Immunomodulatory Effects of Feed-Borne *Fusarium* Mycotoxins in Chickens Infected with Coccidia

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The potential for *Fusarium* mycotoxins to modulate immunity was studied in chickens raised to 10 weeks of age using an enteric coccidial infection model. Experimental diets included: control, diets containing grains naturally contaminated with *Fusarium* mycotoxins, and diets containing contaminated grains + 0.2% polymeric glucomannan mycotoxin adsorbent (GMA). Contaminated diets contained up to 3.8 μg/g deoxynivalenol (DON), 0.3 μg/g 15-acetyl DON and 0.2 μg/g zearalenone. An optimized mixture (inducing lesions without mortality) of *Eimeria acervulina*, *E. maxima* and *E. tenella* was used to challenge birds at 8 weeks of age. Immune parameters were studied prior to challenge, at the end of the challenge period (7 days post-inoculation, PI), and at the end of the recovery period (14 days PI). Total serum immunoglobulin (Ig) A and IgG concentrations in challenged birds fed the contaminated diet were higher than controls at the end of the challenge period. Serum concentration of IgA, but not IgG, was significantly decreased at the end of the recovery period in birds fed the contaminated diet. The percentage of CD4+ and CD8+ cell populations in blood mononuclear cells decreased significantly at the end of the challenge period in birds fed the control or the contaminated diet compared to their percentages prior to challenge. The pre-challenge percentage of CD8+ population was restored at the end of the recovery period only in birds fed the control diet. Interferon-γ (IFN-γ) gene expression in caecal tonsils was up-regulated in challenged birds fed the contaminated diet at the end of the challenge period. No significant effect of diet was observed on oocyst counts despite the changes in the studied immune parameters. It was concluded that *Fusarium* mycotoxins modulate the avian immune system. This modulation involves alteration of gene expression but apparently does not enhance susceptibility or resistance to a primary coccidial challenge. Exp Biol Med 233:1411–1420, 2008

Key words: *Fusarium* mycotoxins; immunomodulation; chickens; coccidia

Introduction

Mycotoxins produced by fungal species of the genus *Fusarium* are frequently found in grains used for animal and poultry feeding. Type B trichothecenes, particularly deoxynivalenol (DON), are the most commonly encountered *Fusarium* mycotoxins (30). Literature reports on the immunomodulatory effect of these mycotoxins are contradictory, and are mainly studied in laboratory animals with very few investigations of possible effects on poultry. The mechanisms by which feed-borne *Fusarium* mycotoxins cause immunomodulation are not fully understood. *Fusarium* mycotoxins appear to act at different cellular and molecular levels that affect viability, proliferation and differentiation of immune system cells. These effects may be a result of inhibited protein synthesis (11) or impairment of the activity or secretory functions of immune system cells (28) as well as synthesis of cytokines that regulate the communication network of the immune system (28, 41).

The immunomodulatory effects of trichothecenes have been found to vary between immunosuppression and immunostimulation, depending on the dose, duration and time of exposure (5). Trichothecene-induced immunostimulation has been described at low doses and immunosuppression has been observed at high doses mainly due to apoptosis of leukocytes (11, 29). Repetitive exposure to high doses of trichothecenes may result in injury to actively dividing cells such as those present in bone marrow, intestinal mucosa, spleen and thymus (11). The capacity to inhibit protein synthesis has been described as a major effect

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of trichothecenes (5, 11). It has also been suggested that toxic manifestations of trichothecenes in laboratory animals are related to de-regulation of cell signaling and consequent alterations in downstream gene expression (31).

Reports have been inconsistent regarding the effects of feeding *Fusarium* mycotoxin-contaminated grains on the performance and health of poultry (13) and there is a lack of knowledge of the potential immunomodulatory effects of feed-borne *Fusarium* mycotoxins in birds. Mycotoxin-induced alterations in immune function may contribute to the signs of mycotoxicoses or predispose birds to infectious diseases (28). The present study was conducted to investigate the effects of feeding grains naturally contaminated with *Fusarium* mycotoxins on some immune parameters of chickens in the absence and presence of an enteric coccidial infection.

The adverse effects associated with feeding mycotoxin-contaminated grains can be reduced by using physical, chemical, nutritional, or biological methods (19). A polymeric glucomannan mycotoxin adsorbent (GMA) has been reported to reduce the adverse effects of feeding blends of grains naturally contaminated with *Fusarium* mycotoxins to different avian species (7, 40, 41, 43). The efficacy of GMA in preventing the immunomodulatory effects of *Fusarium* mycotoxins was also evaluated in the present study.

Materials and Methods

Experimental Birds. One hundred and twenty day-old Ross 308 commercial broiler breeder female chicks were obtained from Aviagen Inc. (Blairsville, GA). Chicks were not vaccinated in the hatchery against coccidiosis. Chicks were raised in the Central Animal Facility Isolation Unit in the Ontario Veterinary College, and were managed as has been prescribed by the Canadian Council on Animal Care with all the Animal Utilization Protocols approved by the Animal Care Committee of the University of Guelph (6).

Experimental Diets. Corn, wheat and soybean meal based control diets were formulated to meet the standard nutritional specifications for starter and grower broiler breeder chickens (27). The mycotoxin-contaminated diets were formulated to the nutrient specifications of the control diets by replacing control corn and wheat by naturally contaminated corn and wheat. Contaminated + GMA diets contained 0.2% GMA (Mycosorb®, Alltech Inc., Nicholasville, KY) added at the expense of corn. No coccidostat was included in any of the experimental diets.

Dietary contents of DON, 3-acetyl DON, 15-acetyl DON, nivalenol, T-2 toxin, acetyl T-2 toxin, iso T-2 toxin, HT-2 toxin, T-2 triol, T-2 tetraol, fusarenone-X, diacetoxyscirpenol, scirpenol, 15-acetoxyscirpenol, neosolaniol, zearalenone, zearalenol, aflatoxin and fumonisin were analyzed by gas chromatography-mass spectrometry as described by Raymond *et al*. (35). Limits of detection were 0.02 μg/g for aflatoxin, 2 μg/g for fumonisin and 0.2 μg/g for the other mycotoxins.

Experimental Design. One-day-old chicks were randomly divided into three groups of 40 birds, each fed one of the three experimental diets. Appropriate temperature, humidity, feeding program and lighting program were followed according to the standards recommended by the supplier. Birds were fed starter diets *ad libitum* from day one to 3 weeks of age, and grower diets on a 4/3 skip-a-day feed restriction program from 3 weeks to 10 weeks of age. Coccidial challenge and sampling were done on a feeding day 4–5 hr after feeding time. At 8 weeks of age, 8 birds from each group ($n = 24$) were selected at random, killed by cervical dislocation and immediately sampled. Collected samples included cardiac whole blood and caecal tonsils. Half of the birds remaining in each group were orally inoculated with a standardized mixture of *Eimeria acervulina* (Guelph strain; 100,000 sporulated oocysts/bird), *E. maxima* (USDA strain 68; 30,000 sporulated oocysts/bird) and *E. tenella* (USDA strain 80; 7,500 sporulated oocysts/bird). Oocyst doses were predetermined to cause measurable lesions and effects on growth without mortality in a pilot study. Birds were reared on the floor to the age of 8 weeks. Upon oral challenge with *Eimeria* and until the end of the experiment, birds were housed in cages (2 birds/cage) with wire mesh floor and solid sides in order to collect daily total droppings and prevent fecal-oral re-infection. The sides of the cages had openings to access feed and water. At day 7 post-inoculation (PI) (the end of the challenge period), 8 birds from each challenged group and 8 birds from each non-challenged group ($n = 48$) were randomly selected, killed and sampled as above. At 14 days PI (the end of the recovery period), the remaining birds ($n = 48$) were similarly killed and sampled.

Cardiac blood was immediately processed for separation of mononuclear cells as described below. Five ml of the collected blood from each bird was simultaneously forwarded to the Animal Health Laboratory at the University of Guelph for determination of total and differential leukocytic counts. The remaining blood was allowed to coagulate and then refrigerated for 2 hr and lightly centrifuged to clarify serum. Caecal tonsil samples were submerged in RNAlater™ (Qiagen, Mississauga, ON, Canada) and were stored at –20°C until analyzed. Sera were also stored at –20°C until analyzed. Total droppings were collected daily from day 5 to day 13 PI from each challenged group to determine total *Eimeria* oocyst outputs.

Body Weight Gain. Birds were weighed individually on a weekly basis throughout the experimental period and daily body weight gain was calculated.

***Eimeria* Oocyst Counts.** Total droppings were collected from each cage (2 birds) every 24 hr and were added to tap water and made up to a volume of 2 liters. The mixture was blended and then poured into a larger beaker and stirred to obtain uniform distribution of oocysts. A 1:10 dilution was prepared using saturated sodium chloride

solution. The total number of oocysts was counted using a McMaster counting chamber according to the following formula:

$$\text{Total oocyst number} = \text{oocyst count} \times \text{dilution factor} \times \frac{\text{fecal sample volume}}{\text{counting chamber volume}}$$

Lesion Scores. Intestinal lesions were scored at the end of the challenge period by examining the mucosal surface of the duodenum, jejunum, ileum and caecum. Lesions were scored from 0 to 4 according to the degree of severity in terms of inflammation and destruction of intestinal mucosa (18). Lesion scores reflect the degree of intestinal damage caused by *E. acervulina* (duodenum), *E. maxima* (jejunum and ileum) and *E. tenella* (caecum). The scoring system (18) can be summarized as follows:

E. acervulina

0: No gross lesions.

+1: Scattered white plaque-like elongated lesions confined to the duodenum; lesions may range up to 5 lesions per cm^2.

+2: Lesions are closer but not coalescent; lesions may extend as far as 20 cm posterior to the duodenum.

+3: Lesions are numerous, smaller and coalescent; lesions may extend as far as the level of Meckel's diverticulum; intestinal wall is thickened.

+4: Completely coalescent grayish lesions; intestine is congested and may contain petechiae; intestinal wall is greatly thickened; creamy exudates accumulates in the intestinal lumen.

E. maxima

0: No gross lesions.

+1: Small petechiae in the mid-intestine; orange mucus may be observed.

+2: Petechiae are numerous and intestine may be filled with orange mucus; little ballooning of the intestine and thickening of the intestinal wall.

+3: Intestinal wall is ballooned and thickened; the mucosal surface is roughened and intestinal contents have pinpoint blood clots and mucus.

+4: Most of the intestine is ballooned; mucus and blood clots are found in the intestine with characteristic putrid odor; intestinal wall is greatly thickened.

E. tenella

0: No gross lesions.

+1: Very few scattered petechiae on the cecal wall.

+2: Lesions are more numerous; blood may be noticed in the cecum; cecal wall is slightly thickened.

+3: Large amount of blood or cecal core; greatly thickened cecal wall.

+4: Cecal wall greatly distended with blood or large cecal core.

Phenotyping Cardiac Blood Mononuclear Cells. Five ml of freshly collected blood were mixed with 10 ml Dulbecco's phosphate buffer saline (D-PBS, Mediatech Inc., Herndon, VA) containing 0.05% sodium azide. Blood samples were then underlain with 5 ml Histopaque®-1077 (Sigma, St. Louis, MO) and centrifuged at $400 \times g$ for 30 minutes at room temperature for density gradient separation of cardiac blood mononuclear cells (CBMC). The cell layer at the Histopaque® interface was collected, washed 3 times with D-PBS at 4°C, and suspended in 1 ml of D-PBS. The viability and counts of CBMC were determined by a Trypan Blue exclusion method (39). Cell viability was consistently above 95%.

Duplicate 50 µl samples containing 1×10^6 suspended CBMC were transferred to each well of a 96-well round-bottom plate and re-suspended with 50 µl of the appropriate antibody (Southern BioTech, AL). Cells were dual stained with fluorescein isothiocyanate (FITC)-conjugated mouse anti-chicken CD4 and phycoerythrin (PE)-conjugated mouse anti-chicken CD8 according to the manufacturer's directions. Cells were stained with mouse anti-chicken IgM followed by goat anti-mouse IgM-PE for labeling B lymphocytes. The plate was shaken for 5–10 seconds and incubated at 4°C for 30 minutes in the dark for each step of antibody labeling. Wells were subsequently washed 3 times with D-PBS containing 1% bovine serum albumin (BSA). Antibody-labeled cells were suspended in 500 µl of D-PBS with BSA and analyzed by a FACSCalibur™ flow cytometer using CellQuest™ software (Becton-Dickinson Biosciences, Mississauga, ON, Canada). Appropriate isotype controls were included in the analysis and subtracted from the sample values.

Immunoglobulin Quantification. Total serum immunoglobulin (Ig) A, IgG and IgM were quantitatively measured by sandwich ELISA using the appropriate chicken Ig quantitation kits (Bethyl Laboratories Inc., Montgomery, TX) according to the manufacturer's protocols. Averages of duplicate readings from each standard, control and sample were used for data analysis using a four parameter logistic curve fit (Microplate Manager® software, version 4.0, Bio-Rad Laboratories, Hercules, CA).

Relative Quantification of Gene Expression. Total RNA was extracted from caecal tonsils using Trizol® (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol. The RNA pellet was dissolved in 20 µl diethylpyrocarbonate (DEPC)-treated water and treated with DNAase using DNA-*free*™ kit (Ambion Inc., Austin, TX). RNA concentration was estimated by spectrophotometry at 260 nm. $Oligo(dT)_{20}$ primers (SuperScript™ III First-Strand Synthesis SuperMix, Invitrogen, Burlington, ON, Canada) were used for synthesis of cDNA according to the manufacturer's instructions. The primers used for amplification of target and reference genes were synthesized by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) and are shown in Table 1.

Reverse transcription PCR (RT-PCR) and real-time PCR were carried out in 20 µl capillary tubes using LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH, Germany). Each real-time RT-

Table 1. Primer Pairs Used for Amplification of Target and Reference Genes[a]

Gene	Primer sequence[b]	Product size (bp)
β-actin	F 5′-CAACACAGTGCTGTCTGGTGG-3′ R 5′-ATCGTACTCCTGCTTGCTGAT-3′	205
IFN-γ	F 5′-CTGAAGAACTGGACAGAGAG-3′ R 5′-CACCAGCTTCTGTAAGATGC-3′	264
IL-10	F 5′-AGCAGATCAAGGAGACGTTC-3′ R 5′-ATCAGCAGGTACTCCTCGAT-3′	215
IL-18	F 5′-GAAACGTCAATAGCCAGTTGC-3′ R 5′-TCCCATGCTCTTTCTCACAACA-3′	216

[a] Primers were synthesized by Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).
[b] F, forward primer; R, reverse primer.

PCR assay was run along with a relevant dilution of the standard, which served as calibrator, as well as a no-template negative control. The thermal cycling parameters were established according to Abdul-Careem *et al*. (1) with slight modifications. In brief, they included pre-incubation (95°C/10 min), 40 cycles of amplification (Table 2), melting curve analysis and cooling (40°C/30 sec).

Statistical Analysis. Means were compared by the Student's *t* test. Comparisons were considered significant at $P \leq 0.05$. For relative mRNA expression of the target genes, treatment groups were compared to non-challenged group of the same age fed the control diet using relative expression software tool (REST©-MCS, 2006). Efficiency-corrected crossing point deviations for interferon-γ (IFN-γ), interleukin (IL)-10 and IL-18 were compared among groups after normalization to the reference gene (β-actin). Expression ratios of the investigated cytokine gene transcripts were then tested for significance by pair-wise fixed reallocation randomization (32).

Results

Dietary Mycotoxin Concentrations. The detected levels of mycotoxins in the experimental diets are shown in Table 3. DON was the predominant *Fusarium* mycotoxin in all experimental diets. Contaminated diet and contaminated + GMA diet contained 15-acetyl DON and zearalenone at relatively lower levels than DON. Control diets had no detectable acetyl DON or zearalenone.

Body Weight Gain, *Eimeria* Oocyst Counts and Lesion Scores. Body weight gain was not significantly affected by diet but was significantly reduced at the end of challenge period in challenged birds compared to non-challenged birds (Table 4). There was no significant effect of diet on fecal *Eimeria* oocyst output. Lesion scores were significantly higher in the duodenum, jejunum and ileum of birds fed the contaminated + GMA diet compared to birds fed either the control diet or the contaminated diet.

Total and Differential Leukocytic Counts. Total and differential leukocytic counts, with the exception of monocyte counts, were not affected by diet in non-challenged birds at any sampling point (data not shown). Blood monocyte counts increased significantly at the end of the challenge period only in birds fed the control diet (Fig. 1). Monocyte counts in those birds significantly decreased at the end of the recovery period to a level not statistically different from those in birds fed the other experimental diets.

Serum Ig Concentrations. No significant differences in serum IgA, IgG or IgM concentrations were observed among experimental groups in the absence of coccidial challenge. Differences were seen in response to coccidial challenge where serum IgA concentrations increased at the end of the challenge period as opposed to pre-challenge concentration regardless of diet. Such an increase was statistically significant in birds fed the contaminated diet or the contaminated + GMA diet. A significant decrease in serum IgA concentration was observed at the end of the recovery period in challenged birds fed the contaminated diet in which IgA concentrations were also lower than the controls (Fig. 2). Serum IgG concentrations were significantly higher in challenged birds fed the contaminated diet and contaminated + GMA diet compared to controls at the end of the challenge period and the end of the recovery period. No differences between diets were observed on serum IgM concentrations at any sampling point. IgM concentrations, however, were significantly lower in birds fed the contaminated diet and contaminated + GMA diet at the end of the recovery period compared to the end of the challenge period (Fig. 2).

CBMC Phenotyping. No significant effects of diet on the percentage of CD4+, CD8+, or B cell populations in the total blood mononuclear cells were observed in the absence of coccidial challenge (data not shown). The percentage of CD4+ as well as CD8+ cell decreased significantly at the end of the challenge period in challenged birds fed the control or the contaminated diet but not the contaminated + GMA diet (Fig. 3). At the end of the

Table 2. Real-Time PCR Amplification and Fluorescence Acquisition Conditions

Gene	Amplification conditions			Fluorescence acquisition
	Segment 1	Segment 2	Segment 3	
β-actin	95°C/10 sec	64°C/5 sec	72°C/10 sec	84°C/3 sec
IFN-γ	95°C/10 sec	64°C/5 sec	72°C/10 sec	84°C/3 sec
IL-10	95°C/10 sec	64°C/5 sec	72°C/5 sec	80°C/3 sec
IL-18	95°C/10 sec	64°C/5 sec	72°C/10 sec	82°C/3 sec

Table 3. Mycotoxin Concentrations (μg/g) Detected in the Experimental Diets[a]

	Experimental diets					
	Control		Contaminated		Contaminated + GMA	
Mycotoxin	Starter	Grower	Starter	Grower	Starter	Grower
DON	1.2	0.9	2.5	3.8	2.4	3.2
15-acetyl DON	ND[b]	ND	ND	0.3	ND	0.3

[a] Limits of detection were 0.02 μg/g for aflatoxin, 2 μg/g for fumonisin, and 0.2 μg/g for the other mycotoxins. Dietary contents of nivalenol, T-2 toxin, acetyl T-2 toxin, iso T-2 toxin, HT-2 toxin, T-2 triol, T-2 tetraol, fusarenone-X, diacetoxyscirpenol, scirpenol, 15-acetoxyscirpenol, neosolaniol, zearalenol, aflatoxin and fumonisin were below the detectable levels.
[b] ND, not detected.

Table 4. Effect of Diet on Body Weight Gain, Oocyst Counts and Lesion Scores (Values Are Means ± Standard Error of Means [SEM])

	Body weight gain[a]		Total oocyst output per bird[b] ($\times 10^8$)	Lesion score[c]			
Diet	Non-challenged	Challenged		Duodenum	Jejunum	Ileum	Caecum
Control	16.86 ± 0.86	8.81 ± 1.11	6.98 ± 0.43	1.57 ± 0.2[d]	1.13 ± 0.23[d]	0.13 ± 0.13[d]	1.38 ± 0.37[d]
Contaminated	15.84 ± 2.34	8.45 ± 2.43	7.20 ± 0.87	1.13 ± 0.13[e]	1.0 ± 0.19[d]	0.13 ± 0.13[d]	2.0 ± 0.33[d]
Contaminated + GMA	14.94 ± 1.75	8.43 ± 0.56	7.02 ± 0.52	2.14 ± 0.26[f]	2.63 ± 0.32[e]	0.63 ± 0.18[e]	1.88 ± 0.23[d]

[a] g/bird/day; calculated over the challenge period from day 0 to day 7 PI.
[b] Total number of oocysts shed per bird from day 5 to day 13 PI.
[c] Lesions scored on a 0 to 4 scale at the end of the challenge period (7 days PI, 9 weeks of age).
[d,e,f] Values within columns with the same superscript letter are not significantly different ($P \leq 0.05$).

recovery period, percentage of CD8+ cell population in the total blood mononuclear cells was significantly higher in challenged birds fed the control diet compared to birds fed the other diets. Percentage of CD8+ cell population at the end of the recovery period in birds fed the control diet was also higher compared to birds fed the control diet at the end of the challenge period. B cell percentages in challenged birds fed the control diet or the contaminated + GMA diet decreased significantly at the end of the challenge period compared to the pre-challenge percentage. Percentages of B cells significantly increased at the end of the recovery period compared to the end of the challenge period in all birds regardless of diet (Fig. 3).

Immune-Related Gene Expression in Caecal Tonsils. IFN-γ gene expression in the caecal tonsils was significantly up-regulated at the end of the challenge period in birds fed the contaminated diet (Fig. 4). Up-regulation of IFN-γ was not observed in the absence of coccidial challenge or at the end of the recovery period regardless of diet. No changes in IL-10 gene expression were detected at any sampling point regardless of diet. Birds fed the contaminated + GMA diet had significantly higher IL-18 gene expression at the end of the challenge period compared to birds fed the other diets (Fig. 4).

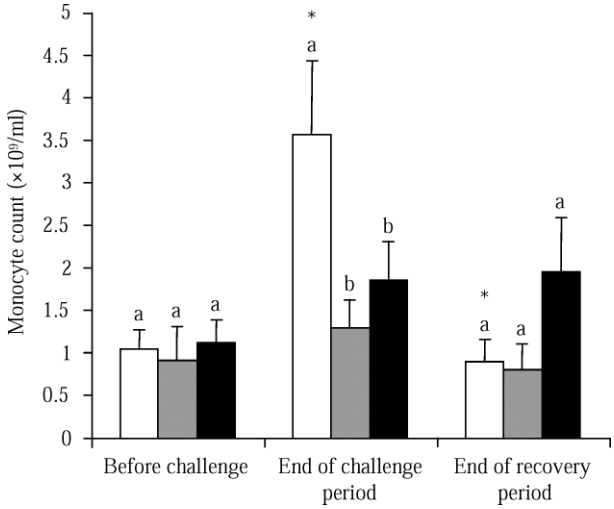

Figure 1. Changes in blood monocyte numbers determined by differential leukocytic counts. Bars represent means ± SEM. White bars represent birds fed the control diet, grey bars represent birds fed the contaminated diet, and black bars represent birds fed the contaminated + GMA diet. Before challenge, 8 weeks of age; end of challenge period, 7 days after challenge (9 weeks of age); and end of recovery period, 14 days after challenge (10 weeks of age). GMA, glucomannan mycotoxin adsorbent. Different letters in each group of bars indicate significant differences. Asterisk indicates significant difference relative to birds fed the same diet at the previous sampling point ($P \leq 0.05$).

Discussion

Reduced body weight gain at the end of the challenge period, induction of intestinal lesions and yield of oocysts

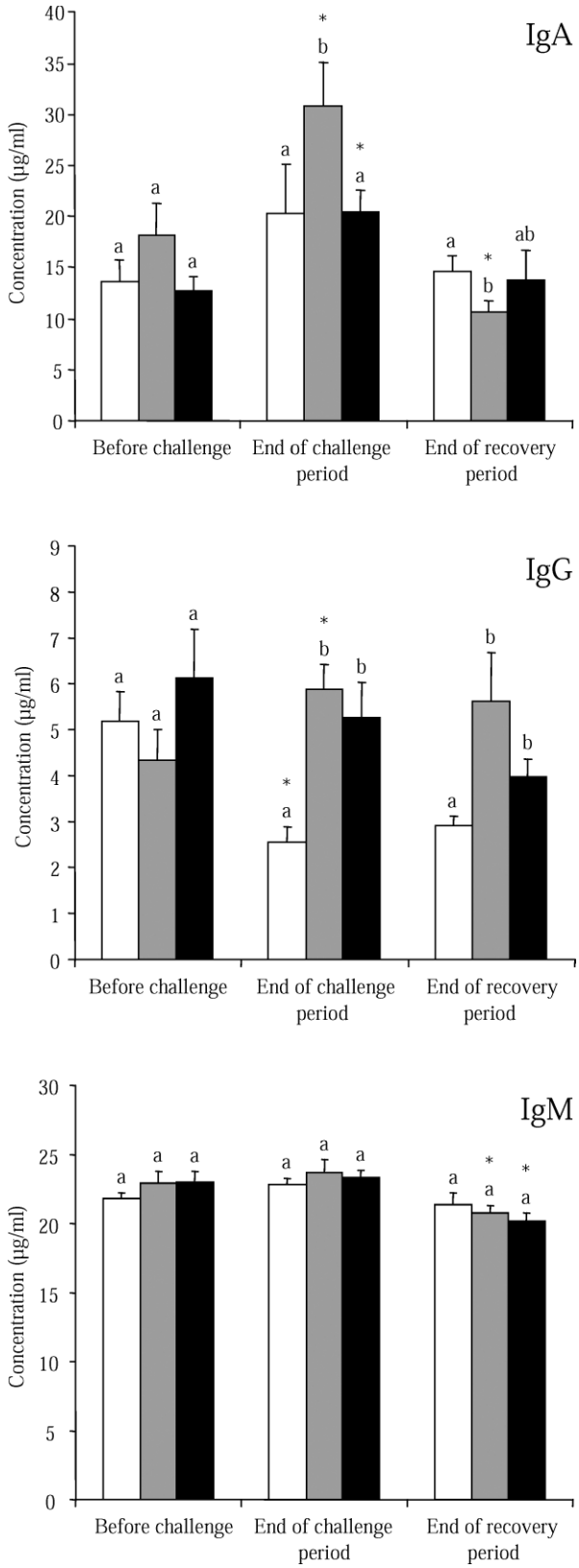

Figure 2. Changes in total serum Ig concentrations measured by ELISA. Bars represent means ± SEM. White bars represent birds fed the control diet, grey bars represent birds fed the contaminated diet, and black bars represent birds fed the contaminated + GMA diet. Before challenge, 8 weeks of age; end of challenge period, 7 days after challenge (9 weeks of age); and end of recovery period, 14 days after challenge (10 weeks of age). GMA, glucomannan mycotoxin adsorbent. Different letters in each group of bars indicate significant differences. Asterisk indicates significant difference relative to birds fed the same diet at the previous sampling point ($P \leq 0.05$).

throughout the post-challenge period indicate an appropriate dose of parasite challenge. Establishment of a dose of coccidia resulting in disease without mortalities was essential in order to study the interactions among coccidia, *Fusarium* mycotoxins and the avian immune system.

The observed lower monocyte numbers in differential leukocytic counts of the blood of challenged birds fed the contaminated diet and contaminated + GMA diet compared to controls at the end of the challenge period is in agreement with a report of decreased blood monocytes in broilers fed *Fusarium* mycotoxin-contaminated grains (41). Monocytes originate in the bone marrow and are released into the blood, where they circulate briefly before migrating into tissues to become tissue macrophages. Macrophages play an integral role in inflammation as well as cell- and antibody-mediated immunity (21). The decreased number of blood monocytes may indicate their reduced generation in the bone marrow or increased removal from the blood and recruitment to the intestinal sites of coccidial infection. It remains to be confirmed whether these changes in monocyte numbers are due to trichothecene-induced decreases in cellular protein synthesis or due to monocyte recruitment to the sites of coccidia replication.

The observation that feeding contaminated diets in the current study did not cause significant changes in serum IgA, IgG or IgM levels in the absence of disease challenge is in agreement with previous reports of studies with avian species (7, 41). It has been suggested, on the other hand, that DON enhances differentiation of IgA-secreting cells in the intestine of mice, consequently resulting in increased systemic level of IgA (29). Similar effect of DON in birds may explain the observed elevation in serum IgA concentrations upon challenge with coccidia in birds fed the contaminated diets. DON has been reported not to act as an adjuvant for inducing IgA when an exogenous antigen was orally administered (34). The current results, however, indicate that *Fusarium* mycotoxins boosted IgA levels upon coccidial challenge, perhaps due to the relatively long time of antigens exposure during replication of the parasite in the intestine. The effect of mycotoxins on antibody production and Ig class switching remains unclear. Serum IgA concentration is known to be proportional to mucosal IgA concentration. The drop in serum IgA concentrations observed at the end of the recovery period in birds fed the contaminated diet may be explained by interference of *Fusarium* mycotoxins with the healing of coccidia-induced damage to the intestine. Such intestinal injury may have decreased the local mucosal and, subsequently, the systemic levels of IgA.

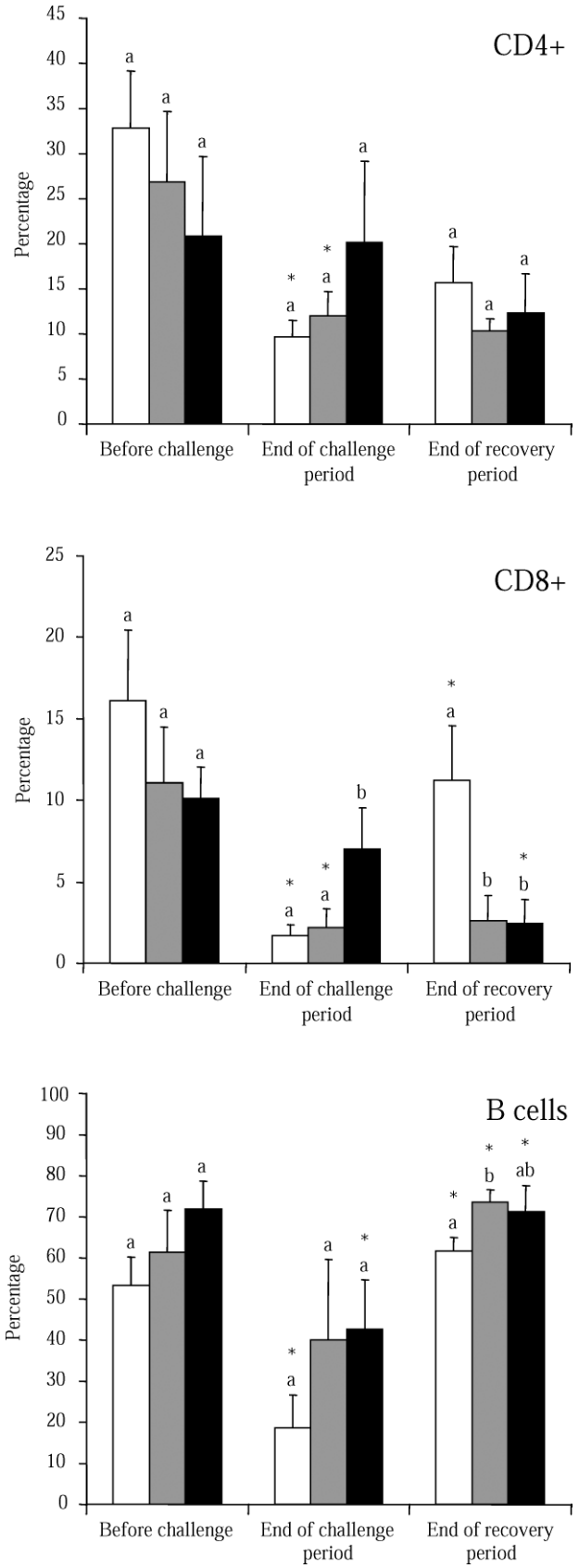

Figure 3. Changes in blood mononuclear cell phenotypes detected by flow cytometry. Bars represent means ± SEM. White bars represent birds fed the control diet, grey bars represent birds fed the contaminated diet, and black bars represent birds fed the contaminated + GMA diet. Before challenge, 8 weeks of age; end of challenge period, 7 days after challenge (9 weeks of age); and end of recovery period, 14 days after challenge (10 weeks of age). GMA, glucomannan mycotoxin adsorbent. Different letters in each group of bars indicate significant differences. Asterisk indicates significant difference relative to birds fed the same diet at the previous sampling point ($P \leq 0.05$).

The decrease in the percentage of mononuclear cell subsets in the blood of challenged birds may be explained by migration of these cells from the peripheral blood to the intestine, the site of coccidia replication. Unlike birds fed the control diet, birds fed the contaminated diets did not restore the pre-challenge percentage of CD8+ cells in their blood at the end of the recovery period. This could reflect prolonged retention of these cells in the gut or delayed replication necessary to replenish this subset in the circulation. Recruitment and stimulation of lymphocytes at the site of coccidial infection contribute to the clearance of the primary coccidial infection and development of immunity to re-infection, mainly through up-regulation of IFN-γ (38).

The authors are not aware of any previously published research on changes in the expression of immune-related genes of birds in association with *Fusarium* mycotoxicoses. Superinduction of cytokine gene expression by protein synthesis inhibitors such as T-2 toxin and cycloheximide has been observed previously in laboratory animals (16, 46) and explained by the ability of these compounds to inhibit synthesis of labile protein repressors of mRNA expression (42). IFN-γ gene expression has been shown to gradually increase throughout primary coccidial challenge in birds reaching peak expression by 6–8 days PI and contributing to the development of resistance to coccidial challenge (30, 44). IL-10 and IL-18 are involved in the cross-regulation of IFN-γ gene expression (26) and secretion (33), respectively. We studied the expression of IFN-γ, IL-10 and IL-18 at 7 days PI (at or near the peak of parasite-induced damage to the intestinal tract) so that cytokine gene expression could be examined in the context of lesion scores. Caecal tonsils were used because of their structural similarity to Peyer's patches where both T and B lymphocytes are present in germinal centers and because they are involved in the local immune response to the coccidial species used (24).

It has been hypothesized that induction of immune-related genes by DON reflects regulatory effects at the transcription level, by increasing the binding activity of transcription factors in leukocytes, and at the post-transcription level by increasing the stability of mRNA (31). This may explain the increased IFN-γ gene expression observed in the current study at the end of the challenge period in challenged birds fed the contaminated diet. IFN-γ mRNA expression in the caecal tonsils of challenged birds fed the contaminated diet may have been more persistent than in the birds fed the control diet. IFN-γ has been reported to be associated with increased resistance to

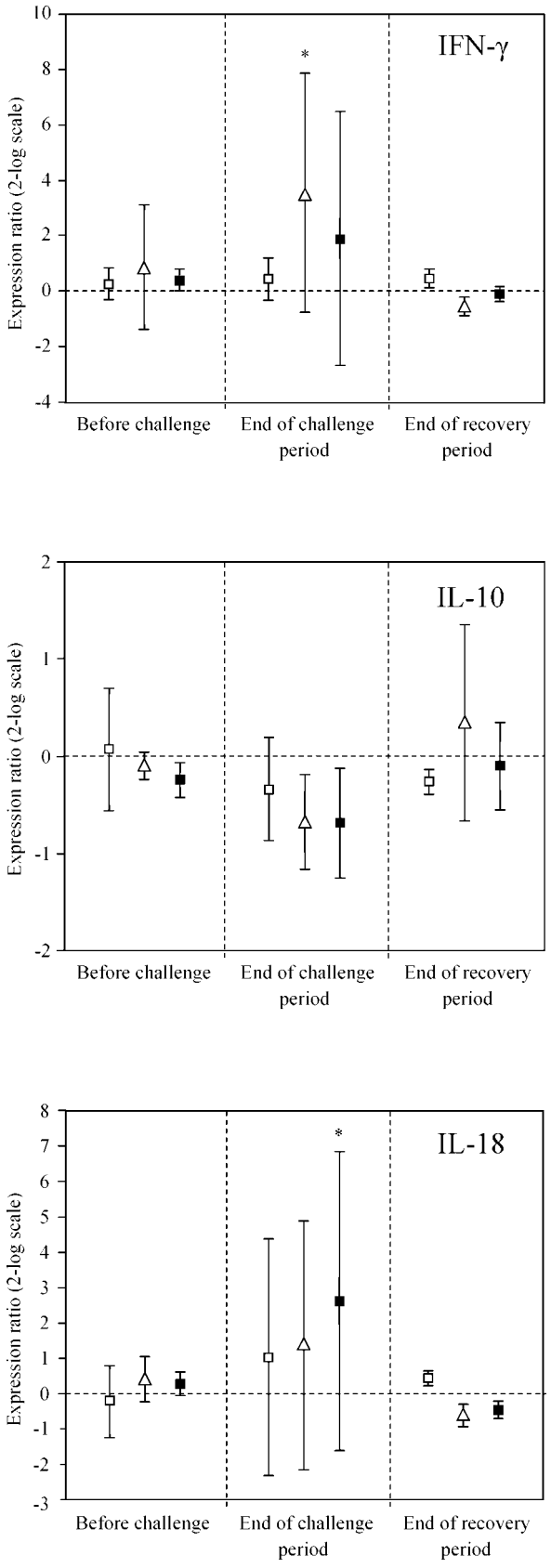

Figure 4. Relative expression of immune-related genes in the caecal tonsils of birds challenged with coccidia compared to non-challenged birds of the same age fed the control diet. Efficiency-corrected

coccidia and lowered oocyst yield during primary infections (12, 17, 25). The observed up-regulation of IFN-γ gene expression in birds fed the contaminated diet in the current study could not, however, be linked to apparent resistance to coccidial infection in terms of changes in oocyst yield. Resistance may be related to the expression of a set of interleukins rather than IFN-γ solely. Changes in IL-18 gene expression detected in birds fed the contaminated diet in the current study were not statistically significant. IL-18 is known to induce IFN-γ release in chicken immune system cells (15, 33). IL-18-induced secretion of IFN-γ in birds does not seem to be regulated at the transcriptional level. IFN-γ is stored in cells as an active protein that is quickly released in response to IL-18 (33). Up-regulated IFN-γ gene expression, therefore, may not necessarily be associated with functional secretion of IFN-γ. This may explain the apparent lack of correlation between oocyst yield and up-regulation of IFN-γ gene expression in birds fed the contaminated diet.

It can be concluded that *Fusarium* mycotoxins alter the interleukin milieu in the presence of enteric infection resulting in altered immune parameters. Inherent differences between *Fusarium* mycotoxins in their capacity to induce IgA response and up-regulate cytokines during enteric viral infections have been described in laboratory animals (22, 23). Previous work has provided evidence of immunostimulation by low levels of *Fusarium* mycotoxins in which increased numbers of T cells were detected in birds fed *Fusarium* mycotoxin-contaminated diet. Those increased numbers of T cells, however, did not alter antibody- and cell-mediated immune response in Tier II immunotoxicity tests (41). Modulated immune response associated with the presence of *Fusarium* mycotoxins in avian diets, even though not associated with profound changes in coccidial lesions, may be important in the field where measuring immune parameters is the main tool used to assess vaccination success. With the presence of *Fusarium* mycotoxins in the feed, immune responses may be misinterpreted.

The effect of mycotoxins on body weight gain might have been masked by the feed restriction program. Due to feed restriction, birds consumed all the allocated amount of feed in all experimental groups. Thus, it is difficult to determine any partial feed refusal of the contaminated diets. It is also not clear whether the enzymatic actions of mycotoxin producing fungi on grains make some nutrients more readily available for digestion. The degree of apparent

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crossing point deviations, after normalization to the reference gene, were compared using REST©-MCS software. The symbols □, △, and ■ on the bars represent birds fed the control diet, birds fed the contaminated diet, and birds fed the contaminated + GMA diet, respectively. Before challenge, 8 weeks of age; end of challenge period, 7 days after challenge (9 weeks of age); and end of recovery period, 14 days after challenge (10 weeks of age). GMA, glucomannan mycotoxin adsorbent. Asterisk indicates significant difference relative to non-challenged birds of the same age fed the control diet ($P < 0.01$).

tolerance or adaptations of the experimental birds to contaminated diets in terms of feed intake and weight gain also remains unclear.

Significantly higher lesion scores observed in the duodenum, jejunum and ileum of infected birds fed the GMA-containing diet may be related to up-regulation of IL-18 via activation of intestinal Toll-like receptors by GMA. The core components of GMA, β-glucans, appear able to activate Toll-like receptors (45). Toll-like receptor activation induces the production of the pro-inflammatory cytokines IL-1β and IL-18 (2). IL-18 has been shown to be associated with persistent inflammation and sometimes pathological changes in the gut (14, 37). It is possible that lesions were not exacerbated in the caecum of infected birds fed the GMA-containing diets because β-glucan-degrading bacteria inhabit the lower part of the gastrointestinal tract of chickens, particularly the caecum (3).

The use of naturally contaminated grains containing multiple *Fusarium* mycotoxins in the current study is more representative of field conditions than feeding individual purified mycotoxins or fungal culture material. Feeding naturally contaminated grains takes into consideration the presence of masked mycotoxins and precursors (4, 36) as well as unidentified fungal metabolites that may contribute to underestimation of the total amount of *Fusarium* mycotoxins detected by routine methods. Differences between the levels of *Fusarium* mycotoxins in the contaminated diets may be due to the lack of uniformity in mycotoxin distribution in contaminated grains or sampling variability (9). The occurrence of DON in the control diets was inevitable. DON has been the most frequent trichothecene contaminant of grains across Canada. For example, a persistent DON problem existed in Ontario grains over 17 years from 1979 to 1995 with annual incidence of 13–100% and mean concentration of up to 1.4 ppm (10). The feeding of grains naturally contaminated with *Fusarium* mycotoxins in the current study represents chronic exposure to relatively low concentrations of mycotoxins rather than an acute challenge. Feeding such naturally contaminated grains mimics field conditions where the toxicity of mycotoxins involves the indirect effect arising from *Fusarium* infection of the grain (8).

In conclusion, the current findings suggest that *Fusarium* mycotoxins may be regarded as subtle modulators of immunity in birds. Their influences were observed at relatively low levels that were not associated with obvious adverse effects on growth, and were exacerbated after the immune system was stimulated by an enteric coccidial challenge. Further research is needed to understand the mechanisms by which *Fusarium* mycotoxins alter cytokine and immunoglobulin production, and how such effects are influenced by the dose and duration of mycotoxin exposure. The findings reported here under experimental conditions assume practical importance because of the common occurrence of *Fusarium* mycotoxins in poultry feeds and their interaction with coccidiosis which is one of the most common diseases in commercial poultry flocks.

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