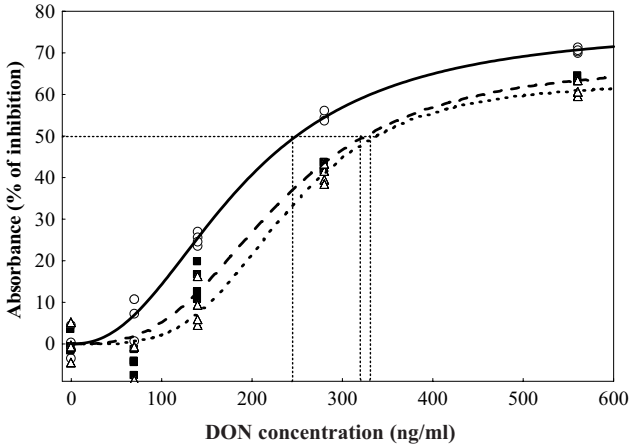

Figure 4:
Inhibiting effect of DON (0, 70, 140, 280, 560 ng/ml) after 76 h incubation with increasing cell counts (1 x 10^5 [r^2=0.99]; 2 x 10^5 [r^2=0.98]; 4 x 10^5 [r^2=0.95]) on *in vitro* proliferation of porcine blood lymphocytes in the MTT assay (570 nm); ····· IC_{50}, inhibiting concentration of 50 %; n = 4 for each DON concentration.

concentrations resulted in a decrease of absorbance compared to nonstimulated cells (Figure 2). Therefore, further tests were conducted with 2.5 µg ConA/ml. The stimulation index (SI) of lymphocytes stimulated with ConA was about two- or hundredfold of the absorbance of non-stimulated cells in the MTT and BrdU assay, respectively (Figure 2). The absorbance of the MTT and BrdU assay were decreased to 63, 76, 77, 72, 132 % and 92, 90, 81, 54, 217 % when 200 ng/ml DON were added to lymphocyte cultures after stimulation with ConA concentrations of 1.25, 2.5, 5, 10 and 20 µg/ml (Figure 2).

Immunoglobulin concentrations of culture supernatant incubated for 72 h with increasing ConA concentrations showed an equal pattern of stimulation between 1.25 and 5 µg ConA/ml, followed by a decrease in Ig concentrations at higher ConA concentrations (Figure 3). IgG concentrations increased only slightly by 1.1-fold and supernatant IgA levels by 1.4- to 1.6-fold, whereas IgM was elevated more than 5-fold (Figure 3). Following incubation with 200 ng DON/ml the immunoglobulin concentration was 35, 33, 30, 45 and 81 % for IgA, 12, 12, 17, 33 and 104 % for IgM, and 82, 68, 71, 82, 117 % for IgG compared to the respective ConA-stimulated control (1.25, 2.5, 5, 10 and 20 µg ConA/ml; Figure 3).

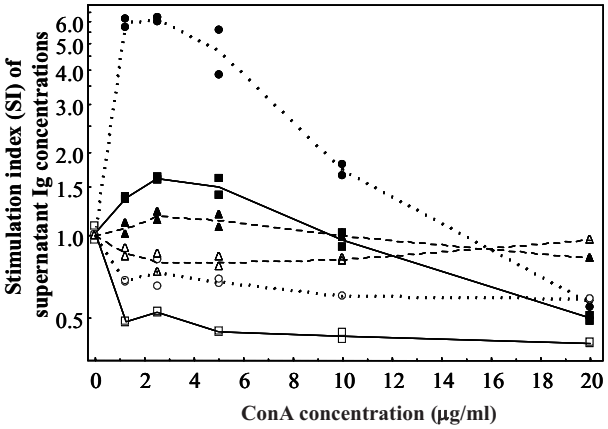

Figure 3:
Effects of increasing ConA concentrations (0, 1.25, 2.5, 5, 10, 20 µg/ml) on the stimulation index of supernatant immunoglobulin concentrations after 72 h incubation *in vitro* in the absence (■ IgA, ▲ IgG, ● IgM) or presence (□ IgA, △ IgG, ○ IgM) of 200 ng DON/ml; n = 2 for each ConA concentration.

3.2 Cell number

Absorbance of ConA-stimulated lymphocyte cultures was 1.5 and 2.5 times higher when 2 x 10^5 and 4 x 10^5 cells were used instead of 1 x 10^5 cells (data not shown). But ConA-stimulated lymphocyte cultures with 1 x 10^5 cells showed a more pronounced dose-response decrease after adding increasing DON concentrations (0, 70, 140, 280, 560 ng/ml) than their counterparts with 2 x 10^5 and 4 x 10^5 cells (Figure 4). The IC_{50} values were 249, 325 and 337 ng DON/ml for the 1 x 10^5, 2 x 10^5 and 4 x 10^5 cells/well, respectively, indicating a higher sensitivity when 1 x 10^5 cells/well were used. For that reason, further tests were carried out with a cell count of 1 x 10^5 lymphocytes/well.

3.3 DON exposure and cell status

Absorbance of freshly processed and frozen lymphocytes were not significantly different, but ConA-stimulated frozen lymphocytes showed a reduction of 13 and 9 % in comparison to the ConA-stimulated fresh lymphocytes in the BrdU and MTT assay, respectively (data not shown). Data of freshly processed lymphocytes were obtained from Goyarts et al (2006) (Figures 5 and 6). In freshly processed lymphocytes, the percentage of

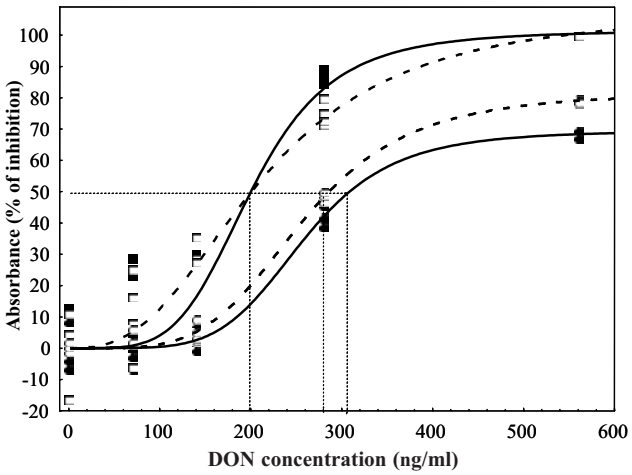

Figure 5:
Inhibiting effect of DON (70, 140, 280, 560 ng/ml) after incubation of 76 h on *in vitro* proliferation of ConA-stimulated porcine blood lymphocytes (fresh and frozen) in the MTT (570 nm; fresh [r^2=0.98]; frozen [r^2=0.99]) and BrdU (450-690 nm; fresh [r^2=0.92]; frozen [r^2=0.94]) assay; ····· IC_{50}, inhibiting concentration of 50 %; n = 4 for each DON concentration; values of freshly processed lymphocytes according to Goyarts et al (2006).

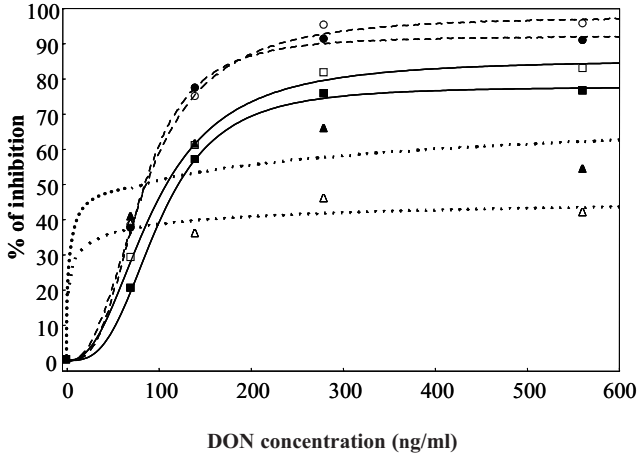

Figure 6:
Inhibiting effects of DON (0, 70, 140, 280, 560 ng/ml) on IgA (□ frozen, ■ fresh), IgG (△ frozen, ▲ fresh) and IgM (○ frozen, ● fresh) concentrations of the supernatants after 72 h incubation of ConA-stimulated porcine blood lymphocytes *in vitro* (n = 2); values of freshly processed lymphocytes according to Goyarts et al (2006).

absorbance compared to the ConA-stimulated control (= 100 %) decreased dose-dependently in the MTT (106, 97, 58, 31 %) and BrdU assay (87, 85, 16, 0 %) after 76 h incubation with 70, 140, 280 and 560 ng DON/ml (Figure 5). Accordingly, a dose-dependent decrease of MTT and BrdU values was detected in ConA-stimulated lymphocytes after a freezing procedure (Figure 5). The absorbance of frozen lymphocytes declined with increasing DON concentrations to 97, 95, 52, and 21 % or 89, 77, 25, and 0 % of the ConA-stimulated control (= 100 %) in the MTT or BrdU assay, respectively (Figure 5). In fresh and frozen processed lymphocyte cultures, the inhibition was significant ≥ 280 ng DON/ml in the MTT and BrdU assay. The stimulation index (SI) after addition of 2.5 µg ConA/ml was 2.2 or 2.7 in the MTT and 212 or 153 in the BrdU assay for both fresh and frozen lymphocytes. DON concentrations of 309 and 286 ng/ml resulted in a 50 % reduction of optical density (IC_{50}) in the MTT assay, while IC_{50} values were 200 and 201 ng/ml in the BrdU assay for fresh and frozen lymphocytes, respectively (Figure 5).

In accordance to the results of proliferation, immunoglobulin levels of lymphocyte culture supernatants showed the same pattern of inhibition, when lymphocytes were frozen or freshly processed (Figure 6). In frozen lymphocytes, after addition of 70, 140, 280 and 560 ng DON/ml immunoglobulin supernatant concentrations decreased by 29, 61, 82 and 83 % for IgA, by 38, 75, 95 and 95 % for IgM, but only by 39, 36, 46 and 42 % for IgG. Therefore, IC_{50} could not be calculated for supernatant IgG. The IC_{50} values were 108 and 84 ng DON/ml for IgA and IgM, respectively, which is in accordance with the IC_{50} obtained from freshly processed lymphocytes (121 and 86 ng DON/ml for IgA and IgM; Goyarts et al, 2006).

4 Discussion

4.1 MTT and BrdU assay

Measurement of DNA-synthesis is closely related to cell proliferation, because the radio-labeled [^{3}H]-thymidine or the non-radioactive BrdU are incorporated into the replicating DNA during the S phase of the cell cycle and measurement of the incorporation is consequently directly proportional to the number of actively dividing cells (Widestrand et al, 1999). As DNA-synthesis can only be measured in reproductively dividing populations, the proliferation of lymphocyte cultures was stimulated by mitogens. Although the immunochemical BrdU method is faster and easier to use and not as time consuming than the traditionally used radioactive [^{3}H]-thymidine incorporation assay, it still includes washing and labeling steps and shows a higher variation than the MTT assay (Figure 5). Since Mosmann (1983) demonstrated that the tetrazolium salt MTT is formed to a dark blue formazan only by living, metabolically active cells, and that this coloration is directly proportional to the number of such cells, it is widely used for determination of cell viability and proliferation because of its convenience and rapidity. However, it has to be kept in mind that the MTT cleavage is only an unspecific measurement of cell proliferation, and under non-ideal cell culture conditions (such as pH and D-glucose concentrations in culture medium), the MTT response may vary greatly in viable cells due to the metabolic state of the cells (e.g., cellular concentration of pyridine nucleotides) (Vistica 1991).

In the present study, inhibiting DON concentrations (IC_{50}) in lymphocytes after fresh and frozen procedure were lower in the BrdU assay (200 and 201 ng DON/ml) compared to the MTT cleavage (309 and 286 ng DON/ml; Figure 5), indicating a higher sensitivity of the DNA-synthesis measurement *in vitro*. This is in accordance with Charoenpornsook et al (1998), who tested the mycotoxin damage (T-2, DON, FB_1 and OTA) on bovine PBL and observed a sensitivity in decreasing order [^{3}H]-thymidine > LDH > MTT > trypan blue uptake. In the same way, exposure of 3T3 mouse fibroblasts to the mycotoxins T-2, HT-2, DON and NIV showed a higher sensitivity of the BrdU assay compared to the MTT and LDH assay (Widestrand et al, 1999). Furthermore, Widestrand et al (1999) suggested a cytostatic rather than cytotoxic effect of the type B trichothecenes (DON and NIV), because the cells maintained a high metabolic activity (MTT assay) at concentrations causing inhibition of DNA-synthesis (BrdU assay). Moreover, Kondo et al (2003) reported that optimal concentrations of mitogens in the MTT assay were two- to four-fold higher (1.25-2.5 mg PHA/ml, 50-100 µg ConA/ml) than those in the [^{3}H]-thymidine uptake and trypan blue exclusion assay and concluded that this may reflect a lower sensitivity of mitochondrial DNA to mitogens compared with that of nuclear DNA.

4.2 Mitogens

Mitogens are substances derived mostly from plant and bacterial extracts that promote mitosis in a non-specific manner. They have been widely used in studying lymphocyte proliferation and have individual stimulatory effects on lymphocytes, e.g., ConA and PHA stimulate T-lymphocytes, and LPS stimulates predominately B-lymphocytes, while PWM (pokeweed mitogen) stimulates both, B-cells and T-cells (Charoenpornsook et al, 1998).

In the present study, the stimulation index (SI) after addition of 2.5 µg ConA/ml was higher in the BrdU (~ 100) compared to the MTT (~ 2) assay, indicating that metabolic activity is not as dependent on stimulation as DNA-synthesis, because quiescent lymphocytes also exhibit metabolic activity, while DNA-synthesis occurs only in proliferating cells. In any case, the stimulation index (SI) in the MTT assay of 1.5-2.25 (Tiemann et al, 2006) are in accordance with the SI in the MTT assay of the present study (Figures 1-2).

Optimal stimulation of porcine PBL ranged from 2.5-20 µg/ml ConA, 0.25-50 µg/ml PHA, 2.5-50 µg/ml PWM and 10-100 µg/ml LPS (Brown-Borg et al, 1993; Davis et al, 2002; Haberstock-Debic et al, 1997; Leshin et al, 1998; Morrow-Tesch et al, 1994a, b; van Heugten et al, 1994, 1997, 2003), indicating a high variance between laboratories due to protocol, time of incubation and type of measurement. In the present study, ConA seemed to be the most potent mitogen of porcine PBL and showed highest stimulation at comparatively low doses (1.25-5 µg/ml ConA) and a decline of stimulation at higher doses (>10 µg/ml ConA) (Figures 1-3). This is in accordance with Leshin et al (1998), who observed the highest proliferative responses of porcine PBL cultures after ConA-stimulation, whereas Buschmann et al (1980) found the stimulation of porcine blood and spleen lymphocytes with PHA and PWM most effective. PHA stimulated both porcine PBL and splenocytes, whereas ConA and LPS were better stimulants of spleen cells. Mosmann (1983) compared the colorimetric MTT and the radioactive [^{3}H]-thymidine incorporation assay after stimulation of mouse spleen cells with various concentrations of the mitogens LPS and ConA. The author observed excellent agreement for ConA-stimulations, and small differences between the endpoint of LPS-stimulations. Furthermore, LPS stimulated mouse spleen cells over an extended concentration range (0.19-200 µg LPS/ml), whereas ConA showed a narrow optimum (1.25-2.5 µg/ml), with little or no proliferation at high or low concentrations (Mosmann 1983). This is in agreement with the present findings that ConA stimulated proliferation between 1.25-5 µg/ml in both assays, whereas higher concentrations showed a decrease of stimulation (Figures 1-3). Accordingly, Kondo et al. (2003) reported that responses of chicken splenocytes were suppressed at mitogen levels exceeding optimal concentrations (1.25-2.5 mg PHA/ml, 50-100 µg ConA/ml) and suggested that concentrated PHA and ConA solutions may be harmful to lymphocyte activity. On the other hand, Hoskinson et al. (1992) found a generally higher lymphocyte proliferation of porcine PBL in response to 20 µg/ml ConA than that with 10 µg/ml ConA, whereas Pang et al. (1987) observed generally lower values at 50 µg/ml ConA. Moreover, Tomar et al. (1986) showed that 3-acetyl-DON suppressed the proliferative response of human PBL to ConA, PWM and PHA, but this effect is less susceptible in PHA induction and the authors concluded that this difference may be associated with the variety of lymphocyte subpopulation that each mitogen activates. Furthermore, the authors asserted that amplifier cells are equally responsive to ConA and PHA, while precursors of effector cells are responsive to ConA (Tomar et al., 1986). In addition, IC_{50} values of human PBL after DON exposure *in vitro* using the [^{3}H]-thymidine assay were comparable for nonstimulated (150 ng DON/ml), ConA-stimulated (100-150 ng DON/ml) and LPS-stimulated (150-200 ng DON/ml) lymphocyte cultures, whereas PHA-stimulated lymphocytes were slightly more resistant (250 ng DON/ml) (Mekhancha-Dahel et al., 1990). Therefore, ConA appeared to be a good stimulant when testing trichothecene mycotoxins in primary lymphocyte cultures.

4.3 Cell number

The MTT assay provides a linear relationship between cell number and formazan production at low and high cell densities (Lewis et al., 1999; Mosmann 1983). On the other hand, Arnould et al. (1990) observed that the mitochondrial activity of cells was not constant and depends on the cell density in the culture prior to harvesting. In addition, it was maximal during the exponential growth and decreased when the culture became confluent (Arnould et al., 1990). Therefore, optimal seeding density had to be established for each cell line and consequently for lymphocyte culture as in the present study. However, the amount of formazan depends, besides the number of cells, upon the cell line and incubation time, with fibroblasts being more efficient than tumor cells (Cory et al., 1991). In the present study, 1 x 10^5 cells per well appeared to be more sensitive to increasing DON concentrations compared to 2 x 10^5 and 4 x 10^5 cells/well.

4.4 DON exposure and cell status

Inhibition of protein synthesis has generally been regarded as the mode of action of trichothecene mycotoxins (Bamburg 1983; Feinberg et al., 1989; Rotter et al., 1996), although inhibition of DNA-synthesis (Rosenstein et al., 1983), damage of the cell membrane (Bunner et al., 1988; Rizzo et al., 1992), altered intercellular communication (Jone et al., 1987), dysregulation of calcium homeostasis (Yoshino et al., 1996) and induction of apoptosis (Holme et al., 2003; Shifrin et al., 1999; Uzarski et al., 2003) are also suggested metabolic effects. Holt et al. (1987, 1988) reported of comparable inhibitory effects of T-2 toxin on Chinese hamster ovary (CHO) and L929 cell cultures on DNA- ([^{3}H]thymidine) and protein synthesis ([^{3}H]leucine) *versus* MTT reduction, whereby lymphocytes were 3 times more sensitive to T-2 than comparable tissue culture cell lines. Furthermore, inhibition of the DNA-synthesis could be explained by the fact that eukaryotic cells depend on newly synthesized protein to enter the S phase of the cell cycle (Feinberg et al., 1989).

In the present study, in the incubation of porcine PBL cultures with 70 – 560 ng DON/ml *in vitro*, the inhibition caused a sigmoid inhibition in the MTT and BrdU assay (Figure 5), indicating that considerable inhibition of lymphocyte proliferation does not occur below 100 ng DON/ml and is saturated at DON concentrations above 500 ng/ml. Although cytotoxicity values other than IC_{50} concentrations can be estimated from the dose response curve, the midpoint cytotoxicity value is considered more reliable than values based on 20 or 80 % inhibition (Arnould et al., 1990). So, Goyarts et al. (2006) reported of IC_{50} values of 309 and 200 ng DON/ml in the MTT and BrdU assay, respectively, when lymphocytes were freshly processed (Figure 5). Following a freezing procedure, lymphocytes of the same control animal were inhibited by the same extent after exposure to 286 and 201 ng DON/ml in the MTT and BrdU assay, respectively (Figure 5). The results of the present investigation therefore demonstrated that frozen PBL as well as fresh PBL could be of use in the proliferation assay, provided that the number of applied viable cells via trypan blue exclusion is equal. In human PBL, DON concentrations of 150 and 100-150 ng/ml inhibited the incorporation of [^{3}H]-thymidine by 50 % in nonstimulated and ConA-stimulated cultures, respectively (Mekhancha-Dahel et al., 1990). These values are lower than the IC_{50} values of porcine PBL observed in the present study, possibly due to the use of a different assay or the dissolving of toxin in DMSO, known as a membrane facilitating substance. Using the MTT assay, in PHA- stimulated human PBL an IC_{50} of 219 ng DON/ml was reported, but in a second study 300 ng DON/ml decreased MTT-absorbance to only 55 % compared to the control (Meky et al., 2001), indicating that human and porcine blood lymphocytes are similarly sensitive to DON. While in the present study a significant inhibition was not observed until 280 ng DON/ml, primary cultures of blood lymphocytes and splenocytes from prepubertal gilts showed a significant inhibition at 140 ng DON/ml in the MTT assay by 34 and 24 %, respectively (Tiemann et al., 2006). As DON was dissolved in DMSO in the latter study, but in NaCl in the present study, it can be suggested that the toxin dissolved in DMSO penetrates the cell more easily and may therefore previously exert toxic effects. On the other hand, in the present study a different animal category was used than in the experiment of Tiemann et al. (2006) (prepubertal gilts *versus* growing pigs). Furthermore, individual variation of lymphocyte proliferation has to be taken into consideration, because Thuvander et al. (1999) found IC_{50} values of 290-700 nM DON/ml [86-207 ng DON/ml] in mitogen-stimulated lymphocyte cultures of 15 human donors.

Although the same conditions were used (1 x 10^5 cells/well, 2.5 µg ConA/ml, 76 h incubation) in both studies, 200 µg DON/ml were found to inhibit the BrdU incorporation only by 10 % in the mitogen study (Figure 2), but by 50 % in the DON-toxicity study (Figure 5). However, it has to taken into consideration that the variation in the BrdU assay is very high, e.g., the stimulation index (SI, Figure 2) ranged between 78.9-126.7 (mean: 105.4) and 77.2-106.4 (mean: 95.2) for the incubation with 2.5 µg ConA/ml without and with 200 ng DON/ml, respectively. On the other hand, the calculated IC_{50} (Figure 5) appeared to be more reliable to describe the inhibiting effect of DON in the BrdU assay, because the other DON doses are also included in this kind of data evaluation.

In PHA-stimulated rat and human lymphocytes IC_{50} values of 90 and 220 ng DON/ml were obtained using the [^{3}H]-thymidine incorporation assay, while acetyl-DON required higher doses of 450 and 1060 ng/ml, respective-

ly (Atkinson et al., 1984). The concentration inhibiting 50 % of the DNA synthesis (IC_{50}) in Swiss mouse 3T3 fibroblasts using the BrdU assay were 444, 4890, 510 and 23300 ng/ml for DON, 3-acetyl-DON, 15-acetyl-DON and de-epoxy-DON, respectively (Eriksen et al., 2004), indicating that acetylation decreased immunotoxicity and deepoxidation leads practically to a detoxification of DON. However, Tomar et al. (1987) found clearly lower IC_{50} values of 230, 187 and 167 ng 3-acetyl-DON/ml after stimulation of murine splenocytes with PHA, ConA and LPS, respectively. For that reason, it can be suggested that primary cultures of lymphocytes are more sensitive to trichothecene mycotoxins compared to cell lines.

Inhibition of cell proliferation at high toxin concentrations (560 ng DON/ml) showed saturation at 60-80 % for the MTT assay and nearly 100 % for the BrdU assay (Figure 5). Likewise, using the MTT assay absorbance of blood lymphocyte cultures exposed to 500 ng DON/ml or 1120 ng DON/ml was still 32 % (human; Meky et al., 2001) or 25 % (prepubertal gilts; Tiemann et al., 2006), respectively. Furthermore, Cook et al. (1989) observed a relative insensitivity of the MTT assay below 10 % cell survival. Therefore, it can be assumed that the MTT assay may underestimate cellular damage, since apoptosis is an active mode of cell death requiring the metabolism of cells, and this test detects cell death only at later stages of apoptosis when metabolic activity of cells is reduced.

4.5 Immunoglobulins

In the present study, IgA, IgG and IgM in supernatant of ConA-stimulated lymphocyte cultures were dose-dependently inhibited by DON (Figure 6). Accordingly, Thuvander et al. (1999) observed IC_{50} values of 119 ± 39, 110 ± 24 and 116 ± 24 ng DON/ml for IgA, IgG and IgM supernatant concentrations of PWM-stimulated human PBL cultures, respectively, which were in the same range as observed for the lymphocyte proliferation determined by [^{3}H]-thymidine incorporation (113 ± 33 ng DON/ml). However, Tiemann et al. (2006) were not able to show an effect of 70-1120 ng DON/ml on IgA concentrations of ConA-stimulated splenic lymphocyte cultures.

The antibody production in PWM-stimulated human PBL was significantly inhibited by 3-acetyl-DON concentrations of ≥ 200 ng/ml, whereas the proliferative response measured by [^{3}H]-thymidine was already inhibited at 100 ng 3-acetyl-DON/ml (Tomar et al., 1986). This difference was explained by the fact that different lymphocyte subsets are involved in the process of antibody production and lymphocyte proliferation (Tomar et al., 1986). On the other hand, immunoglobulin supernatant concentrations of the present investigation seemed to be more sensitive to DON as both proliferations assays, because IC_{50} values were markedly lower than in the proliferation assays (Figures 5 and 6). Therefore, it can be suggested that DON reduced first the production of secretory proteins (immunoglobulins), and thereafter their proliferation (DNA-synthesis, BrdU) to prolong their maintenance requirements (metabolic activity, MTT assay). This will be underscored by Atkinson et al. (1984), who observed no effects of DON on viability in rat and human lymphocyte cultures at doses that significantly inhibited lymphocyte proliferation, indicating that this mycotoxin has little effect on quiescent lymphocytes containing only few polysomes, but inhibits proliferating cells.

5 Conclusions

Porcine blood lymphocytes reacted most sensitively to DON after 72 h incubation (37 °C, 5 % CO_2) when 1 x 10^5 cells/well were stimulated with 2.5 µg ConA/ml. Both frozen and freshly processed lymphocytes can be used. Although the DNA-synthesis (BrdU assay) seemed to be more sensitive compared to the metabolic activity (MTT assay), the variability of the BrdU-results was higher probably due to more working steps. Furthermore, DON was shown to inhibit lymphocyte proliferation and immunoglobulin concentrations of supernatant dose-dependently as a sigmoid curve. Sensitivity to DON increased expressed as IC_{50} values increased from MTT assay (~ 300 ng DON/ml) > BrdU assay (~ 200 ng DON/ml) > supernatant Ig concentrations (~ 100 ng DON/ml).

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