EFFECTS OF AFLATOXIN B₁, OCHRATOXIN A, PATULIN, CITRININ, AND ZEARALENONE ON THE IN VITRO PROLIFERATION OF PIG BLOOD LYMPHOCYTES

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Abstract

The effects of aflatoxin B₁ (AFB₁), ochratoxin A (OTA), patulin (PAT), citrinin (CIT), and zearalenone (ZEA) on in vitro response of pig peripheral blood mononuclear cells to mitogen concanavalin A was assayed after three days of incubation using ³H-thymidine uptake. Dose response curves for each mycotoxin were generated and the concentrations producing 50% inhibition of cell proliferation (IC₅₀) were estimated. AFB₁, OTA, and PAT were the most potent toxins with the IC₅₀ of 0.06, 0.17, and 0.19 µmol/L, respectively (0.2, 0.7, and 0.3 µg/mL, respectively). Based on the molar concentration, the inhibition potencies relative to that of AFB₁ were determined. OTA had 35% and PAT 31% potency to that of AFB₁, but CIT and ZEA had only 1.6 and 1.9 of AFB₁ inhibition potencies.

Key words: swine, mycotoxins, lymphocytes, proliferation assay.

The contamination of food and feed by mycotoxins is a major problem for human and animal health. The ingestion of mycotoxins may cause a range of toxic responses from acute toxicity to long term or chronic health problems, including immunosuppression and even carcinogenesis (reviewed in 2). Certain fungi belonging to the Penicillium and Aspergillus families, which are in worldwide distribution, produce the following mycotoxins: ochratoxin A (OTA), citrinin (CIT), and zearalenone (ZEA) on in vitro response of pig peripheral blood mononuclear cells to mitogen concanavalin A was assayed after three days of incubation using ³H-thymidine uptake. Dose response curves for each mycotoxin were generated and the concentrations producing 50% inhibition of cell proliferation (IC₅₀) were estimated. AFB₁, OTA, and PAT were the most potent toxins with the IC₅₀ of 0.06, 0.17, and 0.19 µmol/L, respectively (0.2, 0.7, and 0.3 µg/mL, respectively). Based on the molar concentration, the inhibition potencies relative to that of AFB₁ were determined. OTA had 35% and PAT 31% potency to that of AFB₁, but CIT and ZEA had only 1.6 and 1.9 of AFB₁ inhibition potencies.

The mycotoxins, which may act as an immunsuppressive agent, may increase disease susceptibility (3). Although the molecular basis for many of the specific immunosuppressive effects of mycotoxins are presently unclear, the inhibition of DNA, RNA, and protein synthesis via a variety of different mechanisms appears to be directly or indirectly responsible for the immunosuppressive action of many mycotoxins.

Some of these toxins have already been identified to be causal agents in the development of renal diseases (32), and affect lymphocyte proliferation in pigs (17) and cattle (33). As the renal morphology of the porcine kidneys is very similar to that of the kidneys in humans, it is expected that humans would also be susceptible to mycotoxins known to have a renal effect in pigs (28). On the other hand, ZEA and its metabolites have oestrogenic and anabolic activities in several species, such as rodents, pigs, and monkeys (16), and are able to cause alterations in the reproductive tract of laboratory animals and dairy cattle, and have reducing effects on the meiotic progression of bovine oocytes (24).

Many mycotoxins may be produced by one mould; in particular, many Aspergillus and Penicillium species can produce several mycotoxins simultaneously, depending on the environmental and substrate conditions. This is particularly true for OTA, OTB (ochratoxin B), CIT, and, occasionally, AFB₁ and PAT. The last has also been associated with alterations in renal function and /or the development of renal pathologies (30).

Immunotoxicology is regarded as an important element when considering the potential adverse effects of mycotoxin exposure, and the effect parameter involving the immune system have been found to be particularly sensitive to various mycotoxins. It is well known that OTA, in addition to its other effects, may suppress the immune system. Immunomodulating effects have also been documented for other Penicillium and Aspergillus mycotoxins selected for this study,
including CIT, PAT, and AFB₁ (6, 35). However, the effects of *Penicillium* toxins on the immune system have been little studied, and other toxins produced by the genus may also be involved in interrupting the immune status.

The present work was undertaken to establish an *in vitro* model for studying the effect of individual mycotoxins on isolated immune cells from pigs, and to use the method to compare the effect of some *Penicillium* and *Aspergillus* toxins - AFB₁, OTA, PAT, CIT, and ZEA - which can potentially occur in animal feed.

**Material and Methods**

**Mycotoxins.** Pure crystalline mycotoxins AFB₁, OTA, PAT, CIT, and ZEA were obtained from Sigma Chemical Co. A standard stock solution was made in HPLC grade DMSO at a concentration of 100 mg/mL for each of the toxins. The working solution of the mycotoxins was prepared in RPMI 1640 medium in a concentration appropriate to the running of the experiments.

**Experimental animals.** Four clinically healthy pigs, 7-week-old, obtained from a local farm were used as blood donors. The animals were fed a commercial pig feed ad libitum throughout the experimental period. The blood samples for the lymphocyte proliferation tests were collected from the vena cava cranialis in sterile heparinised tubes once a week.

**Lymphocyte proliferation test.** The blood samples were diluted with PBS and the peripheral blood lymphocytes were separated by density gradient centrifugation on Histopaque (1.077) at 400 x g for 30 min. The lymphocytes were collected using a Pasteur pipette, washed with 10 ml of PBS solution, and centrifuged at 200 x g for 10 min. The collected cells were counted and resuspended in a RPMI 1640 medium supplemented with 10% calf serum, L-glutamine, and antibiotics. The concentration of the cells was adjusted to 0.5 x 10⁶ per mL. The suspension of the lymphocytes was seeded in 24 well flat-bottomed tissue culture plates containing 100 µl per well of individual mycotoxin solution. Concanavalin A (Con A) solution at a final concentration of 5 µg/mL/well was added immediately before incubation at 37°C in a humidified 5% CO₂ atmosphere for 72 h. DNA synthesis was assessed by the incorporation of ³H-methyl thymidine (specific activity of 20 Ci/nmol) at a concentration of 0.5 µCi/mL, added to each well 16 h before the cell harvest. The controls were prepared in the same manner, but without toxins.

Next, the incubated cells were aspirated with a Pasteur pipette and transferred into plastic tubes, washed with PBS, and precipitated with cold 10% trichloroacetic acid (TCA). The suspension (precipitate) was centrifuged and washed twice with 10% TCA. The suspension (precipitate) was centrifuged and washed twice with 10% TCA. The suspension (precipitate) was centrifuged and washed twice with 10% TCA. The suspension (precipitate) was centrifuged and washed twice with 10% TCA. The suspension (precipitate) was centrifuged and washed twice with 10% TCA. Finally, the pellet was washed with methanol and filtered on glass fibre filters by means of filtration manifold (Millipore Instruments). The filters were dried, put into scintillation liquid, and counted on a scintillation counter (Packard Instruments). The results were expressed as mean counts per minute (cpm).

**Experimental design.** Triplicates of each concentration of the five mycotoxins were tested in the isolated lymphocytes from each of the four pigs. Each plate contained six control wells (cells with no toxin addition); three wells containing Con A, and three wells without Con A. The mean cells proliferation of triplicates of the runs, were used for the calculations. Acceptable dose effect results for a specific mycotoxin were defined as those having a relative standard deviation less than 25% in all triplicates. The mycotoxins’ effect on cell proliferation was calculated as a percentage of the proliferation in the control cells (relative proliferation). Relative proliferation is presented as the mean with a standard error of four pigs.

**Results**

The pigs were clinically healthy during the experiment. The mean radioactivity values in triplicate control cultures without Con A ranged from 200 to 800 cpm, and the corresponding cultures with Con A stimulation ranged from 10,000 to 30,000 cpm. For all the mycotoxins the dose-response curves showed a dose dependent suppression of Con A induced lymphocyte proliferation (Fig. 1). The concentration producing 50% inhibition (IC₅₀) of lymphocyte proliferation was estimated from the curves (Table 1). AFB₁, OTA, and PAT were found to be the most potent inhibitors of lymphocyte proliferation. Based on molar concentration, PAT and OTA showed similar potency. They were approximately 200 times more potent as an inhibitor than CIT and ZEA. AFB₁ served as a model toxin, the strongest inhibitor of lymphocyte proliferation, and it was almost 600 times more potent as an inhibitor than CIT or ZEA.

**Table 1**

The concentration of mycotoxins causing IC₅₀ of Con A induced proliferation of pig lymphocytes estimated from the dose response curves. The results are presented as weight per volume or as molar concentrations. The inhibition potencies relative to that of aflatoxin B₁ are indicated.

<table>
<thead>
<tr>
<th>Mycotoxins</th>
<th>IC₅₀ (µg/mL)</th>
<th>IC₅₀ (µmol/mL)</th>
<th>Relative inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B₁</td>
<td>0.02</td>
<td>0.06</td>
<td>100</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>0.07</td>
<td>0.17</td>
<td>35</td>
</tr>
<tr>
<td>Patulin</td>
<td>0.03</td>
<td>0.19</td>
<td>31</td>
</tr>
<tr>
<td>Citrinin</td>
<td>9.0</td>
<td>36.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Zearalenon</td>
<td>10.0</td>
<td>31.4</td>
<td>1.9</td>
</tr>
</tbody>
</table>
When the inhibition potency of AFB$_1$ was established as 100%, OTA was 35% and PAT 31% less potent than the model inhibitor; however, the gradient of the dose response curves were similar to them. The weakest slope was observed in the curve for CIT and ZEA. These mycotoxins’ concentration range between zero and the maximal inhibition of cell proliferation was approximately x 10 (Figs 1A-B). Generally, CIT and ZEA required a 20 times higher concentration to give the same effect than AFB$_1$, PAT, and CIT.

Discussion

The lymphocyte proliferation test has been a well-accepted method in veterinary immunology for a very long time (19). It determines the activity of cells before differentiation into functional effector cells. The mitogen used, Con A, is a polyclonal mitogen, inducing the proliferation of T lymphocytes in an unspecific manner (7). The mechanism of stimulation is similar to antigen stimulation, although it does not involve the T cell antigen receptor. The toxin induced inhibition of mitogen induced lymphocyte proliferation gives an indication of immune suppression, which may result in a decreased resistance to infections. The demonstrated inhibitory effect on the proliferation of isolated lymphocytes indicates that the mycotoxins studied may also have the potential to inhibit these cells of the immune system in vivo. However, the immune system comprises of both non-specific macrophage and highly antigen-specific humoral and cell mediated responses, which complement each other in immune reactions. Results from in vitro studies, such as lymphocyte proliferation assay, should nevertheless be regarded as valuable supplements to in vivo studies. The lymphocyte proliferation test has been previously used for the toxicity testing of various environmental contaminants, including heavy metals, pesticides, mycotoxins, and many other xenobiotics (9, 33).

Fig. 1. The dose-response curves for the mycotoxins: AFB$_1$, PAT, OTA (A), CIT, and ZEA (B) on the proliferation of Con A stimulated PBMC isolated from 4 pigs.
AFB1. Mycotoxins are fungal metabolites widely present in feed and food crops all over the world. Because long-term exposure to low-levels of mycotoxins cannot be completely avoided, they are likely to be of potential concern for animal and public health. The Aspergillus and Penicillium species are two of the most abundant fungi found in feed and food. From the discovery of aflatoxins, mainly AFB1, their potential carcinogenic effect on number of animal species have been shown and associated with mycotoxicoses in poultry and other domestic animals. Aflatoxin B1 is the most studied mycotoxin up until now, but its effect of AFB1 on lymphocyte proliferation in domestic animals was very seldom investigated. In the present study, we have chosen pig lymphocytes as a target system to investigate the immunotoxicity of AFB1.

In this investigation, we have shown that AFB1 is a very strong inhibitor of lymphocyte proliferation. The concentration of AFB1, which reduced up to 50% of lymphocyte proliferation, was approximately 0.02 µg/mL in 72 h cultures. In primary swine alveolar macrophages, the concentration of AFB1, which gave a 50% inhibition of lymphocyte proliferation, was lower and elevated at 0.1µg/mL (4); however, our results were more consistent with another (36). Many of the biological effects of AFB1 required their bioactivation to the 2.3-epoxide metabolite by monooxygenase; active AFB1-epoxide covalently bound with macromolecules (1). Mononuclear blood cells from several mammalian species are reported to have monooxygenase activity for the activation of an environmental toxicant (27). Our data also reveal that stimulated pig lymphocytes by Con A may have monooxygenase to activate AFB1 to toxic metabolites, thereby causing the inhibition of proliferation, cytotoxicity, and other alterations. Compared to the results obtained from primary hepatocyte or kidney cell culture treated with AFB1, pig lymphocytes apparently have a similar susceptibility to AFB1 toxicity. On the other hand, in comparison with data from mononuclear macrophages treated cultures, AFB1 was at least five times more toxic to lymphocytes. In this study we have shown how big the difference is in the inhibition of lymphocyte proliferation between AFB1 and OTA or PAT, which are also very toxic mycotoxins.

Thus, AFB1 is a very strong inhibitor of lymphocyte proliferation in pigs in vitro. Only 0.02 µg/mL or 0.06 µmol/mL inhibits the proliferation at 50%. According to the results obtained in our experiment, the concentration of 0.08 µg/mL almost completely stop cell proliferation. This can be explained in that pigs are very sensitive species to mycotoxins, and especially to AFB1. Therefore, in our experiment AFB1 was used as the model mycotoxin, which is not only a strong carcinogen but also a very potent inhibitor of lymphocyte proliferation.

OTA. The effect of OTA on the proliferation of swine lymphocytes found in the present study was generally in accordance with the published results, if the same method was used (15, 17). They reported a 60% inhibition at 0.5 mg OTA/L, whereas in our study 50% inhibition was at 0.7 µg/mL. Our estimated IC50 for OTA on lymphocyte proliferation compares to the measured mean concentration of the toxin in the serum of the pig, after 42 daily intragastric doses of 0.02 mg/kg of body weight (29). This daily dose was considered to correspond to the average contamination of OTA in feed in Central Europe, and no clinical signs and pathological deviation was found. Thus, our results confirmed that immune interactions may be sensitive end points for OTA effects in pigs. However, in various surveys on OTA in blood plasma or serum from a pig at slaughter, considerably lower average concentrations have been reported. The mean OTA concentration in porcine plasma or serum varies from 5.1µg/L in a Canadian study (26) to 0.5 µg/L in a Norwegian study (20). Other studies of OTA in in vitro induced human lymphocytes or thymoma cells have demonstrated the effective concentration to be 20-30 times higher than in porcine lymphocytes in the present study (10). Even though the contamination level of OTA in feed, and thus in the corresponding porcine serum concentration, may vary considerably, in vitro results from the toxin in immune cells indicate that such an effective toxin level is probably reached in natural circumstances only in exceptional cases.

PAT. The potency of PAT at a weight dose level in inhibiting lymphocyte proliferation was found to be more than twice that of OTA, and to be similarly effective at a molecular level. A stronger effect on the immune cells of PAT compared to OTA at weight level has been reported previously (23), where the effect on cytokine production and the proliferation in thymoma cells were investigated. Cell proliferation was totally suppressed at 0.5 mg/L, which correspond to 0.3 µg/mL in the present study. A similar response to PAT in vitro has also been described by Escoula et al. (13).

In other in vivo studies, primarily in mice, the effects of PAT on different immune parameters have been observed (6). The authors have concluded that the exposure to PAT at levels consistent with potential human exposure in foods would be unlikely to alter immune responses (21). However, sublethal doses of PAT inhibit cellular and humoral function in mice and rabbits after intra-gastric or intra-peritoneal administration (13). Furthermore, pigs are more sensitive to PAT than mice, and this toxin may occur in a considerable concentration in rotten fruit, particularly in apples, which may be a common ingredient in food waste intended for pig feed (5). Thus, PAT can potentially be an immune toxin of concern for pigs fed such leftovers. Data on PAT concentrations in human or animal blood are not available, but experiments on rats with [14C]-labelled PAT have shown that the radioactivity was absorbed and distributed to various tissues (11).

CIT. The potency of CIT molecules as an inhibitor of lymphocyte proliferation was found to be at least 20 times lower than that of AFB1. The effects of CIT on the immune systems have been investigated to a limited extent. In a recent study on the effect on human T cell function indicated by the expression of cytokines, a stronger effect was found on interferon gamma with 50% inhibition at 8.3 mg/L (37), which is similar to the IC50 in our study. CIT was found to have less effect on
cell vitality using the MTT method (32). The estimated IC_{50} measured using our lymphocyte proliferation method was approximately 100 times the measured mean concentration of CIT in the pig serum (0.11mg/L after 57-dintra-gastric administration in a dose of 0.02 mg/kg b.w.) (29). This dose was considered to correspond to the average CIT contamination of feed in Central Europe, and no clinical signs or histopathological deviations were found. Thus, our results indicate that the inhibitory effect of CIT on pig lymphocytes occurs at higher concentrations than that at the average serum level in ewes. However, the data on CIT levels in feedstuffs are estimations, as no quantitative and accurate method for analysis exists.

**ZEA.** While OTA, PAT, and even CIT are carcinogenic, immunosuppressive and mainly nephrotoxic mycotoxins, ZEA and its metabolites, on the other hand, have oestrogenic and anabolic activities in several species including swine and rodents (i.e. being able to cause alterations in the reproductive tract). A limited number of studies have been carried out to assess the immunotoxicity of ZEA. Concerning bovine lymphocyte proliferation, ZEA is a weak inhibitor of cell proliferation (22). In our study, increasing the concentration of ZEA slightly decreases pig lymphocyte proliferation. We cannot exclude that with a higher concentration of ZEA it would be possible to obtain more evident reduction of pig lymphocyte proliferation. It has been demonstrated that the treatment of lymphocyte cultures with 0.1 μM ZEA for 36 h induces phosphorylation of histone H3, a marker of proliferation activity in MCF-7 cells (14), confirming our results that indicate there are no differences between exposed, and control cultures at 0.1 μM of ZEA. It can be assumed that only ZEA in high concentrations and on a very low scale may yield immunosuppressive effects as tested in the lymphocytes proliferation study.

The authors conclude that the lymphocyte proliferation test used was sensitive and applicable to evaluate the *in vitro* effects of the toxins on a selected part of the immune system, which has a broad range of functions. The discussion of the five selected mycotoxins, particularly as potential contaminants in feed for a relatively sensitive species, i.e. the pig, indicate that some of the toxins may be of importance. AFB_1_, OTA, and PAT are specifically mentioned, as pig feed may be contaminated with various *Penicillium* and *Aspergillus* sp., which may produce a range of mycotoxins. Studies of the effect of mycotoxins in combinations are thus very relevant. Therefore, we intend to study the combined effects of selected mycotoxins in the same test system.

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**References**