Sex-related differences in the immune response of weanling piglets exposed to low doses of fumonisin extract

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Fumonisin B1 (FB1) is a mycotoxin produced by Fusarium verticillioides, a fungus that commonly contaminates maize. Sex-related effects of FB1 have been observed with respect to carcinogenicity in rodents, to performances in pigs and immunosuppression in mice. In the present study the sex-related effect of FB1 on the pig immune response was determined. Female and castrated male piglets received for 28 d either control feed or feed contaminated with 8 mg FB1/kg feed in the form of F. verticillioides culture material. At day 7 and day 21, animals were immunised subcutaneously with a Mycoplasma agalactiae vaccine. Ingestion of FB1-contaminated feed significantly decreased weight gain in males but had no effect in females. No sex-related difference was observed in biochemical parameters, but a higher level of creatinine was noted in toxin-treated animals. FB1 also altered the pig immune response in a sex-specific manner. In males, ingestion of FB1-contaminated feed significantly decreased specific antibody levels after vaccination as well as the mRNA expression level of IL-10. In females, the toxin has no effect on specific antibodies or on cytokine mRNA levels. The results of the present study indicate that FB1 is immunosuppressive in pigs. The magnitude of this FB1-induced immunosuppression is highly dependent on sex, with males being more susceptible than females.

Fumonisin: Mycotoxins: Immunosuppression: Food contamination: Pig: Gender-related effects

Mycotoxins are secondary metabolites of fungi, which may contaminate animal feeds and human foodstuffs. The global occurrence of mycotoxins is considered an important risk factor for human and animal health, as up to 25% of the world crop production may be contaminated (Fink-Gremmels, 1990). Recent surveys of fumonisin contamination of food and feed in Europe and the USA also have raised concerns about the extent of FB1 contamination of maize and its implications for food safety (Murphy et al. 1996; International Programme on Chemical Safety, 2000). FB1 was found in up to 50% of maize samples collected between 1988 and 1991 from the mid-Western USA (Murphy et al. 1993). In this survey, up to 10% of the samples had toxin levels between 10 and 50 parts per million (Murphy et al. 1993). Similarly, another survey of fumonisins in maize gluten and other maize products in the UK found these mycotoxins in almost every sample at concentrations of up to 32 parts per million (Scudamore et al. 1990).

Abbreviations: FB1, fumonisin B1; IFN, interferon; Th, T-helper.

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Howard et al. 2001; Johnson & Sharma, 2001). In a 2-year feeding study, hepatocellular adenomas and carcinomas were induced by FB1 in female mice but not in males. In the same study it was also revealed that male Fischer F344 rats developed renal tumours that were not seen in females (Howard et al. 2001). Sex-specific effects of FB1 have been described in the immune response of mice following subcutaneous injection (Bhandari et al. 2001; Johnson & Sharma, 2001). In females, FB1 treatment reduced relative spleen and thymus weights, splenic cellularity, thymocyte CD4+ / CD8+ double positive cells, lymphocyte proliferation, and IL-2 expression. These effects were not observed in male mice (Johnson & Sharma, 2001). In addition, FB1 administration caused increased expression of TNF-α, IL-12p40, interferon (IFN)-γ, IL-1β, IL-6 and IL-10 in male liver while female mice only showed an increased expression of IL-6 and a down modulation of IFN-γ (Bhandari et al. 2001). In pigs, males are more adversely affected by low doses of purified FB1 than females, as indicated by the effect of the toxin on average daily gain, serum biochemical parameters, pancreas and adrenal weight (Rotter et al. 1996).

In the present study the sex-specific effects of FB1 on the pig immune response was investigated. Ingestion of mycotoxin-contaminated feed altered the immune response of males but did not affect the immune response of females as indicated by the expression of Th-helper (Th) 2 cytokines and by the production of specific antibodies upon vaccination.

Materials and methods

Experimental design

Twenty, 4-week-old, crossbred weanling piglets (ten females and ten castrated males) were studied for 28 d. The animals were from seven different litters and were balanced for litters and ten castrated males) were studied for 28 d. The animals were from seven different litters and were balanced for litters across treatments. They were acclimatised for at least 1 week before being used in the experimental protocol and were given ad libitum access to water and feed. They were fed a maize-soyabean-meal-based diet (Marin et al. 2002) supplemented with or without a fumonisin-containing fungal extract. The extraction procedure following in vitro culture of the F. verticilloides strain NRRL 34 281 has been described (Oswald et al. 2003). Briefly, sterilised maize inoculated with the fungal strain was incubated for 4 weeks at 25°C. The culture extract contained 54 % FB1, 8 %FB2 and 9 %FB3 (Tran et al. 2003). We verified that it did not contain detectable amounts of zearalenone, deoxynivalenol, fusarochromanone or trichothecenes (Oswald et al. 2003). The extract was incorporated into the pig basal diet to provide 8 mg FB1/kg feed. Considering the average feed consumption of the animals, this corresponded to doses of 0·99 and 1·49 mg/kg body weight per d for the first and the second halves of the experiment. Body weights and food consumption were recorded weekly throughout the experiment. Animals were cared for in accordance with the National Institute of Health Guide.

Immunisation and blood sample collection

On day 7 and day 21 of the experiment, all piglets were immunised by subcutaneous inoculation with a 1 ml suspension of Agavac® (Institute Pasteur, Bucharest, Romania) as previously described (Marin et al. 2002). This vaccine consists of a combination of formol-inactivated Mycoplasma agalactiae strains re-suspended in aluminium hydroxide. On day 0, day 20 and day 28 of the experiment, blood samples were aseptically collected by jugular vein puncture. Syringes without anticoagulant were used to collect serum for antibody and biochemical parameter measurements and syringes containing lithium-heparin were used to collect blood for measuring cytokine mRNA expression.

Measurement of blood biochemical parameters

Serum concentrations of Na, K, chloride, Ca, P, total proteins, urea, creatinine, glucose, cholesterol, triglycerides and bilirubin, and concentrations of alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, γ-glutamyl transferase and lactate dehydrogenase were determined on a Vitros 950 IRC analyser (Ortho Clinical Diagnostics, Raritan, NJ, USA) at the Laboratory of Biochemistry (Rangueil Hospital, Toulouse, France).

Measurement of total and specific antibody levels

Total concentrations of the different Ig subsets (IgG, IgA and IgM) were measured by ELISA (Bethyl, Interchim, Montluçon, France) as previously described (Taranu et al. 2005).

Antibodies against M. agalactiae were also measured by ELISA. Briefly, ELISA plates were coated with supernatant fraction from ultrasonicated M. agalactiae culture. Diluted serum samples (1/100) were then added to the plates and the anti-mycobacterial antibodies were detected with peroxidase-labelled anti-pig IgG (Marin et al. 2002). The absorbance at 405 nm was recorded using an ELISA plate reader.

Blood cell culture for cytokine mRNA expression analysis

The mRNA expression of five different cytokines was analysed in the blood samples obtained from the control and FB1-treated animals. Whole blood was cultured as previously described (Marin et al. 2002). Briefly, blood was diluted 10-fold in RPMI 1640 supplemented with 2 mM-L-glutamine, penicillin (100 U/ml), streptomycin (0.1 mg/ml; Sigma, St Louis, MO, USA), 10% fetal calf serum (Hyclone, Perbio, Brebières, France) and then 2 ml of diluted blood was cultivated with phytohaemagglutinin (10 μg/ml). After 24 h of culture, cell pellets were harvested and re-suspended in 1 ml Trizol (Gibco BRL Life Technologies, Cergy Pontoise, France) then frozen at −80°C until used.

Determination of cytokine mRNA expression by semi-quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted following the manufacturer’s recommendations and quantified by spectrophotometry. Semi-quantitative determination of IL-4, IL-10, IL-6, IL-2 and IFN-γ and cyclophilin, chosen as a housekeeping gene, was carried out using RT-PCR performed as previously described (Dozois et al. 1997; Fournout et al. 2000). Briefly, mRNA was reverse transcribed with Moloney leukaemia virus RT (Promega, Charbonnières, France) and amplified with DNA
Taq polymerase enzyme (Invitrogen, Life Technology, Cergy Pontoise, France) using the already published primer sequences (Dozois et al. 1997; Fournout et al. 2000). Semi-quantitative analysis of PCR products was done by hybridisation of $^{32}$P-labelled specific oligonucleotide probes to PCR products immobilised on nitrocellulose membranes by dot blotting (Pié et al. 2004). The DNA probes used for hybridisation of the different cytokines have already been described (Darwich et al. 2003; Pié et al. 2004). The relative amounts of each product were determined by measuring radioactivity with a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA, USA). For each cytokine, the amounts of RT-PCR products were normalised to the values obtained with cyclophilin, which was used as an internal standard for each sample.

Statistical analysis

All data are expressed as mean values and standard errors of the mean. Statistical differences between groups for feed consumption, biochemical parameters, serum immunoglobulin subsets and cytokine expression levels were determined using an ANOVA two-way analysis; the measurements for these parameters were done once at the end of the experiment. A two-way ANOVA with replications was used to analyse the antibody and the average weight gain during the experiment. Further differences between means were determined by the least square difference Fisher procedure. Values of $P < 0.05$ were considered significant.

Results

Sex-related effect of fumonisin on animal performance

We first investigated the sex-related effect of FB$_1$ treatment on clinical signs and animal weight. The feeding of 8 mg FB$_1$/kg feed for 28 d did not produce any detectable alteration in the general status of either male or female piglets. As shown in Fig. 1, in females the feeding of fumonisin had no significant effect on the average daily weight gain ($P = 0.821$). By contrast, in males, throughout the 4 weeks of the experiment, ingestion of fumonisin-contaminated feed decreased ($P = 0.0001$) the average daily weight gain by up to 23% when compared with control. Further differences between control and fumonisin male groups at specific time points were determined by the least square difference Fisher procedure. A significantly treatment effect began after the 3rd ($P = 0.021$) and the 4th ($P = 0.049$) week of intoxication. This lower weight gain was not due to reduced feed intake. The experimental protocol did not allow measurement of individual feed ingestion but group data indicated that feed consumption was 2·6 and 10·6% higher for males compared with females during the first and the second halves of the experiment respectively.

Sex-related effect of fumonisin on biochemical parameters

At the end of the experiment, blood samples were collected from the piglets to investigate the sex-related effect of fumonisin on biochemical parameters. The presence of FB$_1$ in the diet did not alter the activities of serum enzymes including alkaline phosphatase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, γ-glutamyl transferase and lactate dehydrogenase (Table 1). The contamination of pig feed with fumonisin also did not have any influence on the serum concentration of Na, K, chloride, bicarbonate, Ca, P, protein, urea, glucose, bilirubin, cholesterol and triacylglycerol. By contrast, the feeding of fumonisin-contaminated diet significantly increased the creatinine concentrations in the serum of treated animals ($P = 0.035$).

Sex-related effects of fumonisin on total and specific antibody responses

To investigate the sex-related effect of fumonisin on the immune responses, piglets were immunised with Mycoplasma. Serum antibody levels were measured by ELISA after the primary (day 21) and the secondary injections (day 28). As expected, the vaccinal injections increased the antibody levels (Fig. 2). This increase was observed in both males and females receiving either the control or the fumonisin-contaminated diet. Nevertheless a sex-related effect of fumonisin was observed on mycoplasma-specific antibody levels. Repeated-measures ANOVA used for data statistical analysis showed that both ingestion of fumonisin-contaminated feed and the time of exposure significantly decreased the specific antibody synthesis ($P = 0.022$) in males. The further pairwise comparison between the control and FB$_1$ group at each specific time point resulted in a 38% decrease in specific antibody levels ($P = 0.045$) at day 28 of the experiment whereas in females, ingestion of the contaminated feed did not have any
significant effect ($P=0.186$). This sex-related effect was only observed for specific antibody level and no effect of FB$_1$ was observed on total Ig levels, irrespective of the subsets considered (Table 2).

**Sex-related effect of fumonisin on cytokine mRNA expression**

The ability of fumonisin to modulate the cytokine expression in a sex-dependent manner was then investigated on whole-blood samples stimulated with mitogen. The mRNA expression of both Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10 and IL-6) cytokines was measured by RT-PCR at the end of the experiment. The mRNA synthesis of Th1 cytokines (IL-2 and IFN-γ) was not modified by the contamination with fumonisin in either male or female piglets (Fig. 3). Ingestion of fumonisin-contaminated feed induced a sex-dependent decrease in the expression of Th2 cytokines. In males, fumonisin treatment decreased the expression level of mRNA encoding for IL-4, IL-6 and IL-10 (by 31, 51 and 45 % respectively). However, the difference was only significant for IL-10 ($P=0.040$, $P=0.268$, $P=0.159$ for IL-10, IL-6 and IL-4 respectively), due to the small number of animals in each group. By contrast, in female piglets, fumonisin treatment did not induce any changes in Th2 cytokine expression.

**Discussion**

We have studied the sex-related effects of a sub-chronic exposure (28 d) to fumonisin on body-weight gain, blood biochemical parameters and immune response of piglets. In general, males were more adversely affected by the presence of fumonisin in the diet than females. Average daily gain decreased by 23 % in males upon fumonisin treatment, for example, whereas it was not affected in females. Similar results were obtained by Rotter et al. (1996) who demonstrated that castrated pigs were more sensitive to 1–10 mg purified FB$_1$ in the diet than females. Zomborszky et al. (2000), in contrast, did not observe any significant effect on body-weight gain or feed consumption when feeding weanling piglets with 10–40 mg FB$_1$/kg feed for 4 weeks.

The present results indicate that low doses of FB$_1$ had limited effects on the blood biochemical parameters of the piglets (Table 1). In a previous study with purified toxin, biochemical changes were only observed at early (2 weeks) or late (8 weeks) time points (Rotter et al. 1996). No significant effect of FB$_1$ from fungal culture material was observed on blood parameters (Zomborszky-Kovacs et al. 2002).

Sex differences after chronic exposure to FB$_1$ toxicity have been reported for carcinogenic effects in rodents, performance changes in pigs and immunosuppression in mice (Rotter et al. 1996; Bhandari et al. 2001; Howard et al. 2001; Johnson & Sharma, 2001).

In the present study, sex differences in the immune response of pigs after exposure to fumonisin were observed for both antibody and cytokine responses. The feeding of fumonisin was shown to decrease the specific antibody response developed after immunisation with an anti-Mycoplasma vaccine. This effect was only observed in males and was specific for the acquired immune responses. Immunosuppressive effects of FB$_1$ on humoral immune response have been described after immunisation with sheep erythrocytes in male rats receiving 25 mg FB$_1$/kg body weight per d (decreased specific IgM; Tryphonas et al. 1997) and in male mice receiving 0.25–5 mg FB$_1$/kg body weight in one dose (reduced number of specific plaque-forming cells; Martinova & Merrill, 1995). Turkeys treated with 200 mg FB$_1$ for 4 weeks also had significantly lower antibody responses during vaccination against Newcastle disease virus (Li et al. 2000).

**Table 1. Effect of dietary fumonisin administration on blood biochemical parameters in piglets†**

(Mean values with their standard errors)

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Control feed</th>
<th>Fumonisin-containing feed</th>
<th>Control feed</th>
<th>Fumonisin-containing feed</th>
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<td>Mean</td>
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| Na (mM)                | 141 0.1 | 145 0.2 | 146 0.5 | 3.0
| K (mM)                | 8.43 0.05 | 7.83 0.2 | 8.04 0.54
| Chloride (mM)       | 99.8 1.2 | 100.2 1.3 | 100.2 2.7
| Bicarbonate (mM)    | 16.2 1.6 | 17.7 1.0 | 18.4 1.5
| Ca (mM)              | 2.53 0.03 | 2.53 0.09 | 2.40 0.06
| P (mM)              | 4.24 0.51 | 4.44 0.19 | 4.96 0.82
| Total protein (g/l)  | 69.3 1.4 | 73.7 3.9 | 66.6 1.4
| Urea (mM)          | 5.21 0.70 | 4.46 0.73 | 4.46 0.71
| Creatinine (mM)      | 85.2 4.2 | 97.7* 4.8 | 93.6* 3.3
| Glucose (mM)         | 4.26 0.51 | 4.05 0.36 | 4.05 0.17
| Bilirubin (µM)       | 2.9 0.3 | 3.3 0.3 | 3.6 0.9
| Cholesterol (mM)     | 2.77 0.19 | 3.11 0.14 | 2.90 0.23
| Triacylglycerols (mM)| 0.50 0.12 | 0.40 0.01 | 0.32 0.03
| Alkaline phosphatase (IU/l) | 205.2 28.7 | 283.0 88.3 | 266.4 61.8
| γ-Glutamyl transferase (IU/l) | 180.8 20.9 | 140.2 5.1 | 144.4 30.5
| Aspartate aminotransferase (IU/l) | 76.5 1.0 | 60.5 3.6 | 60.6 2.2
| Alanine aminotransferase (IU/l) | 48.4 9.8 | 49.0 8.3 | 46.8 7.0
| Lactate dehydrogenase (IU/l) | 1877 198 | 1383 164 | 1483 103 |

*Mean value was significantly different from that of the animals of the same sex fed the control feed ($P<0.05$).† At the end of the experiment, serum from the piglets (four or five per group) was used to measure the blood biochemical parameters.
They were immunised with a Mycoplasma vaccine on day 7 and day 21 of the experiment. Serum samples were collected on day 0, day 21 and day 28 and levels of vaccine-specific antibody were determined by ELISA. Results are expressed as optical density (405 nm); values are means for five animals, with the vertical bars representing standard errors. Comparison of the specific antibody production in the control and fumonisin B1-treated animals was done using a repeated-measures ANOVA. The presence of fumonisin in the diet, however, significantly decreased the mRNA expression level of IL-10, a Th2 cytokine, in male peripheral blood cells. A tendency for a decrease of other Th2 cytokines, IL-6 (P = 0.68) and IL-4 (P = 0.159), were also observed in males. The lack of significance of these parameters may be due to the small number of animals present in each group (n = 5).

By contrast, ingestion of a high dose of fumonisin-contaminated feed for 8 d, or a low dose of toxin for 3–4 months, did not alter antibody titres against Aujeszky virus (Tornyos et al. 2003).

In the present study the total level of Ig (IgM, IgG or IgA) in piglets at the end of the experiment was not affected by the fumonisin treatment (Table 2). The total Ig in serum did not alter antibody titres against Aujeszky virus (Tornyos et al. 2003).

In the present study the total level of Ig (IgM, IgG or IgA) in piglets at the end of the experiment was not affected by the fumonisin treatment (Table 2). The total Ig in serum did not alter antibody titres against Aujeszky virus (Tornyos et al. 2003).

Few investigations have examined the sex-related effect of FB₁ on cytokine synthesis (Bhandari et al. 2001; Johnson & Sharma, 2001), with no studies on pigs. In male mice, FB₁ treatment increased the expression of TNF-α, IL-12 p40, IFN-γ, IL-1β, IL-6 and IL-10, while female mice showed only an increased expression of IL-6, and a down modulation of IFN-γ and IL-2 (Bhandari et al. 2001; Johnson & Sharma, 2001). In the present study, no effect of fumonisin was observed on Th1 cytokines (IL-2 and IFN-γ). The presence of fumonisin in the diet, however, significantly decreased the mRNA expression level of IL-10, a Th2 cytokine, in male peripheral blood cells. A tendency for a decrease of other Th2 cytokines, IL-6 (P = 0.68) and IL-4 (P = 0.159), were also observed in males. The lack of significance of these parameters may be due to the small number of animals present in each group (n = 5).

Th2 cytokines, especially IL-4, IL-6 and IL-10, are implicated in the development of the humoral immune response and antibody production (Abbas et al. 1996). IL-4 is involved in the stimulation of the antibody production by B cells, promotion of growth and survival of T cells (Nelms et al. 1998). It is a major factor for the differentiation of B lymphocytes that promotes the Ig switch and favours synthesis of IgG. Moreover, IL-4 and IFN-γ play a key role in the regulation of immune response by their mutually antagonistic mechanisms on cytokine synthesis (Abbas et al. 1996). The development of antibody response is also sustained by IL-6, which in addition to its role as activator of the T cells is involved in the final differentiation of the B cells in plasmocyte cells which regulate antibody synthesis (Diehl & Rincon, 2002). IL-10 performs a complex role in the immune response. In cooperation with IL-4, IL-10 stimulates B cells by increasing the expression of class two molecules of the major histocompatibility complex (Abbas et al. 1996) and by stimulating their proliferation and their differentiation to express and produce IgM, IgG and IgA. IL-10 also plays an important role in the cytokine network, by inhibiting cytokine production by Th1 cells.
lymphocytes and monocytes and macrophages (Mocellin et al. 2003). In the present study, the decreased expression of Th2 cytokines (IL-10, IL-4 and IL-6) correlates with a decreased production of specific antibodies (Fig. 2 and Fig. 3). The lower specific antibody synthesis observed in males may, therefore, be a consequence of a lower Th2 cytokine expression.

As mentioned earlier, FB1 is a potent inhibitor of ceramide synthase, an enzyme critical to sphingolipid biosynthesis. The sex-related effect of FB1 could be due to a sex-related difference in sphingolipid metabolism. A higher accumulation of free sphingoid bases was observed in the liver of female mice treated with FB1 when compared with males (Bhandari et al. 2001). In pigs, however, after consumption of diet contaminated with 10 mg FB1/kg, the increase in free sphingoid bases in the lung was more pronounced in males than in females (Rotter et al. 1996). Sphingosine is a potent competitive inhibitor of protein kinase C (Gopee & Sharma, 2003) and, recently, it has been shown that protein kinases are involved in the regulation of cytokine synthesis. In human macrophages, for example, inhibition of protein kinase Cδ selectively suppresses IL-10 production (Foey & Brennan, 2002). Thus, the higher inhibition of IL-10 expression observed in male pigs upon FB1 exposure (Fig. 3) may be due to a higher increase of sphingosine and a greater suppression of protein kinase C.

The species-specific effect of FB1 on sphingolipid metabolism may also explain the contrasting effects on cytokine synthesis observed in mice and pigs. Free sphingoid bases inhibit lymphocyte growth, especially the Th2 subtype (Tokura et al. 1996; Desai et al. 2002). Exposure to FB1 increases free sphingoid bases in male pigs and in female mice (Rotter et al. 1996; Bhandari et al. 2001). By selectively acting on Th2 lymphocytes, these sphingoid bases could decrease synthesis of cytokines (IL-4, IL-6 and IL-10) in a sex- and species-dependent manner. Through its effect on sphingolipid metabolism, FB1 may also modulate the concentration of the different subclass of gangliosides, known to regulate cytokine production. GD1b, GT1b and GQ1b, for example, enhanced IL-2 and IFN-γ production but suppressed IL-4 and IL-5, IL-6 and IL-10 synthesis. GD1a and GM3, however, stimulate IL-10 production (Kanda, 1999; Kanda & Watanabe, 2000, 2001). Further studies are needed to determine the implication of species and sex on lipid metabolism and its modulation by FB1 on the observed variation of cytokine expression.

In conclusion, it has been shown that fumonisin has toxic effects on the immune response and that these effects are more pronounced in males than in females. Due to the possible risk and implications for pig production and human health, this sex specificity requires further investigation, especially in the areas highly contaminated with Fusarium species.

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