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Effects of four *Fusarium* toxins (fumonisin B_1, α-zearalenol, nivalenol and deoxynivalenol) on porcine whole-blood cellular proliferation

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ABSTRACT

The *in vitro* effects of four *Fusarium* toxins, fumonisin B_1 (FB_1), α-zearalenol (α-ZEA), nivalenol (NIV) and deoxynivalenol (DON), on mitogen-induced cell proliferation were determined in swine whole-blood cultures. Considering the lack of sufficient toxicological data both on single and in combination effects, *in vitro* studies may contribute to risk assessment of these toxins. Incubation with increasing concentrations of FB_1 did not produce any consequence on proliferation; in contrast α-ZEA, NIV and DON showed an inhibitory effect. Dose–response curves for each mycotoxin were generated. NIV was found to be the most potent toxin followed by DON and α-ZEA. The effects of both FB_1 + α-ZEA and NIV + DON mixtures were also analysed to investigate possible interactions. The results indicated that combination of FB_1 + α-ZEA produces a synergistic inhibition of porcine cell proliferation; whereas there is no interaction between DON and NIV on porcine whole-blood proliferation, at tested concentrations.

1. Introduction

The most important *Fusarium* toxins that may potentially affect human and animals' health are fumonisin B_1 (FB_1), zearalenone (ZEA) and trichothecenes such as nivalenol (NIV) and deoxynivalenol (DON).

Fusarium fungi are commonly found on cereals grown in the temperate areas of America, Europe and Asia (Creppy, 2002). 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 analysed samples (Schothorst and van Egmond, 2004). *Fusarium* toxins elicit a wide spectrum of toxic effects, including the capacity to modify normal immune functions both in humans and animals. Notably the capacity of these mycotoxins to alter immunity is exerted at levels below those causing over toxicity (Oswald et al., 2005).

Fumonisin B_1 is the etiological factor of several mycotoxicosis in both domestic and laboratory animals (Colvin and Harrison, 1992; Gelderblom et al., 1991; Kuiper-Goodman, 1998) and it has been correlated to the incidence of human oesophageal cancer (Sun et al., 2007). The pathophysiological effects of FB_1 are attributable to disruption of the sphingolipids metabolism since this mycotoxin is inhibitor of ceramide synthase (Ramasamy et al., 1995; Yoo et al., 1996). The effects of fumonisin B_1 on immune system remain controversial: it causes immune-suppression in poultry (Li et al., 2000), swine (Harvey et al., 1995), bovine (Osweiler et al., 2003) and immune-stimulation in rodent species (Dombrink-Kurtzman et al., 2000; Dresden-Osborne et al., 2002; Sharma et al., 2004). Recently, an *in vivo* study showed that FB_1 alters immune functions in broilers by decreasing bursa weight and changing gene expression of cytokines (Cheng et al., 2006).

Zearelenone (ZEA) is a macrocyclic lactone with high binding affinity toward estrogenic receptors and,

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consequently, it causes estrogenic effects in pigs (Dieckman and Green, 1992). It has been suggested as causative agent of infertility and reduced milk production in cattle (D'Mello and MacDonalds, 1997). In particular, α-zearalenol (α-ZEA), a metabolite of ZEA, was found more active than ZEA (Minervini et al., 2001). The immune system is a potential target for estrogenic endocrine disruptors considering that its cells express estrogenic receptors (Igarashi et al., 2001). In spite of that, only few studies have been carried out on the immune effects of zearalenone and its metabolites. In particular immune alterations were found at high concentrations of this *Fusarium* toxin *in vitro* such as reduction of both mitotic index and cell survival of bovine lymphocytes (Lioi et al., 2004; Yu et al., 2005).

Nivalenol and deoxynivalenol belong to the trichothecenes group, very stable compounds both during storage/milling and processing/cooking of food (Grove, 1988). Different effects have been associated to trichothecenes, including impaired delayed-type hypersensitivity, phagocyte activity (Pestka et al., 1994; Rotter et al., 1996) and modulation of host response to enteric infections (Li et al., 2005). *In vitro* analyses showed that trichothecenes can both suppress and stimulate immune functions (Bondy and Pestka, 2000). In particular, NIV inhibited proliferation of human lymphocytes (Forsell and Pestka, 1985; Thuvander et al., 1999).

Despite the major sensitivity of swine to *Fusarium* toxins (Pestka and Smolinski, 2005), a very limited number of studies have been carried out to assess the immunotoxicity of *Fusarium* toxins in this species. *In vitro* investigations on pig peripheral blood mononuclear cells indicated that FB_1 decreased IL-4 and increased IFN-gamma synthesis at both protein and mRNA levels (Taranu et al., 2005) and induced apoptosis of primary swine alveolar macrophages (Liu et al., 2002) and porcine renal epithelial cells (Gopee and Sharma, 2004). Moreover, FB_1 altered the proliferation and the barrier function of porcine intestinal epithelial cells (Bouhet et al., 2004, 2006). Tornyos et al. (2003) showed that FB_1 had no significant effect on the humoral and cellular specific and non-specific immune responses in pig fed with contaminated feed at different levels of mycotoxin; on the other hand Marin et al. (2006) reported that FB_1 was immunosuppressive and its effect was higher in males.

In vivo and *in vitro* studies in pig showed that DON induced a significant inhibition of lymphocytes proliferation and immunogloblulin secretion (Goyarts et al., 2006); whereas Accensi et al. (2006) did not observe any immune effects when pigs were fed with feed contaminated by low doses of DON.

Considering that food and feed commodities are often contaminated by more than one mycotoxin, (Speijers and Speijers, 2004), studying the interactions between different mycotoxins can be useful. It is known that *Fusarium* toxins can exert additive and synergistic effects (Tajima et al., 2002); but mycotoxins may also act as antagonists (Koshinsky and Khachatourians, 1992). Theumer et al. (2003) showed different *in vitro* effects of a mixture of aflatoxin B_1 (AFB_1) and fumonisin B_1 in comparison to the individual toxins. Another study on *Penicillium* mycotoxins showed that the majority of examined mixtures produced *in vitro* less-than-additive effects (Bernhoft et al., 2004).

In the present study we investigated the effects of fumonisin B_1, α-zearalenol, nivalenol and deoxynivalenol on porcine immune response by proliferation assay performed using whole blood. Then, the effects of fumonisin B_1, α-zearalenol, nivalenol and deoxynivalenol were assessed in binary combination.

2. Materials and methods

2.1. Reagents

RPMI-1640 medium, L-glutamine, streptomycin, penicillin, 2-mercaptoethanol and non-essential aminoacids were purchased from Cambrex Bioproducts Europe (Verviers, Belgium). Fumonisin B_1 (F-1147), α-zearalenol (Z-0166), deoxynivalenol (D-0156), nivalenol (N-7769) and concanavalin A (C 0412) were purchased from Sigma (St. Louis, MO, USA).

2.2. Experimental animals

Twenty-six clinically healthy castrated male pigs of Norwegian Landrace breed, 8–10 months old, were used as blood donors. Blood samples for cell proliferation tests were collected from *vena cava cranialis* into heparinized sterile Vacutainer™ tubes (7 ml containing 150 IU Li–Heparin).

2.3. Whole-blood cell proliferation assay

Blood samples (20 ml) were diluted 1:20 (v/v) with RPMI-1640 containing penicillin (100 units/ml), streptomycin (100 μg/ml), 5×10^{-5} M 2-mercaptoethanol, 1% (w/v) non-essential aminoacids and 2% pooled and heat-inactivated (56 °C for 30 min) swine serum.

Diluted blood was plated in 96-well flat-bottomed culture plates at 100 μl/well and single test mycotoxins added at a range of final concentrations (FB_1, 0.5–80 μM; α-ZEA, 0.5–20 μM; DON and NIV, 0.0625–2 μM). In binary combinations mycotoxins were applied at same concentrations (1:1 ratio) at a range of final combinations (FB_1 + α-ZEA mixture, 0.5–20 μM; NIV + DON mixture, 0.0625–1 μM).

FB_1 was dissolved in phosphate buffered saline (PBS), NIV and α-ZEA in methanol, whereas DON in ethanol. Each mycotoxin was then further diluted in complete culture medium. In preliminary studies, it was verified that final concentration of solvents in the culture medium did not affect cell proliferation.

Proliferation was induced by Concanavalin A (Con A) at an optimised concentration of 10 μg/ml (data not shown). The drug concentration in each well was calculated based on the final volume of 200 μl/well. Cell culture was incubated at 37 °C in a 5% CO_2-humidified air incubator for 72 h. All assays were performed in triplicate.

Eighteen hours prior to harvesting, cells were pulsed with 1 μCi/well [^{3}H]-thymidine. Cultures were harvested on filters using a semiautomatic cell harvester (Filtermate, Packard, Danvers, MA). [^{3}H]-thymidine incorporation was assessed by a microplate liquid scintillator (Top Count NXT™, Packard, Danvers, MA). Results were expressed as counts per minute (cpm).