Conjugated linoleic acids alleviate infectious bursal disease virus-induced immunosuppression in broiler chickens

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ABSTRACT The immunoregulatory actions of conjugated linoleic acid (CLA) of relevance to viral disease pathogenesis and immune responses were investigated. To test the hypothesis that CLA ameliorates immunosuppression, we developed a viral challenge model by infecting chickens with infectious bursal disease virus (IBDV). After 14 d of dietary supplementation with either soybean oil or CLA, half of the chickens in each group were challenged with IBDV. We examined the effect of CLA on the development of lesions (i.e., lymphoid depletion and necrosis) and observed the immune responses against IBDV. The IBDV infection depleted lymphocytes in the medullary area and significantly stimulated interferon (IFN)-γ and IL-6 mRNA relative expression of bursa (P < 0.05) compared with the uninfected bursa. Compared with the CLA diet, lymphocytes depletion was more accentuated in chickens fed the control diet, whereas IFN-γ and IL-6 mRNA relative expression were upregulated (P < 0.05). Additionally, histopathological examination of the bursa revealed that the pathological changes tended to be more severe in infected chickens fed the control diet, which also significantly decreased (P < 0.05) on lymphocyte proliferation. Significant interactions were found between infection and diets for lymphocyte proliferation, antibody titers, and IFN-γ mRNA relative expression (P < 0.05). The results of this study indicate that dietary CLA enhanced immune function in chickens, particularly those of the IBDV-immunosuppressive status. Furthermore, at the molecular level, the immunoregulatory functions of CLA on chickens are attributable mainly to the antiinflammatory properties of CLA and are mediated, at least in part, through suppressing IBDV-specific proinflammatory cytokines mRNA relative expression.

Key words: conjugated linoleic acid, immunosuppression, proinflammatory cytokine, chicken

INTRODUCTION

Infectious bursal disease virus (IBDV) is a significant and widespread pathogen in the poultry industry. Infectious bursal disease virus destroys B lymphocytes in the bursa of young chickens and causes an acute, highly contagious and immunosuppressive disease (Rodenberg et al., 1994; Van Den Berg, 2000). During acute infection, chickens exposed to IBDV had enhanced gene expression of proinflammatory cytokines [i.e., interferon (IFN)-γ, IL-8, and IL-6] in the bursa and spleen (Kim et al., 2000; Eldaghayes et al., 2006; Ruby et al., 2006; Khatri and Sharma, 2009). In addition, the virus-induced immunosuppression results in secondary infections, suboptimal response to vaccinations, and growth retardation. Therefore, IBDV has caused enormous worldwide economic loss in the poultry industry. Nutrition strategies may be desirable to manipulate the secretion of proinflammatory cytokines and alleviate immunosuppression in IBDV-exposed chickens.

One strategy to attenuate immunosuppression and the negative effects of proinflammatory cytokines is to supplement the diet with conjugated linoleic acid (CLA). Conjugated linoleic acid refers to a class of positional and geometric conjugated dienoic isomers of linoleic acid, of which cis-9,trans-11-CLA and trans-10,cis-12-CLA are the main isomers (Evans et al., 2000). Conjugated linoleic acid has been shown to alleviate immunosuppression in animals. Our initial research led to the discovery that supplementation with CLA, predominantly the cis-9,trans-11 CLA isomer, enhanced immune function under the cyclosporin A-immunosuppressive status in chickens (Long et al., 2010), which prevents the synthesis of T-cell cytokines

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by blocking a late-stage signaling pathway initiated by the T-cell receptor, which affects the production of IL-2; hence, T-cell proliferation is affected (Hill et al., 1989; Sigal and Dumont, 1992). Bassaganya-Riera et al. (2003) reported that CLA ameliorated viral infectivity in a pig model of type-2 porcine circovirus-induced immunosuppression. Furthermore, CLA has been shown to have antiinflammatory activities. Conjugated linoleic acid decreased the production of inflammatory cytokines such as tumor necrosis factor α and IL-6 in both human and animal models (Turek et al., 1998). Bassaganya-Riera et al. (2006) reported that CLA had implications for the treatment of inflammatory conditions such as inflammatory bowel disease. In this regard, Hontecillas et al. (2002) demonstrated that CLA decreased IFN-γ production in the colonic mucosa of pigs with bacterially induced colitis and ameliorated tissue inflammation and weight loss associated with inflammatory bowel disease in pigs. Lai et al. (2005) showed that the supplementation of CLA suppressed the synthesis of proinflammatory cytokines (i.e., IL-1β, IL-6, and tumor necrosis factor α) in lipopolysaccharide (LPS)-challenged pigs.

Therefore, to investigate the immunoregulation role of CLA, we created a model of IBDV in chickens and determined the effects of dietary CLA on bursal lymphocyte proliferation, antibody titers to IBDV, and mRNA expression of proinflammatory cytokine profiles (i.e., IL-6 and IFN-γ).

MATERIALS AND METHODS

Dietary Treatments and Bird Management

The bird management protocol for this research was approved by the China Agricultural University Animal Care and Use Committee. A total of 80 male Arbor Acre broiler chickens (1 d old) were distributed into 2 dietary treatments: control or CLA-supplemented diet. Chickens were fed the experimental diets for 14 d before the IBDV challenge. After challenge of 40 chickens with IBDV, the design became a 2 × 2 factorial arrangement [dietary treatment (control or 1.0% CLA) and infection treatment (IBDV-infected or uninfected)], which was then separated in individual negative-pressure isolators. The CLA was provided by Loders Croklaan Co. (DP999, Lipid Nutrition, Wormerveer, the Netherlands). A corn–soybean meal diet was used. In the CLA diets, 1.0% CLA was replaced by 1.0% soybean oil to keep the diets isoenergetic within phases. Portions of the bursa and spleen were used as-fed basis (%)

<table>
<thead>
<tr>
<th>Item</th>
<th>Starter (d 1–21)</th>
<th>Finisher (d 22–42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td>55.32</td>
<td>58.77</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>37.26</td>
<td>33.36</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>3.33</td>
<td>4.24</td>
</tr>
<tr>
<td>Conjugated linoleic acid&lt;sup&gt;1&lt;/sup&gt;</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>1.98</td>
<td>1.68</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.18</td>
<td>1.07</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>Mineral premix&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.16</td>
<td>0.13</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.18</td>
<td>0.15</td>
</tr>
<tr>
<td>Lysine-HCl</td>
<td>0.051</td>
<td>—</td>
</tr>
<tr>
<td>Vitamins premix&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Antioxidants</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Nutrition level</td>
<td>2.95</td>
<td>3.05</td>
</tr>
<tr>
<td>ME (Mcal/kg)</td>
<td>21.00</td>
<td>19.50</td>
</tr>
<tr>
<td>CP</td>
<td>1.00</td>
<td>0.90</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.45</td>
<td>0.40</td>
</tr>
<tr>
<td>Available phosphorus</td>
<td>0.50</td>
<td>0.40</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.15</td>
<td>1.00</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.50</td>
<td>0.40</td>
</tr>
</tbody>
</table>
| 1In the conjugated linoleic acid (CLA) diets, 1.0% CLA was replaced by 1.0% soybean oil to keep the diets isoenergetic within phases.
| 2Provided per kilogram of diet: Cu, 8 mg; Zn, 75 mg; Fe, 80 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.35 mg.
| 3Provided per kilogram of diet: vitamin A, 12,500 IU; vitamin D<sub>3</sub>, 2,500 IU; vitamin K<sub>3</sub>, 2.65 mg; thiamin, 2 mg; riboflavin, 6 mg; vitamin B<sub>12</sub>, 0.025 mg; vitamin E, 30 IU; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid, 12 mg; niacin, 50 mg.

The IBDV BC6/85 strain was obtained from the China Institute of Veterinary Drug Control (Beijing, China) and had the median bird lethal dose of IBDV at 105.838/0.25 mL, which was performed as described previously (Sharma, 1986; Brown et al., 2007), and was diluted 1/100 in sterile saline solution. Chickens in the IBDV-infected group were inoculated with 0.2 mL of IBDV dilution by means of nose and eye drops. In the control group, chickens received equal volumes of saline by the same route.

Sample Collection

On d 3, 7, 14, and 28 postinfection, heparinized blood samples were collected from the wing vein and then chickens were killed by exsanguination while under deep anesthesia by an intraperitoneal injection of sodium pentobarbital (30 mg/kg of BW; Marchant-Forde et al., 2008). The bursa, thymus, and spleen were aseptically excised and a portion of the bursa was collected immediately and fixed in 5.0% (vol/vol) oxymethylene solution. A second portion of the same tissues was snap frozen in liquid nitrogen and then stored at −80°C for later cytokine mRNA relative expression analysis. Furthermore, portions of the bursa and spleen were used for lymphocyte proliferation analysis.
**Histopathology Examination**

The fixed bursa samples were dehydrated, embedded in paraffin, serial sectioned (5 mm thickness), and kept at 37°C for more than 12 h. The sections were washed 3 times in xylol for 5 min to remove paraffin and hydrated by 5 washings successively in solutions of 100, 100, 95, 80, and 70% alcohol. Sections were then stained with hematoxylin–eosin solution according to route protocol and the slides were coded to prevent observer bias during examination and evaluation under an Olympus light microscope (Olympus Optical Co., Beijing, China).

**Hematoxylin and Eosin Staining**

For the detection of histopathological lesions, the bursa of Fabricius was collected, fixed in 10% buffered formalin, and stained with hematoxylin and eosin. Lesions were observed microscopically and compared between groups (Sharma et al., 1989; Kim et al., 1999).

**Lymphocyte Isolation and Proliferation Assay**

The bursa was squeezed with a 5-mL syringe plunger to extrude cells. Cell suspensions were filtered through 70-mm nylon (Falcon BD Biosciences, Erembodegem, Belgium) before being washed 3 times in RPMI 1640 medium and resuspended in the same medium supplemented with 2% inactivated fetal calf serum. Heparinized blood samples were collected from the wing vein. Lymphocytes were isolated from peripheral blood using lymphocyte density-gradient centrifugation medium (density = 1.077; HaoYang Biological Manufacture Co. Ltd., Tianjin, China) and were washed 3 times in RPMI 1640 medium before being resuspended in RPMI medium supplemented with 5.0% inactivated fetal calf serum. The lymphocyte proliferation assay was performed as described previously (Lambrecht et al., 2004; Zhang et al., 2005; Rauw et al., 2007).

**Antibody Titers to IBDV by ELISA**

Sera were obtained from IBDV-treated chickens on d 14 and 28 postinfection to quantitate antibody against IBDV. The ProFLOK IBDV antibody test kit (Synbiotics Co., Kansas City, MO) was used as described previously (Kim et al., 1999).

**IL-6 and IFN-γ mRNA Relative Expression Assay**

Total RNA was extracted from bursa tissue on d 3, 7, and 14 postinfection using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Total RNA sample was reverse transcribed using the RevertAid First Strand cDNA Synthesis kit (MBI Fermentas, Hanover, MD) according to the manufacturer’s instructions. Reverse transcription was performed at 42°C for 1 h followed by heat inactivation for 5 min at 70°C. All the cDNA preparations were stored frozen at −20°C until further use.

Quantitative PCR assay was performed with the 7900-HT fluorescence detection system (Applied Biosystems, Foster City, CA) according to optimized PCR protocols using the SYBR-Green qPCR kit (Applied Biosystems). To amplify IL-6, IFN-γ, and β-actin cDNA fragments, the following sequences of PCR primers pairs were used: forward 5′-TTTATGGAGAAGACGGTGAGG-3′, reverse 5′-TGGGCA-GATTGTAACAGAG-3′ for IL-6 (NM_204628); forward 5′-ACTGAGCCATCACAAGAG-3′, reverse 5′-CAATAATAGGCTCCACCGTCA-3′ for IFN-γ (AY705909); and forward 5′-CCACCGCAATGGTCTAAAC-3′, reverse 5′-AAGACTGCGTCTGACTACCTTC-3′ for β-actin (NM_205518). The cDNA was amplified with an initial denaturation step of 1 min at 95°C; 40 cycles of 30 s at 95°C, 30 s at the annealing temperature 60°C, and 50 s at 72°C; and a final extension step of 72°C for 8 min.

Relative standard curve methods were used for quantification of gene expression. Copy numbers were determined from 2 independent cDNA preparations of each sample and were calculated relative to a dilution series of the respective reference plasmids, comprising 103 to 108 copies. The reference plasmids contained cloned real-time PCR products obtained with these primers. The housekeeping gene, β-actin, was used as internal standard for the PCR reaction. The cycle threshold value (number of cycles halfway through the exponential phase) was determined and was used to calculate the relative expression level compared with a known β-actin standard.

**Statistical Analysis**

Data were analyzed by GLM procedure of SPSS 16.0 (SPSS Inc., Chicago, IL). The main effects of challenge (IBDV or saline), dietary treatment (control or 1.0% CLA), and their interactions were analyzed. Differences among each treatment group were tested by Duncan’s multiple comparison when the significant interaction between the main effects was observed. A level of $P < 0.05$ was set as the criterion for statistical significance.

**RESULTS**

**Clinical and Gross Pathologic Observations**

The health and behavior of the nonchallenged chickens fed control diet or CLA diet were consistently normal throughout the experiment; chickens infected with IBDV showed almost normal appetite except that it was slightly decreased d 3 to 14 postinfection. No petechia was shown in the mucosal surface of bursa from the control group. On d 7 and 14 postinfection, gross...
observation showed slight swelling, punctate hemorrhage, and depauperating in the bursa in most of the infected chickens (Figure 1A).

**Histopathologic Lesions in Chickens**

In the control, texture was normal, the boundary among lymphoid nodules was obvious, and there were integrated lymphaticus folliculus and orderly lymphocytes spread in lymphoid nodules. The pathological changes were observed in the bursa of the infected birds. By d 3 postinfection, degeneration and necrosis of lymphocytes in the medullary area of bursal follicles were observed. By d 7 postinfection, red cells had flooded the lymphoid nodes and en masse infiltration of inflammatory cells into the bursal follicles was obvious. All lymphoid follicles were affected by d 7, with typical satellite-like vacuity changes seen in the follicular medullary areas (Figure 1B).

**Bursal Lymphocyte Proliferation**

Chickens infected with IBDV had significantly ($P < 0.05$) decreased bursal cells proliferation in response to concanavalin A (ConA) mitogen on d 7, 14, and 28 postinfection and LPS mitogen on d 14 and 28 postinfection (Table 2). Chickens fed the CLA diets had higher bursal cell proliferation ($P < 0.05$) in response to ConA mitogen on d 7, 14, and 28 postinfection and LPS mitogen on d 28 postinfection than those fed the control diets. The interactions between CLA diets and IBDV infection were significant in the bursal lymphocyte proliferation in response to ConA mitogen on d 7, 14, and 28 and LPS mitogen on d 28 postinfection ($P < 0.05$).

**Antibody Titers to IBDV**

When infected with IBDV, chickens fed the control diet had no antibody titers (negative), whereas chickens fed the CLA diet had antibody titers. Furthermore, chickens fed the CLA diet had higher antibody titers on d 28 postinfection compared with on d 14 postinfection (Figure 2).

**IFN-γ and IL-6 mRNA Relative Expression**

When birds were infected with IBDV, IFN-γ and IL-6 mRNA relative expression were increased (Figure 3). Chickens infected with IBDV had significantly ($P <
0.05) increased IFN-γ and IL-6 mRNA relative expression on d 3 postinfection.

Chickens fed the CLA diets had lower IFN-γ (P < 0.05) and IL-6 (P = 0.101) mRNA relative expression on d 3 postinfection than those fed the control diets. The interactions between CLA diets and IBDV infection were clear in IFN-γ (P < 0.05) and IL-6 (P = 0.078) mRNA relative expression.

**DISCUSSION**

Studies have revealed a critical role of dietary CLA in the modulation of immune function in animals of both normal physiological status and immunosuppressive status (Sugano et al., 1998; Yamasaki et al., 2000; Bassaganya-Riera et al., 2003; O’Shea et al., 2004; Zhang et al., 2005). In this study, to examine whether supplementation of CLA attenuated the inflammation in chickens, we used a model for inducing inflammation in chickens by infecting them with IBDV. Infectious bursal disease is an acute, highly infectious and immunosuppressive disease caused by IBDV, a member of Birnaviridae family affecting mainly young chickens (Tayade et al., 2006). Here, we found that IBDV infection led to typical pathological changes; for example, depauperating, punctate hemorrhage, degeneration and necrosis of lymphocytes, and satellite-like vacuity changes in the in most of the infected chickens. This result is basically consistent with previous studies (Kibenge et al., 1988; Chettle et al., 1989; Tayade et al., 2006). However, in the CLA diet, chickens infected with IBDV showed reduced changes in bursa; for example,

**Table 2.** Bursal lymphocyte proliferation

<table>
<thead>
<tr>
<th>Item</th>
<th>0</th>
<th>1.0% CLA</th>
<th>IBDV</th>
<th>CLA</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>d 3 postinfection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ConA</td>
<td>1.017</td>
<td>1.000</td>
<td>0.997</td>
<td>1.005</td>
<td>0.009</td>
</tr>
<tr>
<td>LPS</td>
<td>1.014</td>
<td>0.999</td>
<td>0.996</td>
<td>0.994</td>
<td>0.012</td>
</tr>
<tr>
<td>d 7 postinfection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ConA</td>
<td>1.160</td>
<td>0.651</td>
<td>1.433</td>
<td>1.383</td>
<td>0.079</td>
</tr>
<tr>
<td>LPS</td>
<td>1.679</td>
<td>1.421</td>
<td>1.869</td>
<td>1.344</td>
<td>0.113</td>
</tr>
<tr>
<td>d 14 postinfection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ConA</td>
<td>1.056</td>
<td>1.010</td>
<td>1.275</td>
<td>1.030</td>
<td>0.025</td>
</tr>
<tr>
<td>LPS</td>
<td>1.094</td>
<td>0.797</td>
<td>1.011</td>
<td>0.927</td>
<td>0.035</td>
</tr>
<tr>
<td>d 28 postinfection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ConA</td>
<td>1.008</td>
<td>1.002</td>
<td>1.104</td>
<td>1.051</td>
<td>0.008</td>
</tr>
<tr>
<td>LPS</td>
<td>1.008</td>
<td>0.999</td>
<td>1.099</td>
<td>1.047</td>
<td>0.007</td>
</tr>
</tbody>
</table>

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*Means in the same row without the same superscript differ significantly (P < 0.05).

†On d 3, 7, 14, and 28 postinfection, bursal lymphocytes were isolated from spleens for proliferation assay; n = 5/treatment group.

‡CLA = conjugated linoleic acid; IBVD = infectious bursal virus disease; minus (−) = IBVD noninfected; plus (+) = IBVD infected; ConA = concanavalin A; LPS = lipopolysaccharide.

§On d 14, half of the chickens in control and CLA diets each were infected.

‖The P-values represent the main effect of the diet (CLA), the main effect of infection (IBDV), and the IBDV interaction between the infection and the dietary treatments (IBDV × CLA).
little depauperating, little punctate hemorrhage, and no typical pathological changes compared with the control diet. These findings suggested the nutritional immune modulation by CLA might prevent clinical signs of IBDV-associated disease.

Chickens infected with IBDV experienced suppression in both humoral (Dohms and Jaeger, 1988; Thompson et al., 1997) and cellular (Confer et al., 1981; Panigrahy et al., 1982; Kim et al., 2000) immunity. Humoral immunosuppression appears to be associated with IBDV-induced B-cell destruction, whereas the mechanism of cellular immunosuppression is largely elusive. For lymphocyte proliferation response, a significant reduction in the proliferation response was observed in the IBDV-infected birds, which was in line with McNeilly et al. (1999). As an immunomodulator, CLA used in animals has been reported. Most studies showed that CLA enhanced lymphocyte proliferation (Cook et al., 1993; Hayek et al., 1999; Bassaganya-Riera et al., 2001). In this study, the supplementation of CLA resulted in increased lymphocyte proliferation. Furthermore, lymphocyte proliferation was increased in infected chickens fed CLA-supplemented diets, which showed immune function enhanced by dietary CLA supplementation under the IBDV-immunosuppressive status in chickens.

At the molecular level, IBDV infection increased IFN-γ and IL-6 mRNA relative expression, which promotes inflammatory reaction. Research in nutritional immunology revealed an important role for dietary CLA in diminishing inflammation-associated diseases
Confer, A. W., W. T. Springer, S. M. Shane, and J. F. Donovan. 1991. Shultz et al., 1992; Lee et al., 1994. In this study, IFN-γ and IL-6 mRNA relative expression were attenuated in infected chickens fed CLA-supplemented diets. These findings suggested that the supplementation of CLA may help attenuate inflammation induced by IBDV challenge. This also indicated that, at the molecular level, the synthesis of these cytokines is controlled in part by the mechanism of transcriptional regulation. This demonstrates for the first time that the supplementation of CLA ameliorates the production and expression of proinflammatory cytokine profiles in IBDV-infected chickens. Consistent with the results of this study, dietary CLA reduced the release of proinflammatory cytokines.

Protection against IBDV may be mediated primarily by anti-IBDV antibodies (Vakharia et al., 1994; Fussell, 1998). The IBDV vaccines used in commercial flocks are selected by the vaccine’s ability to induce vigorous antibody responses (Kibenge et al., 1988; Fussell, 1998). However, the humoral immune responses against IBDV were induced by dietary treatment. The concentrations of IBDV-specific antibody titers were greater in CLA-fed chickens. This finding led to the conclusion that CLA was against the IBDV infection, or involved in immune protection in chickens, or both.

In conclusion, the supplementation of CLA in diet alleviated immunosuppression in IBDV-infected chickens. Conjugated linoleic acid had antiviral actions in part by suppressing IBDV-specific proinflammatory cytokine mRNA relative expression.

ACKNOWLEDGMENTS

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REFERENCES


