Effects of Fumonisins on the Immune System of Sprague–Dawley Rats Following a 14-Day Oral (Gavage) Exposure

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The effects of fumonisin B1 (FB1) on the immune system of Sprague–Dawley rats were investigated. Groups of male and female rats (10 rats/group) were gavaged daily for 14 days with doses of 0, 5, 15, and 25 mg/kg body wt/day and the primary (IgM) response to sheep red blood cells expressed as plaque-forming cell numbers/10⁶ spleen mononuclear leukocytes (PFC/10⁶ spleenocytes) and PFC/spleen was determined. There was a significant dose-related linear trend toward decreased PFC/10⁶ spleenocytes (p = 0.003) and PFC/spleen cells (p = 0.001) in the male rats. Body weights, expressed as a percentage of the control, were significantly reduced (p = 0.002) in the male rats administered 15 and 25 mg/kg doses. The PFC numbers in female rats were not affected significantly by treatment (p > 0.05). For the remaining immunotoxicity studies, groups of male rats (10 rats/group) were gavaged with FB1 doses of 0, 1, 5, and 15 mg/kg body wt/day for 14 days. There was a weakly significant dose-related trend toward increased numbers of serum immunoglobulin class G (p = 0.04). Also a significant dose-related increase (p = 0.013) in Listeria monocytogenes numbers was observed in the spleen at 24 hr postinfection. Treatment did not have a significant effect on organ weights, hematology, mitogen-induced lymphocyte transformation, calcium mobilization, the numbers of leukocytes and T-lymphocyte subsets, the natural killer cell activity, and phagocytosis (p ≥ 0.05). These observations suggested that FB1 may have indirect consequences for human health and warrant further investigations.

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Fusarium mycotoxins, including the T-2 toxin, deoxynivalenol, zearalenone, moniliformin, and fusarin C, are natural contaminants of corn. Recently, a new group of mycotoxins called fumonisins has been isolated from corn contaminated with the fungus Fusarium moniliforme (Beuzidenhout et al., 1988; Cawood et al., 1991; Gelderblom et al., 1988). Of these, fumonisin B1 (FB1) is the major contaminant of corn cultures and of naturally contaminated feed and foodstuffs (Shephard et al., 1990; Sydenham et al., 1991).

FB1 causes leukoencephalomalacia in horses (Marasas et al., 1988b) and pulmonary edema in swine (Harrison et al., 1990). Rats treated with 50 mg FB1/kg diet for 26 months displayed hepatocarcinogenic and hepatotoxic effects (Gelderblom et al., 1991). FB1 has also been associated with an increased risk of esophageal cancer in the inhabitants of rural areas in Transkei, southern Africa (Marasas et al., 1988a; Shephard and Van Schalkwyk, 1992; Sydenham et al., 1990; Rheeder et al., 1992).

While the effects of FB1 are predominantly on the liver and kidneys (Riley et al., 1994; Voss et al., 1993, 1995) the potential effects of this toxin on the immune system and immunosurveillance mechanisms should not be excluded. Effects on the immune system of chicken and calves have been reported for Fusarium moniliforme culture isolates known to produce fumonisin B1 (Marijanovic et al., 1991; Osweiler et al., 1993). Low levels of FB1 (0.5–10 µg/ml) caused significant cytotoxicity in chicken peritoneal macrophages in vitro (Qureshi and Hagler, 1992). Higher levels of FB1 (20, 40, or 100 µg/ml) added to chicken peritoneal macrophages in vitro caused morphological alterations including cytoplasmic blebbing, nuclear disintegration, and decreased phagocytosis (Qureshi and Hagler, 1992). These results suggested that nonspecific parameters of the immune system including effects on the phagocytic lineage of cells may be compromised by FB1. Such effects may ultimately influence other aspects of the immune system including humoral immunity and the ability of the animal to combat infection. In Balb/c mice, humoral immune responses following an ip injection of FB1 were mixed, with no clear trend toward either suppression or stimulation (Martinova and Merrill, 1995).

The present study was undertaken to investigate the potential effects of low levels of FB1 on several aspects of the immune system of Sprague–Dawley rats in a 14-day oral (gavage) study.
MATERIALS AND METHODS

Fumonisin. Fumonisin B₁ was produced at the Plant Research Centre, Agriculture and Agri-Food Canada, as described by Miller et al. (1994). For the experiments, FB₁, 98% pure, was dissolved in sterile 0.9% saline and was prepared fresh each week.

Animals. Specific-pathogen-free male and female Sprague–Dawley rats (60 ± 5 g; Charles River Canada Inc., Montreal, Quebec, Canada) were housed individually in plastic cages under conditions meeting the requirements of the Canadian Council for Animal Care. Purina rat Chow (Woodstock, Ontario, Canada) and water were provided aed libitum. Feed samples analyzed for fumonisins were not found to contain FB₁.

Calcium mobilization. Calcium mobilization was measured using a previously described method with some modifications (Sei and Arora, 1991). Spleen mononuclear cells were adjusted to 5 × 10⁶ cells/ml CM. The probe FLUO-3 was added to the cell suspension at a final concentration of 50 nM.

Leukocyte and lymphocyte concentrations in spleen mononuclear cell preparations were determined using the Coulter Counter Model S-Plus IV (Coulter Electronics Inc., Hialeah, FL) with the white blood cell (WBC) aperture current set at 150 V and the red blood cell (RBC) aperture current set at 190 V. The following parameters were measured: RBC counts (×10⁹/liter); hemoglobin (Hb) concentration (g/liter) hemocrit (Hct); mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) (pg); mean corpuscular hemoglobin concentration (MCHC) (g/liter); red cell distribution width (RDW) (%); and WBC concentration (×10⁹/liter). Leukocyte differential counts included eosinophils, neutrophils, segmented neutrophils, neutrophil bands, basophils, lymphocytes, and monocytes. Leukocyte differentials were obtained by multiplying the calculated percent of cells counted in 200 cells by the total WBC.

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Natural killer cell activity. The natural killer cell activity was measured using a previously detailed 4-hr chromium ($^{51}$Cr) release assay (Smialowicz et al., 1989). Spleen single-cell suspensions were prepared in CM and adjusted to give effector (E) to target YAC-1 (T) cell ratios (E:T) of 10:1, 5:1, and 25:1. Controls included a spontaneous $^{51}$Cr-release control (target cells + medium) (SR) and a total $^{51}$Cr release control (target cells + 10% Tween 20) (TR). Supernatants were harvested using a Skatron supernatant collection system (Mandrell Scientific, Rockwood, Ontario). Radioactivity in CPM was measured using a Beckman 4000 gamma counter (Beckman Instruments). Percentage of cytotoxicity was calculated as:

$$\% \text{ Cytotoxicity} = \frac{\text{Experim. (mean cpm) } - \text{SR (mean cpm)}}{\text{TR (mean cpm) } - \text{SR (mean cpm)}} \times 100.$$  

Phagocytosis. The phagocytic activity of monocytes and granulocytes in peripheral blood was measured using opsonized, FITC-labeled Escherichia coli bacteria according to a commercially available Phagostest kit (Orpegen, New Concept Scientific, Burlington, Ontario). The mean fluorescence intensity (MFI) of bacteria in the phagocytic cells and the percentage of phagocytic cells in peripheral blood were determined using the Becton–Dickinson FACScan.

Listeria monocytogenes infectivity assay. At the termination of treatment, 12 male rats/dose group were injected iv with a sublethal dose (4.55 x $10^8$ bacteria/ml) of L. monocytogenes suspension in physiologic saline according to previously established procedures (Tryphonas et al., 1991). Four rats/group were terminated at 24, 48, and 72 hr postinjection. The spleens were aseptically removed and single-cell suspensions were prepared. Serial twofold dilutions of the spleen/bacteria suspensions were prepared in peptone water and 0.1-ml aliquots were plated on trypticase soy agar. Colony-forming bacteria were enumerated using an automated colony counter (Biotron II, Automated Colony Counter, NBS Model C111, New Brunswick Scientific Co., Inc., Edison, NJ).

**STATISTICAL METHODS**

Average daily food consumption during the study was calculated for each rat. For each rat, the following linear growth curve was fit to the body weight data:

$$\text{body weight} = \alpha + \beta \ \text{day}, \quad (1)$$

where $\alpha$ represents the body weight ($g$) on Day 0 and $\beta$ represents the rate of growth ($g$/day).

The average daily food consumption and the parameter estimates from model (1) were analyzed using a one-way analysis of variance (ANOVA) model with treatment as the main effect. A test for linear trend in the response variables versus dose (Armitage, 1973) was carried out.

A preliminary analysis indicated that terminal body weight was not linearly related to the weight of the brain. Therefore, ANCOVA with body weight as a covariate was used for the analysis of the brain weights (Armitage, 1977).

For the lymphocyte transformation data, a two-way ANOVA model with day and treatment as the main effects and the interaction term was fit separately for each mitogen. Nonsignificant interactions ($p > 0.15$) were then dropped from the model. If an interaction involving treatment was not left in the model and there was a significant treatment effect, pairwise $t$ tests between the control and the 1-, 5-, and 15-$\mu$g dose groups were carried out.

For the calcium mobilization data a statistical analysis was performed for each measure across time by fitting nonlinear models and by analyzing estimates of maximum fluorescence and time to maximum fluorescence.

Data for the NK cell activity, phagocytosis, and L. monocytogenes infectivity assay were analyzed using univariate or multivariate ANOVA models (Morrison, 1976).

In all cases, data were tested for normality (Shapiro and Wilk, 1965) and screened for outliers (Barnett and Lewis, 1978).

**RESULTS**

There were no treatment-related effects for either the mean daily food consumption ($p = 0.16$) or for the parameter estimates from the growth curve model (parameter $\alpha$: initial body weight, $p = 0.65$; parameter $\beta$: growth rate, $p = 0.18$; data not shown). Similarly, there were no treatment-related effects for any of the organ weights ($p > 0.05$).

The covariate analysis of water consumption was significant ($p < 0.001$) indicating that as water consumption increased, so did urine output. The average water consumption was slightly higher for the control (mean $\pm$ SE, 39.03 $\pm$ 1.33) compared to the treated groups (mean $\pm$ SE, 35.67 $\pm$ 1.49; 34.98 $\pm$ 1.50; and 36.27 $\pm$ 0.90 for the 1-, 5-, and 15-mg/kg groups, respectively) although water consumption did not show a significant treatment effect ($p = 0.17$) (data not shown). Similarly, the average urine output was highest for the controls (mean $\pm$ SE, 21.17 $\pm$ 0.86) compared to the treated groups (mean $\pm$ SE, 18.84 $\pm$ 0.89; 17.69 $\pm$ 0.86; and 19.78 $\pm$ 0.73) for the 1, 5, and 15 mg/kg groups, respectively (data not shown).

There were no significant differences between the control and the treated groups for any of the hematological parameters measured or for any of the peripheral blood cell types analyzed with flow cytometry ($p > 0.05$) (data not shown).

For the male rats a significant dose-related linear trend toward decreased numbers of PFC/10$^6$ splenocytes ($p = 0.003$) and PFC/spleen ($p = 0.001$) was noted as the dose of fumonisin increased (Table 2). Pairwise comparisons (paired $t$ test) indicated a significant decrease in the percent-
Effects of a 14-Day Oral (Gavage) Exposure to Fumonisin B₁ on Serum Immunoglobulin (Ig) Levels of Male Sprague-Dawley Rats

<table>
<thead>
<tr>
<th>Dose (mg/kg body wt/day)</th>
<th>Ig Class</th>
<th>Mean (log₂ µg/ml ± SE)</th>
<th>Linear trend p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>IgG</td>
<td>3.14 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>IgG</td>
<td>3.06 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>IgG</td>
<td>3.19 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>(without rat No. 23)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>IgG</td>
<td>3.11 ± 0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.24 ± 0.05</td>
<td>0.04* (without rat No. 23)</td>
</tr>
</tbody>
</table>

A significant reduction in the number of PFC/10⁶ splenocytes (p = 0.004) and PFC/spleen (p = 0.002) was observed in the cyclosporin A-treated group compared to the control (Table 2). Similarly, significantly decreased spleen weights (p = 0.05) and adjusted spleen weights (p < 0.001) were observed in the cyclosporin A-treated group compared to the control (data not shown).

There were no significant treatment-related effects on the mitogen-induced lymphoproliferative activity of rat spleen mononuclear cells (p > 0.05) or on the intracellular Con A-induced calcium levels (p > 0.05) (data not shown). Similarly, there were no significant treatment-related effects on the NK cell activity (p > 0.05) or on the phagocytic activity of peripheral blood mononuclear phagocytes (p > 0.05) (data not shown).

A significant dose-related trend toward increased numbers of bacterial colonies was observed at 24 hr postinfection with L. monocytogenes (p = 0.013) (Fig. 1).

**DISCUSSION**

The present study indicated that the humoral immune response of Sprague-Dawley male rats was significantly affected by FB₁ treatment. In this study, the IgM response to SRBC, measured by enumerating PFC/10⁶ splenocytes and

<p>| TABLE 2 |
|--------------------------|-------------|----------------------|---------------------|
| Effects of a 14-Day Oral (Gavage) Exposure to Fumonisin B₁ on the Plaque-Forming Cell (PFC) Numbers of Male Sprague-Dawley Rat Splenocytes |</p>
<table>
<thead>
<tr>
<th>Dose (mg/kg body wt/day)</th>
<th>PFC/10⁶ spleen cells Log mean ± SE Percentage decrease</th>
<th>PFC/spleen Log mean ± SE Percentage decrease</th>
<th>Terminal body weight mean (g) ± SE Percentage decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.15 ± 0.18</td>
<td>13.61 ± 0.22</td>
<td>215.56 ± 4.58</td>
</tr>
<tr>
<td>5</td>
<td>6.64 ± 0.29</td>
<td>13.34 ± 0.30</td>
<td>211.50 ± 3.53</td>
</tr>
<tr>
<td>15</td>
<td>6.43 ± 0.41</td>
<td>12.82 ± 0.45</td>
<td>203.91 ± 4.32*</td>
</tr>
<tr>
<td>25</td>
<td>5.61 ± 0.54*</td>
<td>11.94 ± 0.51*</td>
<td>181.69 ± 5.48*</td>
</tr>
<tr>
<td>Linear trend for treatment effects</td>
<td>p = 0.003</td>
<td>p = 0.001</td>
<td>p = 0.001</td>
</tr>
</tbody>
</table>

Cyclosporine A (mg/kg body wt/day)

| Effect of dose | p = 0.004 | p = 0.002 |

* Statistically significant at p = 0.04 for linear trend when rat No. 23 which had a high IgG value was omitted from the analysis.
FUMONISIN B, IMMUNOTOXICITY IN SPRAGUE–DAWLEY RATS

FIG. 1. Effect of a 14-day oral (gavage) exposure to fumonisin B, on clearance of L. monocytogenes by the spleen of male Sprague–Dawley rats. Listeria were injected on Day 0 and the number of colony-forming bacteria was enumerated in the spleens 24, 48, and 72 hr postinfection. Plots are means ± SE of the log colony-forming bacteria/spleen (four rats/group/day). A statistically significant (p < 0.013) dose-related trend toward increased numbers of bacterial colonies was observed at 24 hr post infection with L. monocytogenes.

the PFC/spleen, was affected in a dose-related fashion with the treated groups having uniformly lower anti-SRBC PFC numbers compared to the control. A pairwise comparison between each of the treated groups and the control indicated that the PFC numbers were significantly different from the control only at the 25 mg/kg body wt/day dose. At this dose level the terminal body weights of rats also decreased significantly compared to the control suggesting that toxicity may have, in part, influenced the PFC numbers indirectly.

Similar effects on humoral immunity have also been reported by Marijanovic et al. (1991) and by Martinova and Merrill (1995). Marijanovic et al. (1991) fed culture isolates of moniliforme, which are known producers of FB, to chicken for 6 weeks. A reduction in serum levels of antibodies to SRBC and Brucella abortus were reported for the treated chicken compared to the control (Marijanovic et al., 1991). Martinova and Merrill (1995) reported on the effects of FB, on the PFC numbers in mice immunized with SRBC either concurrently with or prior to treatment initiation. In these experiments, however, the direction of the response to SRBC was related to the time the animals were immunized relative to treatment and the duration of treatment. Thus, a significant increase in the response to SRBC over the control was evident when SRBCs were administered prior to treatment with varying doses of FB, while a decrease in the response to SRBC was observed when a single dose of FB, was administered concurrently with SRBC immunization. Furthermore, the response to SRBC in the untreated (control) group was unusually low which raises the question of whether the SRBC dose used for immunization was optimized for maximum antibody production. For these reasons, interpretation of these data can only be speculative.

In the present study, the effect on the PFC numbers were observed in the male rats only suggesting that the effects of fumonisin may be gender related. Similar sex-associated differences between male and female rats have also been reported for other toxicological endpoints (Bondy et al., 1996). While the reason for the differences in sensitivity to fumonisin treatment between male and female rats is not clear, it has been previously shown that after a single oral dose of FB, most of the toxin appears in the feces of rats within 24 hr with trace amounts of the administered toxin appearing in the urine, liver, kidneys, and red blood cells (Shepard et al., 1992a, 1992b). Although FB, is rapidly cleared from the blood, elevated levels of free sphingosine and sphinganine and elevated free sphinganine:sphingosine ratios have been detected in the kidney and liver of male compared to female rats (Riley et al., 1994). Since FB, is a potent inhibitor of ceramide synthase, an enzyme critical to sphingolipid biosynthesis, and since sphingolipids play multiple roles in cellular function (Merill et al., 1996), it is likely that the observed functional alterations of the immune system are related to disruption of these pathways.

While the data unequivocally indicate that FB, treatment resulted in immunosuppression in the male rats, the mechanism of action of FB, on the multistage events involved in T lymphocyte-dependent immune responses to antigens such as SRBC are not well understood. It is possible that fumonisin may have affected the function of the phagocytic cells since FB, treatment had a profound effect on the clearance
of *L. monocytogenes* by FB₁-treated rat spleens at 24 hr following injection with the bacteria at which time clearance by the phagocytic mononuclear lineage of cells is most crucial (Cheers et al., 1978). While there were no significant effects on the in vitro phagocytic activity of peripheral blood monocytes and granulocytes in the present study, others (Qureshi and Hagler, 1992) have noted morphological alterations including cytoplasmic blebbing and nuclear disintegration in chicken peritoneal macrophages incubated in vitro to 0.5, 5.0, and 10.0 Mg FB₁/ml. It is possible, therefore, that the observed lag in the clearance of *Listeria* during the first 24 hr as observed in the present study was a consequence of a defect in the cell’s ability to enter the respiratory burst activity phase.

In summary, results of this study indicated that FB₁ significantly affected the humoral immunity of male but not female rats. This was shown by a dose-related decrease in the SRBC PFC numbers of rats gavaged daily with doses of FB₁ ranging from 5 to 25 mg/kg body wt. The ability of male rat splenocytes to clear the *L. monocytogenes* from the circulation was also compromised at the early stages of infection suggesting that the mononuclear phagocytic cell function may be affected by treatment. However, at 72 hr all groups of rats were successful in eliminating the bacteria and thus overcoming the *Listeria* infection. This, combined with the observation that several other immune related parameters investigated in the same rats who were administered FB₁ doses up to 15 mg/kg body wt of FB₁ were not affected by treatment suggested that the observed effects may have not been of clinical significance. However, the potential consequences the observed immunosuppression would have on rat and indirectly on human health following long-term exposure to FB₁ is not known. Consequently, further research focused on the potential immunotoxic effects of FB₁ using long-term feeding strategies is required.

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**REFERENCES**


