Immune response gene expression in spleens of diverse chicken lines fed dietary immunomodulators

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ABSTRACT Vaccines, antibiotics, and other therapeutic agents used to combat disease in poultry generate recurring costs and the potential of residues in poultry products. Enhancing the immune response using alternative approaches such as selection for increased disease resistance or dietary immunomodulation may be effective additions to the portfolio of strategies the industry applies in poultry health management. The objective of this study was to characterize the effects of dietary supplementation with 3 immunomodulators [ascorbic acid, 1,3–1,6 β-glucans from baker's yeast, and corticosterone] on cytokine gene expression in the spleen of 3 distinct genetic lines of chickens. Relative mRNA expression levels were determined using quantitative reverse transcriptase PCR for IL-1β, IL-2, inducible nitric oxide synthase, and toll-like receptors 4 and 15, all of which play important roles in chicken immune function. Expression data were analyzed by mixed model analysis. The only significant effect detected was sex effect ($P < 0.04$) on expression of IL-1β. The present findings suggest the need for further investigations into the effects of dietary immunomodulators on cytokine gene expression in chickens so as to generate a better understanding of the immunomodulation process.

Key words: chicken, diet, immunomodulator, cytokine, expression

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INTRODUCTION

Present strategies to control diseases in poultry include vaccination, antibiotics, biosecurity, and husbandry programs. Alternative strategies are being pursued, including dietary immunomodulation (Chae et al., 2006). Understanding the genetic regulation of protective functions by immunomodulators may lead to improvement of the safety and economics of poultry production and reduce the potential for development of resistance to antimicrobial treatments. Feed additives have been extensively explored for use in chickens as growth promoters or immunomodulators or both. Immunomodulating properties of ascorbic acid (Gross, 1992; Andreasen and Frank, 1999), β-glucans (Guo et al., 2003; Lowry et al., 2005; Huff et al., 2006), and corticosterone (Virden et al., 2007; Shini et al., 2008) have been demonstrated, but the mechanisms and genetic control of response to those immunomodulators in chickens are not fully understood.

Avian innate immunity provides a first line of host defense to microbial infections. Cytokines are essential effector molecules of innate and acquired immunity and are crucial signaling molecules in cellular communication. Relative expression levels of genes encoding proinflammatory IL-1β, T helper cell type 1 IL-2, inducible nitric oxide synthase (**iNOS**), toll-like receptor (**TLR**) 4, and TLR15 were assayed in the current study. Chicken IL-1β, which belongs to the IL-1 superfamily of cytokines, was one of the first chicken cytokines described. Chicken IL-1β mediates an inflammatory response and increases antibody production, similar to its mammalian counterpart (Leutz et al., 1989; Sterneck et al., 1992). Expression of IL-1β gene after parasitic infestation differed between chicken inbred lines disparate for the MHC (Kim et al., 2008). Interleukin-2, a cytokine signaling molecule, is instrumental in the body's response to microbial infection. Interleukin-2 facilitates immunoglobulin production by B cells and induces the differentiation and proliferation of natural killer cells (Waldmann and Tagaya, 1999; Waldmann, 2006). Inducible nitric oxide synthase is an enzyme that produces nitric oxide from the amino acid L-arginine (Alderton et al., 2001; Bogdan, 2001). It is produced by macrophages stimulated with by cytokines or microbial

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components. Nitric oxide plays a powerful role in immune responses because of its antimicrobial and antitumor functions (MacMicking et al., 1997; Blanchette et al., 2003). Expression of the iNOS gene in chickens varies with genetic background (Dil and Qureshi, 2003) and also after *Eimeria* infection (Kim et al., 2008).

The TLR belong to a multigene family responsible for detection of pathogen-associated molecular patterns expressed by microorganisms. Stimulation of TLR4 by lipopolysaccharide (**LPS**) results in the expression of the proinflammatory cytokine IL-1β. Toll-like receptor 4 detects the presence of pathogen (Akashi et al., 2001), stimulates bacterial killing mechanisms, and induces proinflammatory cytokines (Kogut et al., 2005). Allelic variation in the TLR4 gene is linked to susceptibility of chickens to infection with *Salmonella enterica* serovar Typhimurium (Leveque et al., 2003). The TLR4 expression level varies by genetic line (Dil and Qureshi, 2002, 2003). The expression of TLR15 mRNA, a novel, avian-specific TLR, was highest in bone marrow, bursa, spleen, and cecum, and expression was increased in the cecum by infection with *S. enterica* serovar Typhimurium (Higgs et al., 2006). Expression of TLR15 varies in heterophils from different chicken lines in response to stimulation with *Salmonella enterica* serovar Enteritidis, but not in nonstimulated cells (Nerren et al., 2009).

Understanding the genetics of basal expression levels of genes related to innate immunity is mandatory for effective genetic selection for immune response. Swaggerty et al. (2008) demonstrated that, in broiler chickens, sires with higher or lower than average proinflammatory cytokine (IL-1β and IL-6) and chemokine (CXCLi2 and CCLi2) mRNA expression levels produced progeny with similar profiles. The effect of dietary immunomodulators and genetic line on innate immunity is poorly understood at the molecular level, a gap that the current study was designed to address.

MATERIALS AND METHODS

Experimental Birds, Diets, and Tissue Sampling

Three genetic lines of chickens used in this study included the following: an outbred broiler line, which originated from a commercial broiler breeder line (Kaiser et al., 1998) that has undergone selection for rapid, efficient growth and high muscle percentage; a highly inbred Egyptian Fayoumi line, which was imported to the United States because of reported resistance to leukosis; and a highly inbred Leghorn layer line (Zhou and Lamont, 1999). A total of 56 chicks (24 broiler, 16 Leghorn, and 16 Fayoumi) of both sexes were used and birds of each line and sex were divided equally across 4 diet treatments. The chicks were fed one of 4 corn- and soy-based diets for 21 d, starting at 8 wk of age, except that the corticosterone-fed birds were returned to the basal diet after 14 d. The diets differed in a single ingredient that constituted 0.10% of the diet (Table 1). The basal diet had 0.10% washed sand; the β-glucan diet had 0.10% Macrogard (Immunocorp AS, Oslo, Norway), a highly purified β-glucan isolate from *Saccharomyces cerevisiae*; the ascorbic acid diet had 0.10% Vitamix-Stay C (DSM, Parsippany, NJ) as a source of ascorbic acid in lipid-coated beads; and the corticosteroid diet had 0.01% anhydrous corticosterone (Sigma, St. Louis, MO) and 0.09% washed sand. The anhydrous corticosterone was dissolved in the corn oil before mixing in the diet.

Birds were maintained in floor pens with 2 (Leghorn and Fayoumi) or 4 (broiler) replicate pens per diet treatment group. Birds were maintained with a 16-h light, 8-h dark cycle in a temperature-controlled environment with ad libitum access to feed and water. Birds were killed by cervical dislocation at 11 wk, and spleens were aseptically removed and placed in RNAlater (Ambion, Austin, TX) and kept at −70°C until analysis.

Gene Expression Studies

Total RNA was isolated from spleen using the RNAqueous Kit (Ambion) and then treated using DNA-free kit (Ambion), both protocols according to the manufacturer's instructions. The concentration of RNA was estimated with a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE) and samples were diluted to 50 ng/μL for use. Five immune response genes (IL-1β, IL-2, iNOS, TLR4, and TLR15) were assayed. Primer sequences have been previously reported (Kaiser et al., 2000; Higgs et al., 2006; Cheeseman et al., 2008; Abasht et al., 2009). The relative quantitative real-time reverse transcription PCR were performed using the QuantiTect SYBR-Green system (Qiagen, Valencia, CA) on an Opticon 2 (MJ Research, Waltham, MA) using the protocol described in Redmond et al. (2009).

Table 1. Composition of diets

Ingredient	Value (%)
Corn	62.29
Soybean meal (48% CP)	29.30
Corn oil	3.95
Experimental supplement[1]	0.10
DL-Methionine	0.32
L-Lysine HCl	0.12
Dicalcium phosphate (18% P)	1.51
Calcium carbonate	1.48
Salt (iodized)	0.28
Trace mineral premix	0.30
Vitamin premix	0.35
Total	100

[1]The basal diet had 0.10% washed sand, the β-glucan diet had 0.10% Macrogard (Immunocorp AS, Oslo, Norway), the ascorbic acid diet had 0.10% Vitamix-Stay C (DSM, Parsippany, NJ), and the corticosteroid diet had 0.01% anhydrous corticosterone (Sigma, St. Louis, MO) and 0.09% washed sand.

Table 2. Effects of genetic line, diet, and sex on mRNA expression of immune-related genes (*P*-values)

		Gene[1]				
Source of variation	df	IL1-β	IL2	iNOS	TLR4	TLR15
Line	2	0.58	0.66	0.52	0.86	0.51
Diet	3	0.74	0.72	0.67	0.28	0.34
Sex	1	0.04	0.25	0.27	0.75	0.24
Line × diet	6	0.66	0.17	0.67	0.31	0.70

[1]iNOS = inducible nitric oxide synthase; TLR4 = toll-like receptor 4; TLR15 = toll-like receptor 15.

Statistical Analysis

Expression levels of mRNA, as adjusted cycle threshold values for each gene (Redmond et al., 2009), were analyzed using SAS 9.1 with PROC Mixed model Type 3 (SAS Institute, Cary, NC). The model included line, sex, and diet as main fixed effects, hatch and plate as random effects, and the line by diet interaction effect. The individual bird was the experimental unit for gene expression analysis.

RESULTS AND DISCUSSION

The effects of dietary immunomodulators and other main factors on mRNA expression are presented in Table 2. The mRNA expression did not differ significantly among diets or genetic lines for any of the genes studied. However, the expression of IL-1β was significantly ($P < 0.05$) affected by sex (Table 2). Males exhibited higher ($P < 0.05$) IL-1β expression than females (Table 3). Although statistically nonsignificant, a higher level of expression was also observed in males for all other genes tested (Table 2). Diet main effect was not significant at $P < 0.05$ on mRNA expression, although chicks fed the corticosterone diet consistently demonstrated the highest numerical levels of expression for all 5 genes (Table 3).

In this study, we assessed the effects of diets containing β-glucan, corticosteroid, or ascorbic acid on basal levels of selected splenic genes to better define the potential roles of each feed ingredient in chicken immune response. The complexity of organismal responses and limited understanding of pathways involved in dietary immunomodulation has been observed in different species (Clarke and Mullin, 2008). Reported effects of dietary immunomodulators on immune function and cytokine expression patterns generally vary by the treatment and type of the cytokine studied. In the current study, feeding ascorbic acid, β-glucan, and corticosterone did not cause observable effects on splenic expression of selected genes (IL-1β, IL-2, iNOS, TLR4, and TLR15). This observation is similar to the findings reported by Ferdous et al. (2008), who observed no significant effects of diets containing vitamin C and corticosterone fed to chicks between 2 and 4 wk of age on expression of IL-1β and other proinflammatory genes in thrombocytes. Expression of IL-1β and IL-2 was not significantly changed after dietary vitamin E supplementation to chicks from 1 d to 4 wk of age (Leshchinsky and Klasing, 2003). The cited experiments are similar

Table 3. Splenic gene expression by line, diet, and sex in diverse lines of chickens fed immunomodulators[1]

	Gene[2]				
Item	IL-1β	IL-2	iNOS	TLR4	TLR15
Line					
Broiler	7.0 ± 1.0	8.1 ± 1.9	26.5 ± 0.7	26.2 ± 0.6	24.4 ± 0.3
Leghorn	7.7 ± 1.1	7.2 ± 1.9	26.3 ± 0.7	26.1 ± 0.6	24.8 ± 0.4
Fayoumi	7.2 ± 1.1	7.6 ± 1.9	26.1 ± 0.7	26.0 ± 0.6	24.2 ± 0.4
Diet[3]					
Ascorbic acid	7.2 ± 1.1	7.1 ± 2.0	26.1 ± 0.7	25.6 ± 0.6	24.1 ± 0.4
Basal	7.1 ± 1.1	7.4 ± 2.0	26.4 ± 0.7	26.0 ± 0.6	24.3 ± 0.4
β-Glucan	7.1 ± 1.1	7.6 ± 2.0	26.3 ± 0.7	26.1 ± 0.6	24.4 ± 0.4
Corticosteroid	7.9 ± 1.1	8.3 ± 2.0	26.6 ± 0.7	26.6 ± 0.6	25.1 ± 0.4
Sex					
Female	6.60 ± 1.0[b]	7.1 ± 1.9	26.2 ± 0.7	26.0 ± 0.6	24.2 ± 0.3
Male	8.0 ± 1.0[a]	8.1 ± 1.9	26.5 ± 0.7	26.1 ± 0.6	24.7 ± 0.3

[a,b]Means within a variable (line, diet, sex) with different superscripts differ significantly ($P < 0.05$).

[1]Values are least squares means ± SE of adjusted cycle threshold values.

[2]iNOS = inducible nitric oxide synthase; TLR4 = toll-like receptor 4; TLR15 = toll-like receptor 15.

[3]The ascorbic acid diet had 0.10% Vitamix-Stay C (DSM, Parsippany, NJ), the basal diet had 0.10% washed sand, the β-glucan diet had 0.10% Macrogard (Immunocorp AS, Oslo, Norway), and the corticosteroid diet had 0.01% anhydrous corticosterone (Sigma, St. Louis, MO) and 0.09% washed sand.

to the work reported here in that the dietary treatments were prolonged, which may have contributed to the lack of an observed proinflammatory response. Guo et al. (2003) reported that chicken macrophages showed IL-1 and proliferative responses to in vitro exposure to β-glucans, suggesting that the inflammatory response is observed earlier in the treatment period.

Many studies have explored the potential of dietary supplements to alter responses to other stimulants of the immune system. Other studies have demonstrated an effect of dietary immunomodulators on gene expression by increasing the response to immune stimulation. Immunomodulation by dietary supplementation with glycine in 1-d-old broiler chicks fed for 14 d demonstrated less increase in the mRNA expression of IL-1β, IL-6, TNF-like ligand 1A, interferon-γ, iNOS, and TLR4 in the spleen, collected after LPS injection, compared with the birds fed a basal diet (Takahashi et al., 2008). Interleukin-1β is reported to mediate LPS-induced iNOS expression (Dil and Qureshi, 2003). One-day-old specific-pathogen-free chickens were fed either an ascorbic acid-supplemented diet or a control diet from hatch, then vaccinated against infectious bursal disease virus at 7 d of age and challenged orally with 50% embryo-lethal-dose infectious bursal disease virus 14 d later; higher IL-2 production was found in the ascorbic acid-supplemented group (Wu et al., 2000). These experiments demonstrate that prolonged dietary immune modulation can be effective if there is an immune stimulant, such as LPS (Dil and Qureshi, 2003; Takahashi et al., 2008) or viral challenge (Wu et al., 2000). Studies on the oral administration of β-glucan showed no significant effect on the splenic gene expression of mice (Hashimoto et al., 1991), which is in accordance with our findings in chickens. Treatment with β-glucan maintains production levels of broilers under *Escherichia coli* challenge (Huff et al., 2006). Taken together, the cited works suggest a mechanism for dietary immune modulation in which there is an early increase in inflammatory response and a subsequent augmentation of the response to pathogenic stimuli.

Research conducted in our laboratory has already shown the effect of β-glucan, corticosteroids, or ascorbic acids on splenocyte gene expression from the same genetic lines, resulting in significant differential expression of IL-4 and IL-8 in the Fayoumi line. However, diet did not significantly affect broiler gene expression. Splenic expression of IL-6 was induced in Leghorns when fed the basal or ascorbic acid (Redmond et al., 2010). Additionally, the specific genetic lines used in the present study have been examined in previous, related studies in which significant genetic line effects on mRNA expression were detected. The genetic lines demonstrated significant differences in cytokine mRNA expression in spleen and cecum after challenge with *S.* Enteritidis (Cheeseman et al., 2007) and in heterophils after in vitro exposure to *Salmonella* Enteritidis (Redmond et al., 2009). Samples derived from these lines revealed differential TLR expression in spleen and cecum, induced by *Salmonella* Enteritidis challenge (Abasht et al., 2009). The lack of significant differential expression of the assayed genes in the present study might be an indication of an immune system that had returned to homeostasis by the time that samples were collected.

The current investigation has expanded knowledge of the basal splenic expression levels of the studied immune-related genes in 3 diverse genetic lines of chickens fed different immunomodulators. At the time tested, no significant differences in the assayed genes were detected, suggesting little alteration in basal state of expression and the need for expanded studies with stimulants (such as pathogens) to determine whether the diet treatments prime different responses in gene expression.

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