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EFFECT OF DIETARY FUMONISIN B_1 ON CERTAIN IMMUNE PARAMETERS OF WEANED PIGS

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Only few data are available on the effect of fumonisins on the immune response. The aim of the present study was to examine whether dietary fumonisin B_1 (FB_1) has any effect on the humoral and cellular immune response in weaned pigs, depending on the dose and the time of toxin exposure. *Fusarium moniliforme* fungal culture was added to the experimental animals' diet to ensure an FB_1 intake of 1, 5 and 10 ppm (first experiment) or 100 mg per animal per day (second experiment). The control animals were fed a toxin-free diet. In order to determine the immune response, the animals were vaccinated against Aujeszky's disease with inactivated vaccine (Aujespig K, Phylaxia-Sanofi, Budapest, Hungary). Specific and nonspecific *in vitro* cellular immune response was measured by the lymphocyte stimulation test (LST) induced by PHA-P, Con A, LPS and inactivated suspension of the Aujeszky's disease virus. Humoral immune response, e.g. specific antibody titre, was measured by the virus neutralisation (VN) test. None of the immunological parameters examined showed significant differences between groups. It could be concluded that fumonisin B_1 had no significant effect on the humoral and cellular specific and nonspecific immune response when fed in a high dose (100 mg/animal/day for 8 days) or in a low concentration even for a longer period (1, 5 and 10 ppm for 3–4 months).

Key words: Fumonisin B_1, immune response, lymphocyte stimulation test, pig

Immunotoxicity can be manifested either in immunosuppression, when the function of the immune system is suppressed, or in stimulation of the immune functions. The first results in increased susceptibility to infections, the second in hypersensitivity or autoimmune-like disorders. Mycotoxin-induced immunosuppression may be manifested as depressed T or B lymphocyte activity, suppressed antibody production, and impaired macrophage and/or neutrophil-effector functions (Oswald and Coméra, 1998). Suppressed immune functions may decrease resistance to infectious diseases and acquired immunity induced by vaccination and may increase reactivation of chronic infections. Mycotoxins also affect the inflammation process. Stimulation of the immune system also occurs in the pres-

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ence of mycotoxins; however, this stimulation is not always desirable because it can lead to immunopathology, hypersensitivity and allergic reactions (Oswald and Coméra, 1998).

Most *Fusarium* toxins (T-2, HT-2, deoxynivalenol [DON], nivalenol, etc.) exert a negative effect on the function of the immune system. Rafai (2001) found that T-2 toxin negatively affected the proliferation and differentiation of immunocompetent cells even in a low concentration (0.5 mg/kg). The ratio of T lymphocytes, the antibody production and the rate of blastogenic transformation of T lymphocytes induced by nonspecific mitogens (PHA, Con A) decreased significantly in pigs.

Many mycotoxins have been found to affect humoral immunity. Of particular interest for its effect on antibody synthesis is DON, which depresses serum IgM and/or IgG, and induces an elevation of IgA. Aflatoxin B_1 has the most extensively studied immunomodulating effect, focused on cell-mediated immunity (Oswald and Coméra, 1998).

According to the international literature, mycotoxins capable of modulating the inflammatory processes include aflatoxin B_1, ochratoxin A, patulin and gliotoxin. Fumonisins form a relatively newly described group of mycotoxins produced by *Fusarium moniliforme*. They were discovered in 1988, in South Africa, where a high correlation was found between the occurrence of oesophageal cancer (OC) and the rate of fumonisin B_1 (FB_1) exposure (Rheeder et al., 1992; Myburg, 1998). FB_1 is the most toxic compound and has been shown to promote pulmonary oedema in pigs, leukoencephalomalacia in horses, hepatic cancer and kidney degeneration in rats (Riley et al., 1996). A relatively high percentage of grains used as animal feed in Hungary, mainly maize, is contaminated with fumonisins (Fazekas et al., 1997).

The mode of action of FB_1 is not completely understood yet. Wang et al. (1991) demonstrated that the toxin disrupts the sphingolipid metabolism by inhibiting the sphingosine N-acyltransferase (ceramide synthase) enzyme. It results in accumulation of sphingoid bases, alteration of signalling and disruption of normal cell cycling (Hussein and Brasel, 2001).

Although several toxic and carcinogenic effects of FB_1 are known, data available on its effect on the immune system are limited. The aim of the present study was to examine whether FB_1 has any effect on the humoral and cellular immune response in weaned pigs, depending on the dose and the time of toxin exposure.

Materials and methods

Experimental design, animals, feeding

Two experiments were carried out, each with 20 weaned castrated pigs of identical genotype and approximately 12–14 kg body weight.

In the first experiment the pigs were divided into four groups (n = 5 in each). After a 5-day adaptation period, a *Fusarium moniliforme* fungal culture containing 500 mg/kg FB_1 was added to the experimental animals' diet to ensure an FB_1 intake of 0 (control), 1, 5 and 10 ppm.

In the second experiment two groups (experimental group, n = 14 and control group, n = 6) were formed. The FB_1 intake in this case was 100 mg per animal per day.

The fungal culture was produced in the Veterinary Institute of Debrecen according to the method of Fazekas et al. (1998). The FB_1 content of the fungal culture and feed was determined by the method of Fazekas et al. (1998). The control animals were fed a toxin-free diet (T-2, F-2, DON and ochratoxin A were not detectable in the diet).

The animals were placed in individual metabolic cages and marked individually with ear-tags. They were fed a basal diet corresponding to their age, which contained 187 g/kg crude protein, 12.8 MJ/kg metabolisable energy (ME) and 13.1 g/kg lysine (LYS). They were fed twice a day; drinking water was provided *ad libitum*.

In order to determine the immune response, animals were vaccinated against Aujeszky's disease with an inactivated vaccine (Aujespig K, Phylaxia-Sanofi, Budapest, Hungary). Blood samples were taken from the vena cava cranialis according to the protocol shown in Table 1.

Table 1

Vaccination and blood sampling protocol

Procedure	First experiment	Second experiment
1st blood sampling	Day 0	Day 0
Start of feeding the FB_1-containing diet	Day 1	Day 1
1st vaccination	Day 90	Day 2
2nd blood sampling	Day 97	Day 8
2nd vaccination	Day 104	–
3rd blood sampling	Day 111	–
4th blood sampling	Day 125	–

Immunological analyses

Specific and nonspecific *in vitro* cellular immune response was measured by the lymphocyte stimulation test (LST). Lymphocytes were separated from 2 ml of heparin- (Sigma-Aldrich) treated peripheral blood carefully layered onto a bilayered Histopaque solution (1.119 and 1.077 g/ml, respectively). After spinning, lymphocytes were separated using sterile syringes, washed twice in PBS, and resuspended in RPMI-1640 solution containing 10% fetal bovine serum. In the *in vitro* LST lymphocyte density was adjusted to 5×10^6 cell/ml, cell viability

was determined by toluidine blue staining using Buerker chambers, and after adjustment 100 µl cell suspension was plated into the wells of flat-bottomed 96-well microplates. The ratio of dead cells never exceeded 10% of the total cell count after separation. Cell cultures were kept in a CO_2 incubator (5% CO_2, 100% humidity, 37 °C). In order to investigate nonspecific cellular immune responsiveness, blastogenic transformation of lymphocytes was induced by 12.5 µg/ml phytohaemagglutinin-P (PHA-P, Sigma-Aldrich), 12.5 µg/ml concanavalin A (Con A, Sigma-Aldrich) and 20 µg/ml lipopolysaccharide (LPS, Sigma-Aldrich). Specific cellular immune response was induced by the inactivated suspension of Aujeszky's disease virus (10^7 $TCID_{50}$/ml). Mitotic activity was tested after 72 h of stimulation by MTT (methylthiazole-tetrazolium, Sigma-Aldrich) conversion test, a fast colorimetic method used to determine the dehydrogenase enzyme activity of the lymphocytes (Denizot and Lang, 1986), which is correlated with the rate of blastogenic transformation. Stimulation index was calculated on the basis of the optical density of the control and the mitogen-induced samples (Stites, 1987).

Humoral immune response, e.g. specific antibody titre was measured by virus neutralisation test (VN). Serum samples were diluted in binary logarithmic value. 100 $TCID_{50}$ virus suspension (suid herpesvirus, SHV-1) was added to the diluted serum samples, then incubated on 37 °C for 1 h. Monolayer cell cultures (PK-15) were inoculated with the samples and the effect of the virus was detected daily. Experimental data of the VN test were evaluated and the highest dilution of the serum, which showed the effects of the virus in 50% of the cultures, was expressed in binary logarithmic value.

Results

Effect of long-term FB_1 exposure (first experiment)

Responses to PHA-P showed that the majority of animals had been in a state of slight immunodeficiency in the third month of the trial (Table 2). This had probably resulted from climatic factors (sultry summer weather) or from continuous blood sampling as a stress factor. Results of the control groups, based on the third and fourth blood samplings, improved a little but no significant differences occurred either between groups or between blood samplings. Lipopolysaccharide (LPS) and concanavalin A (Con A) induced nonspecific cellular immune response measured by LST showed similar responses (data not shown).

Cellular responses to specific antigen (inactivated Aujeszky's disease virus, SHV-1) indicated higher activity (Table 3). It showed that vaccination had been successful but no significant differences were found between groups.

In trial 1, after the first vaccination, no antibodies of detectable level occurred in the blood. In certain animals after seven days, at the second blood sam-

pling, a low level of antibodies was detected, indicating that the immune response had started. The second immunisation resulted in a further increase of antibody level that reached the minimum level required for protection. No significant difference between groups was observed (Table 4).

Table 2

Phytohaemagglutinin-P (PHA-P) induced nonspecific cellular immune response measured by LST[(1)]

FB_1 concentration (ppm)	LST PHA-P[(1)]			
	1st sampling	2nd sampling	3rd sampling	4th sampling
0 (control)	1.03 ± 0.01	0.94 ± 0.07	1.26 ± 0.10	1.21 ± 0.11
1	1.09 ± 0.07	1.03 ± 0.11	1.07 ± 0.07	1.08 ± 0.03
5	0.93 ± 0.11	1.04 ± 0.07	1.02 ± 0.08	1.02 ± 0.05
10	0.99 ± 0.07	1.05 ± 0.14	1.02 ± 0.07	1.05 ± 0.06

Table 3

Suid herpesvirus-1 (SHV-1) induced specific cellular immune response measured by LST[(1)]

FB_1 concentration (ppm)	LST SHV-1[(1)]			
	1st sampling	2nd sampling	3rd sampling	4th sampling
0 (control)	0.98 ± 0.03	1.11 ± 0.06	1.29 ± 0.10	1.30 ± 0.10
1	0.99 ± 0.08	1.06 ± 0.05	1.11 ± 0.10	1.17 ± 0.09
5	0.97 ± 0.04	1.04 ± 0.07	1.22 ± 0.08	1.21 ± 0.07
10	0.98 ± 0.07	1.08 ± 0.09	1.19 ± 0.11	1.25 ± 0.28

Table 4

Results of virus neutralisation test expressed in binary logarithmic value

FB_1 concentration (ppm)	VN test results			
	1st sampling	2nd sampling	3rd sampling	4th sampling
0 (control)	2.08	3.06	7.03	6.20
1	2.14	2.16	4.00	6.70
5	2.22	3.22	8.83	8.16
10	2.36	3.14	7.39	8.00

Effect of 100 mg/day/animal FB_1 (second experiment)

Animals fed 100 mg FB_1 per day became depressed, lost their appetite, and their feed intake decreased on the 5th–6th day. They showed severe dyspnoea, and the mucous membranes exhibited signs of cyanosis. Clinical symptoms developed rapidly and pulmonary oedema led to death within 0.5–1 day after the first signs.

Among the haematological parameters examined, elevated red blood cell count (9.3–10.8 × 10^6/μL), haemoglobin concentration (15.8–17.2 g/dL) and haematocrit value (54–66%) and decreased MCH value (14.3–16.6 pg) were observed. This could presumably be due to a compensatory functioning, i.e. more intensive red blood cell and haemoglobin production. Among the clinicochemical parameters examined the high aspartate aminotransferase activity (116–330 U/L) revealed hepatic injury.

The thoracic cavity of the pigs contained varying amount (15–390 mL) of yellow exudate with a tendency to coagulate. The animals had severe pulmonary oedema. The lungs were enlarged, compact and less elastic to the touch, they did not collapse when the thorax was opened. The interlobular septa were widened because of the serious oedematous infiltration. The trachea and the bronchi contained a white and frothy substance. In some cases haemorrhagic infiltration of the peribronchial lymph nodes was found.

In this trial, responses to PHA-P were the most explicit (Table 5). None of the mitogens showed significant difference between the groups. The relatively small changes in the SI values induced by the specific antigen (SHV-1) showed that the animals were not able to adapt within this short time. More blood samplings and second vaccination could not be carried out as the animals died of acute pulmonary oedema and hydrothorax.

Table 5

Immunological parameters measured in the second experiment

Fumonisin B_1 concentration	1st sampling	2nd sampling
LST LPS[(1)]		
0 (control)	1.22 ± 0.07	1.20 ± 0.06
100 mg/animal/day	1.20 ± 0.07	1.18 ± 0.05
LST PHA-P[(2)]		
0 (control)	1.68 ± 0.21	1.65 ± 0.16
100 mg/animal/day	1.60 ± 0.14	1.61 ± 0.11
LST ConA[(3)]		
0 (control)	1.01 ± 0.05	1.06 ± 0.03
100 mg/animal/day	1.05 ± 0.06	0.99 ± 0.03
LST SHV-1[(4)]		
0 (control)	1.06 ± 0.04	1.09 ± 0.10
100 mg/animal/day	1.04 ± 0.06	1.08 ± 0.08

[(1)]Lipopolysaccharide (LPS), [(2)]phytohaemagglutinin-P (PHA-P) and [(3)]concanavalin A (Con A) induced nonspecific cellular immune response measured by LST; [(4)]suid herpesvirus-1 (SHV-1) induced specific cellular immune response measured by LST

The measured antibody titres were 1:2 or lower on day 8; no significant difference between groups was detectable (data not shown).

Discussion

At the moment there are considerable data indicating that fumonisin-induced disruption of sphingolipid metabolism leads to altered cell growth, differentiation and cell injury. However, the consequences of altered sphingolipid metabolism are cell type specific (Riley et al., 1998). Clarifying the role of sphingolipid metabolism in immune function remains a task for future research. According to Qureshi and Hagler (1992) the mechanism by which FB_1 may alter immune function can be inhibition of molecular synthesis, alterations in metabolic processes or alteration in membrane structure and function.

Data concerning the effect of FB_1 on the immune system are controversial according to species, sex, mode of toxin application, dose, etc.

The effect of subchronic dietary exposure to FB_1 was investigated in male Wistar rats (Theumer et al., 2002). After feeding a diet containing 100 ppm of FB_1 for 12 weeks no significant effect on mitogen induced *in vivo* and *in vitro* proliferation of spleen mononuclear cells was found. However, changes in the interleukin profile and some functions of macrophages in antitumour activity was observed.

The results of Johnson and Sharma (2001) indicate that FB_1-induced immunosuppression is highly dependent on sex, with females being more susceptible than males. After five daily subcutaneous injections of 2.25 mg/kg/day of FB_1 phytohaemagglutinin (PHA-P) induced T-lymphocyte and LST-induced B-lymphocyte proliferation was reduced in female mice.

When turkey poults were fed diets containing 200 ppm FB_1 or 200 ppm FB_1 + 100 ppm moniliformin (M), significantly lower primary and secondary antibody response (anti-NDV) was measured after 2–3 weeks (Li et al., 2000). The proliferative response to mitogens also decreased significantly. It could be concluded that FB_1 alone or in combination with M is immunosuppressive in poults; however, neither synergistic nor additive effects between FB_1 and M were observed.

The findings of Qureshi and Hagler (1992) showed that FB_1 exposure induced morphological and functional alterations in chicken macrophages, which may result in increased susceptibility to bacterial infection.

Lymphocytes play a major role in specific and nonspecific immune responses. Dombrink-Kurtzman et al. (1992*a*) reported reduced lymphocyte viability in chickens fed diets containing 62 ppm FB_1 and 15 ppm FB_2. Fumonisin B_1 turned out to be cytotoxic *in vitro* to turkey lymphocytes (Dombrink-Kurtzman et al., 1992*b*). Negligible effects of FB_1 on human lymphocyte proliferation were observed using the MTT assay (Meky et al., 2001).

In our experiments none of the immunological parameters examined showed significant differences between groups. It could be concluded that fumonisin B_1 had no significant effect on the humoral and cellular specific and nonspecific immune response when fed in a high dose for a short period (100 mg/animal/day for 8 days), or in a low concentration even for a longer period (1, 5 and 10 ppm for 3–4 months).

Our experiment provides useful results mainly for the practice, indicating that long-term exposure to low concentrations (1–10 ppm) of FB_1, occurring frequently in Hungary, does not pose a significant hazard as regards the immune response of weaned pigs. However, animals were kept under proper nutritional and hygienic conditions in this study. Other environmental immunomodulating effects may have synergistic or additional effects or may modulate the effect of FB_1.

It is very probable that mycotoxins have more pronounced effect on the mucosal lymphoid tissue than on the systemic immunity. For example, Smith et al. (1996) reported that FB_1 can inhibit the action of pulmonary intravascular macrophages in the removal of particulate matter and pathogens from the circulation in pigs, making animals more susceptible to disease. Thus additional investigations of the effects on the local immune response should also be carried out.

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