Effects of dietary vitamin E type and level on lipopolysaccharide-induced cytokine mRNA expression in broiler chicks

M. G. Kaiser,* S. S. Block,† C. Ciraci,* W. Fang,* M. Sifri,‡ and S. J. Lamont*1

*Department of Animal Science, Iowa State University, Ames 50011; †ADM Research, Decatur, IL 62521; and ‡ADM Alliance Nutrition, Quincy, IL 62301

ABSTRACT Vitamin E modulates the immune response, in part by reducing inflammation. The bacterial component lipopolysaccharide (LPS) can induce an inflammatory response in chickens. The objective of this study was to evaluate immunomodulatory effects of dietary type and level of vitamin E on response of broilers to LPS. One-day-old broiler males (n = 96) were placed in a vitamin E-type (synthetic, natural) × vitamin E level (22, 220 IU/kg) × LPS (LPS, saline) block design. At 22 d, LPS (or saline) was injected subcutaneously. Spleens were harvested for RNA isolation at 3 and 24 h postinjection. Relative levels of RNA expression were measured for the immune-related genes: avian β defensin 10 (AvBD10), interleukin 6 (IL6), interferon-γ (IFN-γ), inducible nitric oxide synthase (iNOS), interleukin 10 and transforming growth factor-β1 (TGF-β1). Avian β defensin 10 and iNOS are innate antimicrobial proteins. Interleukin 6 and IFN-γ are pro-inflammatory cytokines, whereas interleukin 10 and transforming growth factor-β1 are anti-inflammatory cytokines. There were significantly higher splenic levels of IL6, IFN-γ, iNOS, and IL10 RNA expression at 3 h postinjection in chickens receiving LPS than in chickens 24 h post-LPS injection or saline-injected birds at either time. These data suggest that LPS induced an immune response that was regulated by both pro- and anti-inflammatory cytokines. Birds fed natural-type (versus synthetic) vitamin E had a significantly lower LPS-induced inflammatory response, as indicated by lower IL6 RNA expression levels, suggesting a protective effect from natural-type vitamin E when a chicken encounters a bacterial component.

Key words: natural-type vitamin E, immunity, mRNA expression, lipopolysaccharide

INTRODUCTION

Level of dietary vitamin E alters immune function in poultry, including innate cellular oxidative burst (Perez-Carbajal, 2010), humoral response (Friedman et al., 1998; Ruiz-Feria and Abdulkalykova, 2009; Zhao et al., 2010), immune cell populations (Erf et al., 1998), and cytokine expression (Leshchinsky and Klasing, 2003). The pro-inflammatory chemotaxin myelomonocytic growth factor RNA expression was downregulated by dietary vitamin E in chickens that had received intravenous lipopolysaccharide (LPS: Leshchinsky and Klasing, 2003). In mammals, dietary vitamin E is proposed to lower pro-inflammatory cytokine expression by altering the NF-κB pathway (Sen and Packer, 1996). The immunocompetence of broilers under heat stress is enhanced by dietary vitamin E (Niu et al., 2009). Industrial poultry diets standardly use a synthetic form of vitamin E. An alternate dietary source of vitamin E is the natural source, RRR-α-tocopheryl acetate. The degree to which natural-source vitamin E differs from the synthetic form as an immunomodulator is not known.

Lipopolysaccharide is an outer membrane component from gram-negative bacteria and, as such, is a member of a class of antigens known as pathogen-associated molecular patterns (PAMP). Injection of LPS in chickens can be used to effectively model an inflammatory immune response to a bacterial infection without the added complications of live-pathogen challenge (Leshchinsky and Klasing, 2001). The LPS binds to the toll-like receptor 4 (TLR4), a pattern-recognition receptor (PRR), which initiates a host innate immune response signaling pathway and results in a pro-inflammatory response involving cytokines such as interleukin 6 (IL6) and gamma interferon (IFN-γ) and anti-microbial proteins such as avian β defensin 10 (AvBD10) and enzymes such as inducible nitric oxide synthase (iNOS; Hussain and Qureshi, 1997; Kogut et al., 2005; van Dijk et al., 2008) that generate antimicrobial compounds.

Avian β defensin 10 is a cationic-antimicrobial peptide that is an important component of the innate immune response (Milona et al., 2007). There are 14 documented AvBD genes within a relatively small clus-

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1Corresponding author: sjlamont@iastate.edu

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ter (Lynn et al., 2007). The cytokine network is vastly complex, with cytokines having many pleiotropic effects, including autocrine, paracrine and endocrine signaling (Klasing, 1998). Pro-inflammatory cytokines are produced in defense against pathogen challenge; however, over-production of these cytokines can also slow anabolic growth (Gabler and Spurlock, 2008). The inflammatory response is kept in balance by counteracting T cell regulatory (Treg) cytokines, such as transforming growth factor-β1 (TGF-β1; formerly known as TGF-β4 in chickens) and the T helper cell 2 (Th2) cytokine IL10. Furthermore, the pro-inflammatory cytokines initiate the transition from innate to adaptive immune response, with the adaptive response being characterized by IFN-γ (produced by T helper 1 cells; Th1) and IL10 (produced by Th2) cytokine expression. Of the cytokines, IL6 may have the most endocrine activity, including involvement in functions as diverse as metabolism (Gabler and Spurlock, 2008), obesity (Straub et al., 2000), and sleep (Vgontzas et al., 2000).

As a secondary immune organ, the spleen is a site for both innate and adaptive immune response (Olah and Verville, 2008). As such, the spleen is a heterogeneous tissue with a variety of immune-related cells. The spleen is highly populated with T and B cells and monocytes, which can differentiate into antigen-presenting cells (e.g., macrophages, dendritic cells) within a region known as the white pulp. Peripheral antigen-presenting cells migrate to the white pulp where they activate T and B cells. Thus, several cell types that produce cytokines are present in the spleen.

The objective of this study was to determine the immunomodulatory effects of diets supplemented with different levels of natural and synthetic types of vitamin E on response to LPS in broilers, as characterized by splenic cytokine production.

**MATERIALS AND METHODS**

**Experimental Design**

One-day-old commercial broiler chicks (n = 96) were placed into a 2 × 2 × 2 factorial randomized block design of vitamin E type × vitamin E level × LPS. Vitamin E types were synthetic (dl-α-tocopheryl-acetate) or natural source (γ-α-tocopheryl-acetate). The natural-source vitamin E was obtained from ADM ANI (Quincy, IL). The basal diet-added vitamin E level for either the synthetic or natural source is 22.00 IU/kg of diet, which equates to 22.00 mg of synthetic and 16.17 mg of natural source. The experimental diet-added vitamin E level for either the synthetic or natural source was 220.00 IU/kg of diet, which equates to 220.00 mg of synthetic and 161.70 mg of natural source. Birds had ad libitum access to feed (Table 1) and water throughout the test. The LPS factor consisted of injecting LPS or saline. At 22 d of age, chicks were either injected subcutaneously in the thigh with *Escherichia coli* 0111:B4 LPS (100 μg/kg of BW; Sigma, St. Louis, MO) or saline. The LPS concentration was 42.85 μg/mL and, therefore, the approximate volume to achieve the dose of 100 μg/kg of BW was 0.50 to 1.00 mL. Spleens were harvested at 3 and 24 h postinjection from 6 chicks of each LPS treatment × vitamin E type × vitamin E level.

**RNA Isolation**

Tissue samples were held overnight at 4°C in RNAlater (Ambion, Austin, TX) followed by decanting the RNAlater and storing the tissue at −80°C until RNA was isolated. Spleen tissue was homogenized with a polytron homogenizer (Brinkmann, Mississauga ON, Canada) and the RNA was isolated with an RNAqueous kit (Ambion) as previously described (Abasht et al., 2004).

**Quantitative Real-Time PCR**

Relative RNA expression levels were measured for 6 genes. Assays for *IL6*, *IFN-γ*, *iNOS*, *IL10*, and *TGF-β1* were previously reported (Kaiser et al., 2000; Jarosinski et al., 2002; Kogut et al., 2003; Rothwell et al., 2004). Primers were designed for *AvBD10* from the corresponding accession number (F 5′AACTGCTGT-GCCAAGATTTCC3′, R 5′TTTGATGCTCATTTGCAGAG3′, NM_00101609). Quantitative real-time PCR (qRT-PCR) was performed using the Quantitect

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**Table 1. Formula and composition of basal diet**

<table>
<thead>
<tr>
<th>Item</th>
<th>Basal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient, %</td>
<td></td>
</tr>
<tr>
<td>Corn ground</td>
<td>57.96</td>
</tr>
<tr>
<td>47.5% soybean meal</td>
<td>30.30</td>
</tr>
<tr>
<td>Corn gluten meal, 60%</td>
<td>4.45</td>
</tr>
<tr>
<td>Soy oil</td>
<td>2.30</td>
</tr>
<tr>
<td>Calcium carbonate 38</td>
<td>1.60</td>
</tr>
<tr>
<td>Phos monocal 21</td>
<td>1.55</td>
</tr>
<tr>
<td>Premix*</td>
<td>1.25</td>
</tr>
<tr>
<td>Salt</td>
<td>0.45</td>
</tr>
<tr>
<td>DL-Methionine, 99.5%</td>
<td>0.13</td>
</tr>
<tr>
<td>Lysine-HCl, 98%</td>
<td>0.13</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
</tr>
</tbody>
</table>

*Added levels: copper, 14.52 ppm; iodine, 0.64 ppm; iron, 50.40 ppm; manganese, 60.80 ppm; selenium, 0.30 ppm; zinc, 72.00 ppm; choline, 1,295 mg/kg; vitamin A, 24,244 IU/kg; vitamin D3, 12,122 IU/kg; vitamin E, 22.00 IU (as dl-α tocopheryl acetate)/kg; vitamin K, 4.80 mg/kg; biotin, 1.45 mg/kg; folic acid, 4.84 mg/kg; niacin, 218.00 mg/kg; pantothenic acid, 66.00 mg/kg; pyridoxine, 40.00 mg/kg; riboflavin, 29.00; thiamine, 19 mg/kg.
SYBR Green RT-PCR kit (QIAGEN, Valencia, CA) with each sample assayed in triplicate as previously described (Kaiser et al., 2006). The relative RNA expression levels were adjusted for PCR efficiency and the starting template concentration was normalized with 28S rRNA qRT-PCR values and expressed as adjusted Ct (Adj. Ct):

\[
\text{Adj. Ct} = 40 - \left[ \text{Mean Ct}_{\text{test gene}} + (\text{Median Ct}_{28S} - \text{Mean Ct}_{28S}) \times (\text{Slopetest gene}/\text{Slope}_{28S}) \right],
\]

where Mean Ct_{test gene} is the triplicate mean of test gene for a given chick; Median Ct_{28S} is the overall median of Ct_{28S} of the complete data set; Mean Ct_{28S} is the triplicate mean of the 28S gene for a given chick; Slope_{test gene} is the slope from the regression equation generated from qRT-PCR 10-fold serial dilutions of the test gene standard; Slope_{28S} is the slope from the regression equation generated from qRT-PCR 10-fold serial dilutions of pooled RNA.

**Statistical Analysis**

Effects of vitamin E type, vitamin E level, LPS, and collection time on immune-related relative RNA expression level were fitted into a GLM analysis using JMP software (SAS Institute, 2006).

The RNA expression model is as follows:

\[
y = \mu + \text{Vitamin E level} + \text{Vitamin E type} + \text{LPS} + \text{Collection time} + \text{PCR plate} + 2\text{-way interactions} + e,
\]

where fixed effect of Vitamin E level is the dietary level of vitamin E, Vitamin E type is the natural or synthetic source, LPS is the LPS or saline injection, Collection time is the time postinjection of sample collection and random effect of PCR plate, and e is the random error term. Two-way interactions of fixed effects were included in the final model for a gene’s analysis when the interaction \(P < 0.10\). Comparisons within significant variables were ranked by Tukey’s honestly significant difference test (SAS Institute, 2006). Results were considered statistically significant when \(P \leq 0.05\).

**RESULTS**

The main effect of dietary vitamin E type on cytokine RNA expression levels in spleen was significant for one of the 6 genes tested, IL6. No significant effects of vitamin E level were detected (Table 2). Lower levels of IL6 RNA were expressed in spleens of chickens fed natural-type vitamin E (Adjusted Ct LSmean = 11.34) than chickens fed the synthetic-type vitamin E (Adjusted Ct LSmean = 11.87). Of the 30 interactions with vitamin E (5 per cytokine; Table 2) as a variable, only 2 (vitamin E type × LPS and vitamin E level × vitamin E type) were significant. Birds fed either level of the synthetic vitamin E diet had lower iNOS RNA expression after saline injection (\(P = 0.0375\); Adjusted Ct LSmean = 14.79) than birds on the same diet that received LPS injection (Adjusted Ct LSmean = 16.41). The birds fed natural-type vitamin E did not differ in iNOS RNA expression levels regardless of whether they received LPS or saline injections (Table 2). Birds fed the 220.00 IU/kg diet of the natural-type vitamin E had greater TGF-β1 RNA expression (\(P = 0.0143\); Adjusted Ct LSmean = 16.86) than birds fed the 220.00 IU/kg diet of the synthetic vitamin E (Adjusted Ct LSmean = 16.32). Effect of LPS injection and postinjection collection time were both significant on 5 of 6 genes tested (IL6, IFN-γ, iNOS, TGF-β1, and IL10; Table 2). For each gene, injection of LPS resulted in higher RNA expression level than saline injection (Table 3; Figure 1), and 3-h collection time RNA expression level was higher than 24-h collection time (Table 3; Figure 1). The interaction of LPS/saline injection and collection time was consistently observed across genes, with significant

### Table 2. Fixed effects of diet vitamin E level, diet vitamin E type, lipopolysaccharide (LPS) treatment, collection time postinjection, and the 2-way interactions on avian β defensin 10 (AvBD10), interferon-γ (IFN-γ), interleukin 6 (IL6), interleukin 10 (IL10), inducible nitric oxide synthase (iNOS), and transforming growth factor-31 (TGF-β1) RNA expression levels in spleens of commercial broilers

<table>
<thead>
<tr>
<th>Variable</th>
<th>AvBD10</th>
<th>IFN-γ</th>
<th>IL6</th>
<th>IL10</th>
<th>iNOS</th>
<th>TGF-β1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin E level (22, 220 IU/kg)</td>
<td>0.9391</td>
<td>0.5205</td>
<td>0.1630</td>
<td>0.3587</td>
<td>0.9515</td>
<td>0.5939</td>
</tr>
<tr>
<td>Vitamin E type (natural, synthetic)</td>
<td>0.1607</td>
<td>0.3347</td>
<td>0.0290</td>
<td>0.2811</td>
<td>0.1261</td>
<td>0.2293</td>
</tr>
<tr>
<td>LPS (0.0, 100 μg/kg of BW)</td>
<td>0.2354</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0003</td>
<td>&lt;0.0001</td>
<td>0.0339</td>
</tr>
<tr>
<td>Collection time (3, 24 h PI)</td>
<td>0.4233</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0013</td>
</tr>
<tr>
<td>qPCR plate (random)</td>
<td>&lt;0.0001</td>
<td>0.0022</td>
<td>0.1996</td>
<td>&lt;0.0001</td>
<td>0.0008</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Vitamin E level × vitamin E type</td>
<td>NS¹</td>
<td>0.0713</td>
<td>NS</td>
<td>0.0760</td>
<td>NS</td>
<td>0.0143</td>
</tr>
<tr>
<td>Vitamin E level × LPS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Vitamin E level × collection time</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Vitamin E type × LPS</td>
<td>NS</td>
<td>0.0943</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.0375</td>
</tr>
<tr>
<td>Vitamin E type × collection time</td>
<td>NS</td>
<td>NS</td>
<td>0.0833</td>
<td>NS</td>
<td>NS</td>
<td>0.0555</td>
</tr>
<tr>
<td>LPS × collection time</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0018</td>
<td>&lt;0.0001</td>
<td>NS</td>
</tr>
</tbody>
</table>

¹NS = \(P > 0.10\); interaction was removed from the final model for that gene.
interactions detected for *IL6*, *IFN-γ*, *iNOS*, and *IL10* (Table 2). For these 4 genes, LPS-injected chickens had higher levels of RNA expression at 3 h than the other 3 injection-by-time groups (Figure 1). No significant differences in RNA expression level of *AvBD10* were detected, and adjusted Ct values were low in all treatment groups for this gene.

**DISCUSSION**

Type of dietary vitamin E, both as a main effect and as an interaction, had a significant effect on broiler splenic immune response to LPS as measured by relative RNA expression levels. As a main effect, chicks fed natural-type vitamin E had a reduced inflammatory response compared with birds fed synthetic-type vitamin E, as indicated by significantly less *IL6* RNA expression level. Interaction of vitamin E type and LPS had a significant effect on innate response measured by *iNOS* relative RNA expression level, where a less pronounced response was observed in birds fed the natural vitamin E diet than birds fed the synthetic vitamin E diet. At the higher dose level, the natural-type vitamin E enhanced anti-inflammatory (TGF-β1) response in contrast to the synthetic vitamin E. The beneficial effects of vitamin E have been demonstrated in mice (Yu et al., 1996) and in poultry (Friedman et al., 1998; Leshchinsky and Klasing, 2003; Zhang et al., 2009) to be dose-dependent, and adverse effects at high concentrations (≥150 mg/kg of feed) have been reported. The current data suggest modest benefit from the natural-source vitamin E, which appears more effective at tempering an LPS-induced inflammatory response than the synthetic vitamin E. Splenic and plasma protein levels of inflammatory cytokines in response to abdominal injection of LPS (250 μg/kg of BW at d 16, 18, and 20 of age) were lessened by 30 or 50 mg/kg (roughly equivalent to 41 and 68 UI/kg, respectively) dietary levels of α-tocopherol in broilers (Zhang et al., 2010). Dietary α-tocopherol reduced plasma protein levels of IFN-γ, IL-1β, IL-2, IL-6, IL-4, and IL-10 while only reducing IFN-γ, IL-2, IL-6, and IL-10 splenic levels (Zhang et al., 2010). Therefore, both RNA expression levels in the current study and protein levels reported in Zhang et al. (2010) of pro-inflammatory IL-6 were lower after addition of natural-source dietary vitamin E. The current study did not detect differential RNA expression of IFN-γ or IL10, both of which had lower protein levels for diets with vitamin E in the Zhang et al. (2010) study. Commercial poultry production typically generates dust particles, which have been shown to activate the immune system while suppressing growth in broilers (Lai et al., 2012). Although inflammation is necessary for an immune response, a chronic inflammatory state is detrimental to the host and negatively impacts production traits in food animals (Klasing, 2007). Dietary supplementation of vitamin E may help to mediate the immune response when animals are exposed to immune stressors.
Injection of LPS increased the splenic relative RNA expression level to a broad spectrum of immune-response genes in the current study, including innate (iNOS, AvBD10), pro-inflammatory (IL6, IFN-γ), Th1 (IFN-γ), Th2 (IL10), and Treg (TGF-β1). Chickens given intravenous injections of LPS have previously been shown to have elevated splenic RNA expression levels of IL-1β, myelomonocytic growth factor, and IFN-γ while TGF-β1 expression level was unchanged (Leshchinsky and Klasing, 2003). In vitro LPS stimulation of heterophils upregulated pro-inflammatory (IL1β, IL6, and IL18) cytokines; however, that study did not measure anti-inflammatory cytokine responses (Kogut et al., 2005). In the current study, higher relative levels of immune-response gene RNA expression in birds injected with LPS occurred at 3 h postinjection than at 24 h postinjection, and in saline-injected birds at either postinjection sampling time. Within the initial 3-h period postinjection, a complex range of immune-response genes are being activated distally from the site of LPS injection. The level of LPS-induced RNA activation appeared to return to noninduced levels by 24 h postinjection. A pathogenic challenge, such as a live bacterium, would likely provide a longer-term stimulation of the immune system than the LPS model, in which the host would likely upregulate IL6, IFN-γ, iNOS, TGF-β1, and IL10 over a longer time period to maintain a balance of inflammatory response while minimizing host tissue damage.

A previous study of tissue-specific baseline expression demonstrated that AvBD10 is highly expressed in liver, gall bladder, cloaca, kidneys, and testis but weakly expressed in spleen (Lynn et al., 2007). The current study confirmed the low baseline expression level of AvBD10 RNA in the spleen and the apparent lack of inducible expression of AvBD10 after LPS injection.

In summary, the measured host immune responses to LPS injection fit within the expected inflammatory model, in which both pro- and anti-inflammatory cytokines were expressed in cohort. Type of vitamin E appeared to modulate the immune system, with natural-type vitamin E lessening the LPS-induced inflammatory response. These data suggest that supplemen-

Figure 1. Interaction of lipopolysaccharide (LPS) injection [subcutaneously with Escherichia coli 0111:B4 LPS (100 μg/kg of BW) or saline] and postinjection collection time (3 or 24 h) on splenic RNA expression levels of AvBD10 (A), IFN-γ (B), IL6 (C), IL10 (D), iNOS (E), and TGF-β1 (F) in commercial broilers (n = 96). Bars are least squares means ± SE of broilers receiving either LPS or saline injections. Within each graph, bars within time point with different letters are significantly different at $P \leq 0.05$ as determined by Tukey's honestly significant difference test.
tal natural-type dietary vitamin E may assist birds in minimizing the inflammatory response upon bacterial challenge.

REFERENCES


