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Host resistance to *Trypanosoma cruzi* infection is enhanced in mice fed *Fusarium verticillioides* (= *F. moniliforme*) culture material containing fumonisins

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Abstract

Fumonisins, metabolites of *Fusarium verticillioides* (= *F. moniliforme*) and related fungi that occur naturally on corn, elicit various organ- and species-specific toxicities. However, immunologic effects of fumonisins are not well characterized. BALB/c mice were fed diets containing *F. verticillioides* culture material (CM) providing 50 (LD) or 150 (HD) ppm fumonisins ($FB_1 + FB_2$) beginning 1 week before and continuing 5 weeks after challenge with the myotropic Brazil strain of *T. cruzi*. A control group (ZD) was fed a diet lacking CM. The LD and HD diets caused increases in tissue sphinganine/sphingosine ratios and minimum to mild hepatotoxicity, both of which are typically induced by fumonisins. Nitric oxide (NO) production by peritoneal macrophages from HD mice was significantly higher than by peritoneal macrophages from ZD mice on day 14 after challenge. NO production also was stimulated in macrophages from ZD mice, but the peak response did not occur until day 26 after challenge. Compared with ZD mice, LD and HD mice exhibited reduced parasitemia and decreased numbers of pseudocysts in cardiac muscle. Thus, the CM increased host resistance to *T. cruzi* by accelerating NO production by macrophages or otherwise enhancing the immune response. The findings provide additional evidence that fumonisins modulate immune function.
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Keywords: Fumonisin; *Fusarium verticillioides*; Host resistance; Macrophage; Nitric oxide; *Trypanosoma cruzi*

1. Introduction

Fusarium verticillioides (= *F. moniliforme*), a common fungus found in corn, produces a variety of mycotoxins that include fumonisins (Gelderblom et al., 1988). Fumonisins occur in corn products intended for human or animal consumption and have been detected (primarily FB_1) in high levels in corn from South Africa (Rheeder et al., 1992), China (Yoshizawa et al., 1994) and elsewhere (Dutton, 1996; Marasas, 2001). Animal diseases caused by fumonisins include leukoencephalomalacia in horses (Marasas et al., 1988), pulmonary edema in swine (Harrison et al., 1990) and liver and kidney cancer in rats and mice (Gelderblom et al., 1988; Howard et al., 2001). In addition, consumption of corn contaminated with fumonisins has been implicated as a cause of human esophageal cancer (Sydenham et al., 1991; Rheeder et al., 1992; Yoshizawa et al., 1994; Marasas, 2001).

Immunosuppressive effects of *F. verticillioides* culture material (CM), *F. proliferatum* CM, or purified FB_1 fed

Abbreviations: CM, *F. verticillioides* culture material; FB_1, fumonisin B_1; FBS, fetal bovine serum; FDT, fibroblast derived trypomastigotes; HD, high dose group; LD, low dose group; LIT, liver infusion tryptose medium; LPS, lipopolysaccharide; MPS, mononuclear phagocytic system; NO, nitric oxide; iNOS, inducible nitric oxide synthase; PFC, plaque-forming cell; PI, post-infection; PKC, protein kinase C; Sa/So, sphinganine/sphingosine; SRBC, sheep red blood cells; ZD, control group.

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to chickens included decreased antibody production, macrophage functions and lymphocyte proliferation (Marijanovic et al., 1991; Qureshi et al., 1995; Li et al., 1999). In addition, in vitro exposure of chicken macrophages to FB_1 induced both morphological and functional alterations (Qureshi and Hagler, 1992; Chatterjee and Mukherjee, 1994). With rodents, FB_1 decreased responses to sheep red blood cells (SRBC) (Martinova and Merrill, 1995), increased serum IgG, decreased splenic clearance of *Listeria monocytogenes* (Tryphonas et al., 1997), and increased secretion of TNF-α by peritoneal macrophages (Dugyala et al., 1998). Further, a murine macrophage cell line produced elevated concentrations of nitric oxide (NO) upon exposure to FB_1 (Rotter and Oh, 1996).

Infectious challenge models have been used to assess the immunotoxic effects of many environmental toxins (Bradley and Morahan, 1982). *Trypanosoma cruzi*, the etiologic agent of Chagas' disease in humans, was selected as the challenge organism for this host resistance study because the complex immune response of the murine host to the parasite's intracellular (amastigote) and intercellular (trypomastigote) life cycle stages is well characterized and involves both cellular and humoral immune components. In addition, *T. cruzi* is an excellent model as a result of the similarity in disease produced in mice and humans (Hoff and Boyer, 1985).

Although fumonisin-induced liver and kidney toxicities in rodents are well documented, effects of fumonisins on the immune system are largely unknown. Considering the inhibitory effect of fumonisins on sphingolipid biosynthesis (Wang et al., 1991) and the importance of sphingolipid intermediates as cellular messengers (Merrill et al., 1997), fumonisins are likely to affect one or more components of the immune system (Martinova and Merrill, 1995). Following a strategy that has been used successfully to show that carcinogenicity and other in vivo effects of *F. verticillioides* were due to fumonisins, CM was used as the fumonisin source for this initial investigation on host resistance to *T. cruzi*. The specific objectives of the study were: (a) to examine host resistance to *T. cruzi* infection in mice fed *F. verticillioides* CM and (b) to measure NO production by peritoneal macrophages from the infected mice.

2. Materials and methods

2.1. Preparation of diets

Diets were prepared by mixing CM (moldy corn produced from a single fungal isolate under laboratory conditions) of *F. verticillioides* strain MRC 826 and a certified rodent diet (Tek-lad #8604) using a Patterson Kelley V blender with a stirring intensifier bar. Dietary fumonisin (FB_1 plus FB_2) concentrations were confirmed by HPLC (Voss et al., 1996). Diets were stored at 0 °C.

2.2. Animals

Five to 6 week-old female BALB/c mice (Harlan Sprague-Dawley, Indianapolis, IN, USA) were housed in groups of four in plastic cages with filter-barrier tops and Cell Sorb Plus recycled paper bedding (Fangman Specialties, Inc., Cincinnati, OH, USA). Mice were acclimated for 1–2 weeks and were maintained in a controlled environment (12-h light/dark cycle, 23 °C, 50% relative humidity) with food and water provided ad lib.

2.3. Parasites

The myotropic Brazil strain of *T. cruzi* (provided by Dr. Rick Tarleton, The University of Georgia, Athens, GA, USA) was maintained as culture epimastigotes in liver infusion tryptose medium (LIT) at 28 °C or as bloodstream trypomastigotes by serial passage in mice every 4–5 weeks. For in vitro experiments, fibroblast-derived trypomastigotes (FDT) were obtained from cultures of 3T3 murine fibroblasts as previously described (Davis and Kuhn, 1990).

2.4. Experimental design

Mice were randomized into three groups of 24, designated ZD, LD and HD (zero, low and high dose, respectively), which were fed diets containing 0, 50 or 150 ppm fumonisins, respectively, as provided by the CM. Animals were observed daily. Body weight and food consumption were monitored twice weekly. Mice were maintained on their respective control or test diets beginning 6 days (day −6) before parasite inoculation, at which time (day 0) 10^3 bloodstream trypomastigotes of the Brazil strain of *T. cruzi* were administered IP (Table 1). In addition, four uninfected mice per group served as controls for fumonisin toxicity ("satellite" group).

Five mice per group were euthanized by cervical dislocation under CO_2 narcosis on days 0, 14, 26 and 36, and all uninfected mice were euthanized on day 35. Peritoneal cell exudate and organs (liver, kidney, heart and skeletal muscle) were collected from each. Liver and kidney were weighed and portions of each frozen at −80 °C for sphingolipid analysis. Portions of liver, kidney and other organs were fixed in 10% neutral buffered formalin for standard microscopic examination.

2.5. CM toxicity

The toxicity of CM was assessed by microscopic examination of liver and analysis of liver and kidney

Table 1
Experimental design. BALB/c mice randomized by weight into three groups were fed *F. verticillioides* CM to provide 0, 50 or 150 ppm fumonisins in the diet, beginning 6 days prior to infection with *T. cruzi* and initiation of toxicity assessments[a]

Day	Treatment/assessment					
−6	CM					
0	CM	TC	BT	SL	LP/WT	PP/NO
8	CM		BT			
14	CM		BT		LP/WT	PP/NO
20	CM		BT			
26	CM		BT		LP/WT	PP/NO
31	CM		BT			
36	CM		BT	SL	LP/WT	PP/NO

CM = initiation and continuation of CM feeding ad lib. TC = injection (ip) of mice with 10^3 trypomastigotes of *T. cruzi*. For each of the following assessments, five mice/group were used on the designated day: SL = measurement of kidney and liver free sphinganine and free sphingosine. LP/WT = assessment of liver pathology and liver and kidney weights. PP/NO = enumeration of tissue parasites and nitric oxide production. BT = enumeration of bloodstream trypomastigotes.

[a] Four uninfected mice per dose group were maintained as "satellite" control groups, that were euthanized on day 35 with assessments of SL, LP/WT and NO.

sphinganine and sphingosine. Evidence of fumonisin-induced liver injury was subjectively scored on a scale of 0–3 using previously established morphologic criteria: 0 = no sign of fumonisin exposure, 1 = minimum changes, 2 = mild but more obvious changes, and 3 = moderate changes (Voss et al., 1995; Sharma et al., 1997). Tissue sphinganine and sphingosine were measured by HPLC using previously described methods (Riley et al., 1994).

2.6. Parasitemia and tissue parasites

Blood was collected from the retro-orbital sinus from five mice (lightly anesthetized) per dose group on days 8, 14, 20, 26, 31 and 36. Following dilution of 10 μl blood suspension with 90 μl ACK lysing buffer, trypomastigotes were counted in four corner squares (1 mm×1 mm×0.1 mm) on two sides of a Neubauer hemacytometer (for a total volume of 0.8 μl) using light microscopy.

Numbers of *T. cruzi* pseudocysts containing amastigotes in cardiac and skeletal muscle were determined by microscopic examination of hematoxylin and eosin stained sections. For each organ, nine sections (three serial sections taken from three sites) were examined by counting the number of pseudocysts in 40 fields per section (10× ocular and 40× objective).

2.7. Nitric oxide production by peritoneal macrophages

Peritoneal macrophages were collected from mice immediately upon euthanization by peritoneal lavage using complete RPMI-1640 medium (Cellgro, Herndon, VA, USA) supplemented with 20 mM HEPES, 1 mM L-glutamine, 5% fetal bovine serum (FBS) (BioWhittaker, Walkersville, MD, USA), 20 U/ml penicillin, and 20 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO, USA). Peritoneal cells (>95% viability as determined by trypan blue exclusion) were seeded in 96-well tissue culture plates to contain 2×10^5 cells per well in 100 μl of complete RPMI-1640 medium. Following cell adherence for 12 h at 37 °C and 5% CO_2 in a humidified chamber, each well was washed with warm sterile PBS to remove non-adherent cells. To each well, 100 μl of fresh complete RPMI-1640 medium containing 10 μg/ml lipopolysaccharide (LPS) (Sigma) was added to stimulate the production of NO. Cell cultures were incubated at 37 °C and 5% CO_2 for 72 h. At the end of the incubation period, a 50-μl aliquot of supernatant was collected from each well for quantification of NO.

Nitric oxide was measured indirectly as nitrite by the Griess reaction (Ding et al., 1988). Peritoneal cell supernatant (50 μl) was incubated for 10 min with equal volumes of 1% sulfanilamide and 0.1% *N*-1-naphthylethylenediamine. Optical density (OD) was read at 540 nm with a microplate reader (Titertek Multiscan MCC/340), and nitrite concentration was determined by comparison with a standard curve of nitrite (0–200 μM).

2.8. In vitro studies with T. cruzi and fumonisin B_1

Fibroblast derived trypomastigotes (4.25×10^4 FDT/ml) were incubated with 0, 10, 25 or 50 μM FB_1 (98% pure; Sigma) in complete RPMI-1640 medium at 37 °C. Viable trypomastigotes (motile as viewed by light microscopy, 400×) were counted after 6, 24, 48 and 72 h of incubation using a Neubauer hemacytometer.

To assess the effects of FB_1 on the ability of FDT to invade mammalian cells, 10^5 FDT previously incubated for 48 h with FB_1 (0, 10, 25 or 50 μM FB_1 in complete RPMI-1640 medium) were added to adherent 3T3 fibroblasts (Dr Lisa Bain, Clemson University) in 24-well plates (20,000 cells/1 ml/well). After 4 h, cells were washed to remove parasites that had not invaded cells. At the end of the 72-h incubation period, cells were fixed with methanol, stained with Giemsa, and observed by light microscopy. Percent of infected cells was determined by counting 200 cells per well.

Intracellular replication of *T. cruzi* in 3T3 cells exposed to FB_1 was monitored by adding 2×10^5 FDT to adherent 3T3 fibroblasts (20,000 cells/1 ml/well in complete RPMI-1640 medium) in 24-well tissue culture plates. Parasites were allowed to invade for 4 h before cells were washed to remove free parasites and fresh complete RPMI-1640 medium containing 0, 10 or 50 μM FB_1 was added. After 3 or 5 days, cells were fixed with methanol, stained with Giemsa, and numbers of parasites per infected cell recorded, with at least 200 cells per well being counted using light microscopy.

As trypomastigotes cannot be maintained in culture medium, transitional intercellular epimastigote forms of *T. cruzi* (10^3 parasites/ml) were exposed to 0, 5 or 10 μM FB_1 in 5 ml of complete LIT supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Parasites were incubated at 28 °C for 24 days and viable parasites counted every 3 days using a Neubauer hemacytometer and light microscopy.

2.9. Statistical analysis

Continuous data were analyzed by ANOVA followed by Duncan's multiple range test or Fisher's least significant differences test. Non-parametric variables were analyzed using the Kruskal-Wallis test (Ott, 1993; Voss et al., 1996). Significance was judged at $P < 0.05$.

3. Results

3.1. CM toxicity

CM had no effect on survival, appearance or food consumption. Body weights of HD mice infected with *T. cruzi* were slightly decreased (10.5–11.9% lower than ZD mice) only on days 21 and 25 (data not shown), and no differences in body weight were found between the ZD and LD groups.

Relative liver weights (% body weight) of LD or HD mice infected with *T. cruzi* did not differ from infected ZD mice at any time except day 26. At that time, relative liver weights of HD (6.2%) mice were increased compared to the LD (5.1%) and ZD (4.4%) mice. No significant differences in relative liver weights were found among uninfected ZD, LD and HD mice. Relative kidney weights of HD mice (1.8%) were increased compared to the other groups (1.4–1.5%) on day 0 (prior to infection). Thereafter, relative kidney weights of all groups were similar.

On day 0, prior to *T. cruzi* inoculation, liver sphinganine/sphingosine (Sa/So) was increased in the HD group (Fig. 1). Similar dose-dependent increases in liver Sa/So were found in the uninfected "satellite" mice and *T. cruzi*-infected mice at the end of the study. Kidney Sa/So followed a similar pattern (data not shown).

The CM caused obvious but mild hepatocellular apoptosis and anisocytosis (differences in cell size) in the liver of both uninfected "satellite" and infected mice (Table 2). These lesions were consistent with the known effects of fumonisins (Voss et al., 1995; Sharma et al., 1997). Although developing earlier in the HD mice, the severity of these lesions in LD and HD animals was

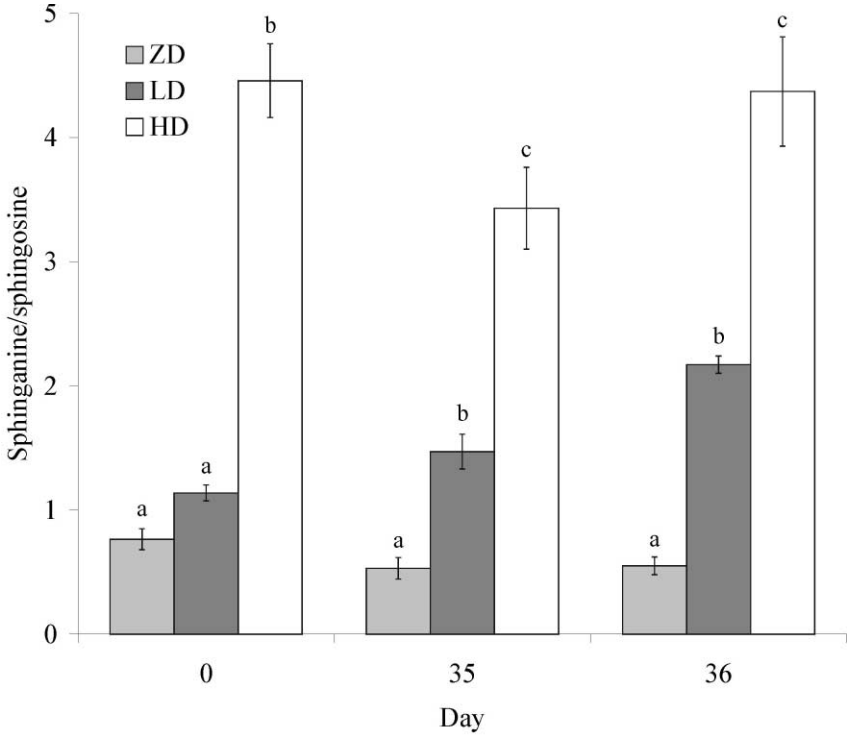

Fig. 1. Liver sphinganine/sphingosine ratios of BALB/c mice on days 0, 35 (uninfected mice) and 36 (*T. cruzi* infected mice). The animals were fed control diets (ZD) or diets containing 50 (LD) or 150 (HD) ppm fumonisins provided by *F. verticillioides* CM beginning 6 days (day −6) before infection (day 0) with *T. cruzi*. Bars represent the group means with S.E.M. ($n = 3$–5). Different letters indicate significant differences among groups on the same day ($P < 0.05$).

Table 2
Liver pathology scores for apoptosis, anisocytosis and focal chronic inflammation of *T. cruzi*-infected BALB/c mice fed *F. verticillioides* CM containing fumonisins[a]

	Fumonisin dose group	Days post-infection[b]				
		0	14	26	36	35[c]
Apoptosis	ZD	0	0.4	0.2	0[d]	0
	LD	0	0.4	1.8	1.2	0.75
	HD	0.4	1.2	1.4	1.2	1.0
Anisocytosis	ZD	0	0	0	0[d]	0
	LD	0	0	0.2	0.6	0
	HD	1.0	0.6	0.2	0.8	1.0
Focal chronic inflammation	ZD	0	1.0	0.8	0.75[d]	0
	LD	0	1.0	1.0	1.2	0
	HD	0	1.2	1.0	1.2	0

0 = No sign of fumonisin exposure, 1 = minimal changes, 2 = mild but more obvious changes, 3 = moderate changes.
[a] Values indicate group mean score ($n = 5$).
[b] Feeding of diets containing CM began on day −6; mice were infected with *T. cruzi* on day 0.
[c] Day 35 represents uninfected "satellite" control group mice ($n = 4$).

comparable at the end of the study. Focal chronic inflammation was found only in infected ZD, LD and HD mice (Table 2). These lesions, not typically seen in fumonisin exposed mice, were of similar intensity in all groups and attributed to *T. cruzi* infection.

3.2. *Parasitemia and* T. cruzi *muscle pathology*

Numbers of trypomastigotes counted in peripheral blood did not differ among groups on days 8, 14, 20 or 36 (Fig. 2). On day 26, parasite numbers for ZD mice

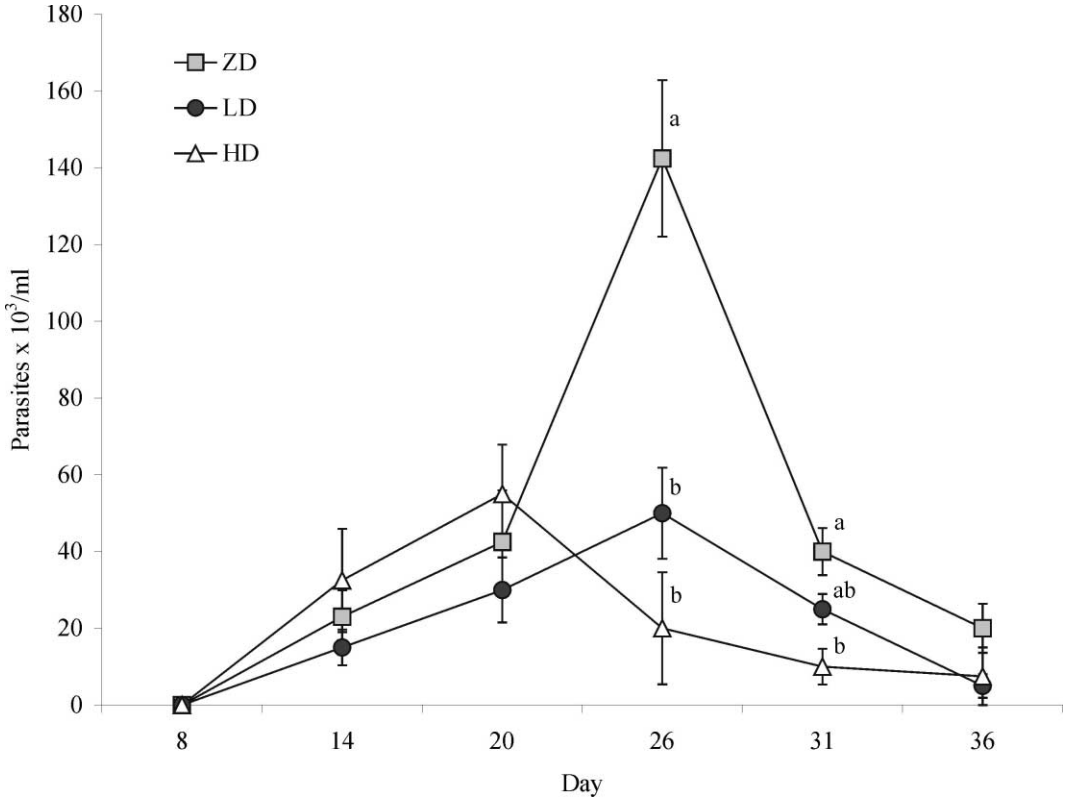

Fig. 2. Numbers of trypomastigotes in the blood of BALB/c mice during acute infection with *T. cruzi*. Animals were fed control diets (ZD) or diets containing 50 (LD) or 150 (HD) ppm fumonisins provided by *F. verticillioides* CM beginning 6 days (day −6) before infection (day 0) with *T. cruzi*. Values are group means with S.E.M. ($n = 5$ for each group). Different letters indicate significant differences among groups on the same day ($P < 0.05$).

peaked, were significantly higher than for both LD and HD mice, and remained significantly higher than HD mice on day 31. Peak parasitemia for HD mice was observed on day 20, almost a week earlier than for the peak observed for ZD mice. Parasitemia decreased thereafter for HD mice, and by day 26 only two of five HD mice displayed bloodstream parasites. Similar to ZD mice, parasitemia of LD mice peaked on day 26. Parasite numbers for both the HD and LD mice remained relatively low throughout the infection, with peak parasitemias of fumonisin-exposed mice being only 40% that of control (ZD) mice. On day 36, no significant differences were noted among groups; however, circulating parasites were observed in four of five ZD mice, but in only one of five HD and two of five LD mice.

Density of *T. cruzi* pseudocysts (intracellular aggregates of amastigotes) in cardiac muscle appeared to correlate with parasitemia, as less tissue colonization by the parasite was observed in LD and HD mice than in ZD mice (Fig. 3). While numbers of pseudocysts were only significantly greater for ZD mice as compared to both LD and HD mice on day 26, tissue pseudocyst counts in the ZD group were two times (or more) higher than those in the latter groups on day 14. Parasites were not observed in cardiac muscle on day 36. No significant differences in mean pseudocyst counts in skeletal muscle were noted among groups, with values ranging from 0.28 ± 0.05 to 0.77 ± 0.89.

3.3. NO production by peritoneal macrophages

Production of NO by peritoneal macrophages did not differ among groups on day 0 (Fig. 4). A dose-related increase in NO production was observed in LD and HD mice on day 14; however, statistical significance was established only in the HD group (three-fold higher than ZD). In contrast, on day 26, NO production by macrophages from ZD mice was two-fold higher than that of HD macrophages. Peak levels of NO produced by HD macrophages on day 14 and ZD macrophages on day 26 were similar. At the end of the study (day 36), no differences in NO production by ZD, LD and HD macrophages from infected mice were observed. Likewise, there were no differences in NO production by macrophages collected from the uninfected "satellite"

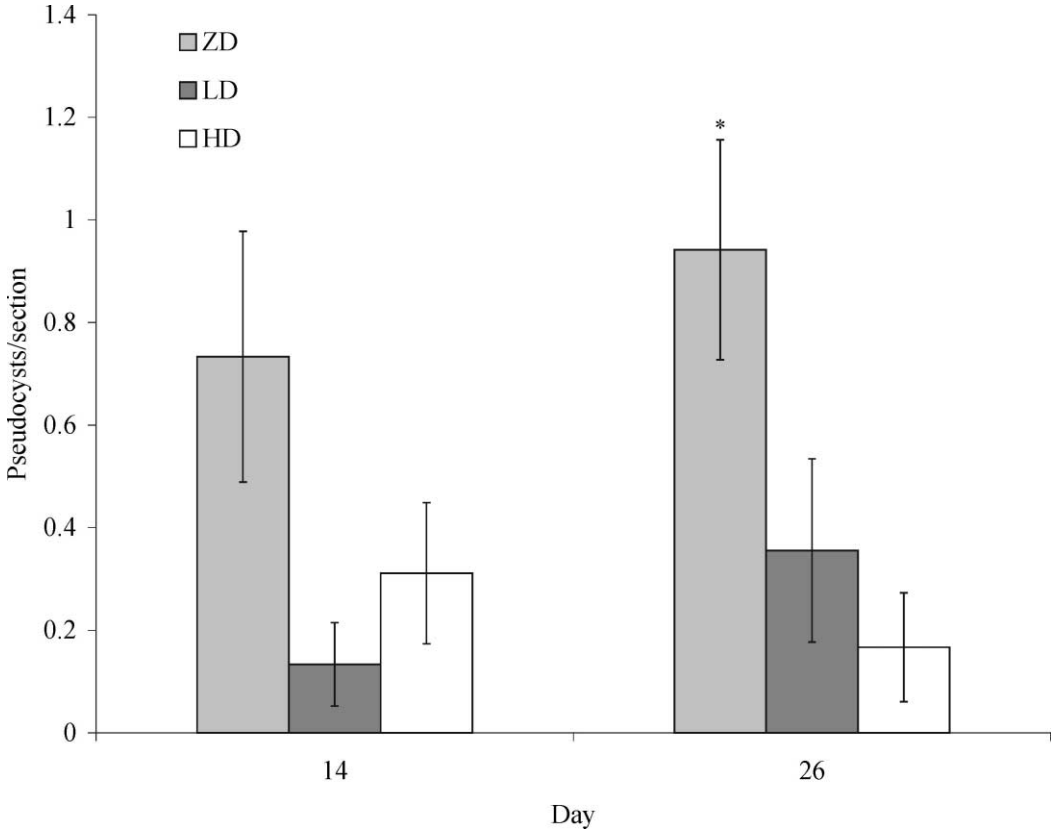

Fig. 3. Mean numbers of *T. cruzi* pseudocysts counted per section (40 fields at ×40) in three serial sections taken from three different sites of cardiac muscle from BALB/c mice during acute infection with *T. cruzi*. Animals were fed control diets (ZD) or diets containing 50 (LD) or 150 (HD) ppm fumonisins provided by *F. verticillioides* CM beginning 6 days (day −6) before infection (day 0) with *T. cruzi*. *Indicates significant differences among groups on the same day ($P < 0.05$).

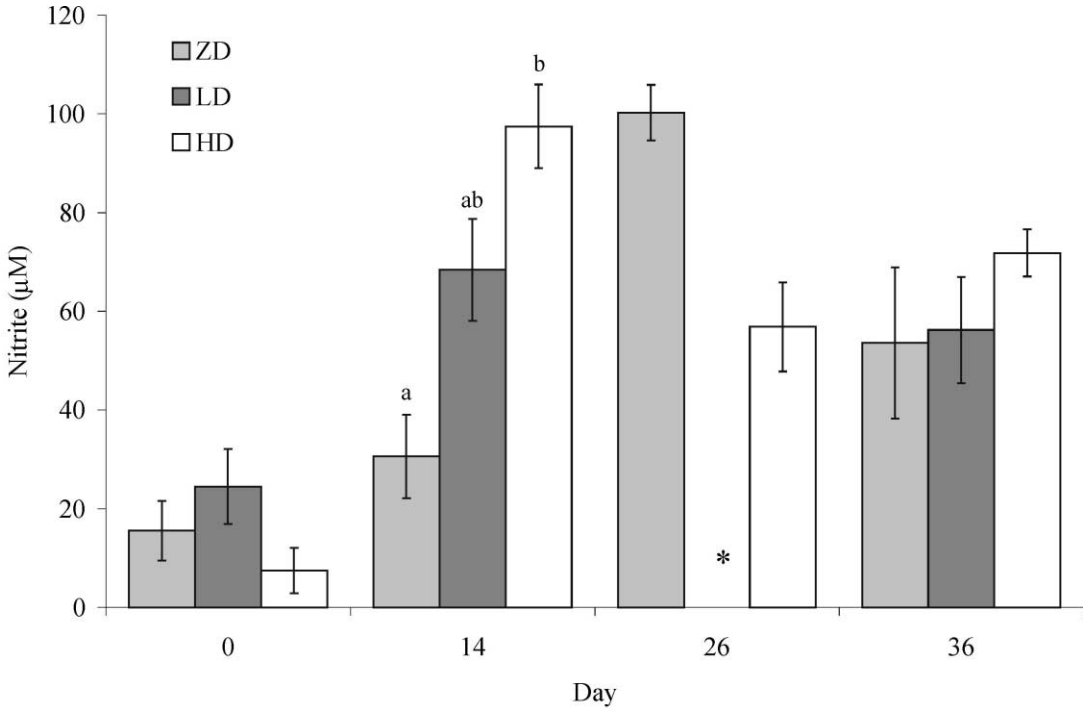

Fig. 4. Nitric oxide production (measured indirectly as nitrite following stimulation with 1 μg/ml LPS for 72 h) by peritoneal macrophages harvested from *T. cruzi* infected BALB/c mice on days 0, 14, 26 and 36. Groups were fed control diets (ZD) or diets containing 50 (LD) or 150 (HD) ppm fumonisins provided by *F. verticillioides* CM beginning 6 days (day −6) before infection (day 0) with *T. cruzi*. Different letters indicate significant differences among groups on the same day ($P<0.05$). Values are group means with S.E.M. indicated by the error bars ($n=3$–5 for each group). Different letters indicate significant differences among groups on the same day ($P<0.05$). *Indicates no sample available due to contamination.

groups (day 35, data not shown). NO production from ZD, LD and HD macrophages from infected mice was consistently two- to three-fold higher than NO production by macrophages from uninfected mice on days 0 and 35 (data not shown).

3.4. *In vitro studies with* T. cruzi *and* FB_1

Incubation with 0–50 μM FB_1 was not toxic to non-replicating FDT, in terms of motile trypomastigote numbers, over a 72-h exposure period. Further, pre-incubation of the FDT with 0–50 μM FB_1 for 48 h had no effect on their ability to infect 3T3 fibroblasts. With intracellular *T. cruzi* amastigotes, replication in 3T3 cells (numbers of amastigotes/host cell) did not differ among FB_1 dose groups on days 3 or 5. Proliferation of *T. cruzi* epimastigotes in LIT medium was affected by exposure to FB_1, but not significantly so until day 9 when numbers of epimastigotes in control medium (13.38×10^5 epimastigotes/ml) were nearly twice that of parasites exposed to 5 and 10 μM FB_1 (6.25×10^5 and 7.63×10^5 epimastigotes/ml, respectively). Although growth patterns for all dose groups were similar from day 9 through day 24, numbers of parasites not incubated with FB_1 remained significantly higher than those incubated with 5 or 10 μM on days 12, 18, 21 and 24.

4. Discussion

Most previous reports suggest that *F. verticillioides* and fumonisins are immunotoxic. Reduced antibody titers were observed when chickens fed *F. verticillioides* CM of isolate MRC 826, the same fumonisin producing strain used in the current study, were challenged with *Brucella abortus* (Marijanovic et al., 1991). Immunosuppressive effects also were found in broiler chicks fed FB_1 (decreased secondary antibody titers, decreased lymphocyte proliferation in response to mitogens, and higher numbers of bacterial colonies in blood, spleen and liver) (Li et al., 1999) and White Leghorn Cornell chicks fed *F. proliferatum* CM (suppressed immunoglobulin levels and decreased phagocytic activity of macrophages) (Qureshi et al., 1995). In rats, fumonisin exposure (gavage of 25 mg FB_1/kg body weight/day) resulted in decreased ability to clear *Listeria monocytogenes* from spleen and decreased plaque-forming cell (PFC) response to SRBC (Tryphonas et al., 1997). Additional evidence for the immunotoxicity of fumonisins was reported by de Nij et al. (1999), who found decreased resistance to *Trichinella spiralis* infection in rats given 0.75 mg/kg FB_1 via the drinking water for 10 weeks. Decreased production of anti-*T. spiralis* IgG also was found in rats given equal to or greater than 0.19 mg/kg FB_1.

Interestingly, Martinova and Merrill (1995) reported that the PFC response of BALB/c mice was decreased following a single ip injection of 5–100 μg FB_1 plus SRBC, yet was increased significantly when 1–50 μg FB_1 was administered for 4 consecutive days following the initial ip injection of FB_1 and SRBC. Thus, immunological effects of fumonisins may depend on a number of variables, including species, dose, length of exposure, nature of the challenge organism, and other experimental circumstances. Results of the current in vivo study, like those of Martinova and Merrill (1995), suggest an enhanced immune response in BALB/c mice exposed to fumonisins.

Activation of the murine mononuclear phagocytic system (MPS) is known to be involved in host destruction of *T. cruzi* (Kierszenbaum et al., 1974), with macrophages playing a key role in the complex host–parasite relationship. Macrophages are stimulated to produce NO, which has cytotoxic activity against many pathogens (MacMicking et al., 1997) and directly kills *T. cruzi* in vitro (Vespa et al., 1994). In studies by Munos-Fernandez et al. (1992), in vitro activation of macrophages by IFN-γ and TNF-α led to significant increases in NO production and clearance of *T. cruzi*. In vivo studies indicated that mice deficient in inducible nitric oxide synthase (iNOS) were unable to produce NO and did not survive acute *T. cruzi* infection (Holscher et al., 1998). Following treatment with FB_1, a murine macrophage cell line (RAW 264.7) and rat splenic macrophages exhibited increased NO production upon stimulation (Rotter and Oh, 1996; Dombrink-Kurtzman et al., 2000). Similarly, iNOS expression was enhanced in LPS-stimulated J774.A1 murine macrophages treated with FB_1 (Meli et al., 2000). Further, peritoneal macrophages harvested from mice injected with FB_1 secreted increased amounts of TNF-α, a cytokine that amplifies NO production (Dugyala et al., 1998). Our results are consistent with these effects of FB_1 on NO production by macrophages and provide direct evidence that the *F. verticillioides* CM and fumonisins enhance macrophage sensitivity to NO-inducing stimuli in vivo.

Previous studies with macrophages from *T. cruzi*-infected mice have shown that NO production increased along with parasitemia and then decreased as parasitemia was controlled (Vespa et al., 1994). In the current study, peak NO production by ZD macrophages coincided with peak parasitemia (day 26) as expected. However, peak NO production by HD macrophages occurred earlier, on day 14. Because NO production was not measured on day 20, at which time peak parasitemia occurred in the HD mice, it is not known whether NO production preceded or occurred simultaneously to parasitemia in this group. Nevertheless, the findings indicate that an enhanced macrophage response did occur earlier in CM-exposed mice and, given the importance of NO for controlling *T. cruzi* infection, suggest that the accelerated NO response was critical for increasing host resistance. The long-term effects of CM and fumonisins on *T. cruzi* resistance need to be established. Decreased parasitism during acute *T. cruzi* infection, as observed here, is likely to increase host survival; however, enhancement of macrophage activation and NO production could also cause significant chronic tissue damage by intensifying the host inflammatory response.

Fumonisins are known to competitively inhibit ceramide synthase, resulting in disruption of cellular sphingolipid synthesis (Wang et al., 1991), and to increase liver and kidney Sa/So in mice (Martinova and Merrill, 1995; Tsunoda et al., 1998; Sharma et al., 2000). Sphingolipids are found in all cells and have multiple cellular functions, including regulation of cell growth and mediation of signaling pathways (Merrill et al., 1997). Therefore, disruption of sphingolipid metabolism by fumonisins could have enhanced macrophage production of NO by altering cell signaling pathways, such as those leading to transcription of iNOS.

It must be recognized that other, non-immunologic effects of *F. verticillioides* and fumonisins may also contribute to increased host resistance. For example, prior studies demonstrated that entrance of infective *T. cruzi* trypomastigotes into mammalian cells is initiated by attachment of parasite membrane components to the host cell membrane (Nogueira and Cohn, 1976; Villalta et al., 1999). Therefore, disruption of sphingolipid metabolism by fumonisins may have interfered with invasion by *T. cruzi* by depleting critical sphingolipid cell membrane components. Secondly, a report by Villalta et al. (1999) suggested that activation of protein kinase C (PKC) correlated with an up-regulation of trypanosome entry into macrophages. Interestingly, repressed expression of PKC by FB_1 was reported in CV-1 cells (Huang et al., 1995), probably due to an increase in sphingosine, which is a known inhibitor of PKC (Merrill et al., 1997). Despite these possibilities, our parasitemia data strongly suggest that the parasite invaded and replicated within all groups of mice to a similar extent through day 20. Furthermore, our in vitro studies demonstrated that concentrations of FB_1 cytotoxic to host cells (10–50 μM) were not toxic to *T. cruzi* trypomastigotes and did not affect the ability of *T. cruzi* to infect and replicate in murine fibroblasts. Although exposure to FB_1 decreased proliferation of *T. cruzi* epimastigotes, these forms occur primarily in the invertebrate intermediate host. The toxicokinetics of FB_1 in mice are not known, but have been studied in a number of species (reviewed by Voss et al., 2001). In the rat and other species, only small amounts of fumonisins are found in tissues and blood after oral exposure (Norred et al., 1993) and, based on our in vitro observations, probably cannot reach concentrations high enough to affect *T. cruzi* in the vertebrate host.

In conclusion, this study provides additional evidence that fumonisins affect immune function in vivo and indicates that mice infected with *T. cruzi* provide a useful model for studying the immunomodulatory effects of fumonisins. Host resistance coincided with early induction of NO production by peritoneal macrophages, indicating that the fumonisin-containing CM increased sensitivity of the macrophages to NO producing stimuli. Additional studies are needed to determine the immunomodulatory effects of purified fumonisins and their immediate and long-term consequences on host resistance.

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