Profile of Chicken Cytokines Induced by Lipopolysaccharide Is Modulated by Dietary α-Tocopheryl Acetate

T. V. Leshchinsky and K. C. Klasing[1]

Department of Animal Science, University of California, Davis, California 95616

ABSTRACT Previously, we found that 25 to 50 IU/kg of dietary vitamin E (VE) had very different immunoregulatory effects than high VE levels (200 IU/kg), and we hypothesized that this difference was due to different cytokine profiles. Chicks were fed 0, 30, or 200 IU/kg supplemental VE and percentages of $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$, and $CD4^-CD8^-$ lymphocytes, and the ratio of $CD4^+/CD8^+$ lymphocytes was determined. The expression of chicken splenic interleukin-1β (IL-1β), myelomonocytic growth factor (MGF), interferon (IFNγ), and transforming growth factor-β (TGFβ) mRNA was determined by reverse transcription (RT)-PCR after intravenous injection of lipopolysaccharide (LPS). Due to a tendency for increased $CD4^-CD8^+$ lymphocytes at 30 IU/kg VE (P = 0.072), the $CD4^+/CD8^+$ ratio was significantly lower for 30 IU/kg VE compared with 0 IU/kg VE (P = 0.041). The VE dose of 200 IU/kg decreased the constitutive (prior to LPS) expression of TGFβ. The LPS caused an increase in IL-1β, MGF, and IFNγ expression at all VE concentrations and had no effect on IL-2 and TGFβ mRNA expression. Dietary VE decreased MGF mRNA (P = 0.049) in a dose-dependent manner but had no effect on the expression of other cytokines. The decreased expression of MGF could explain the immunomodulatory effect of VE in inflammation.

(*Key words*: chicken, cytokines, inflammation, lipopolysaccharide, vitamin E)

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INTRODUCTION

Research on the biology of avian cytokines and their role in regulating immunity has lagged behind the pioneering work in mouse and human systems. Attempts to analyze avian cytokines with mammalian bioassays systems have encountered major difficulties (Rath et al., 1995), as chicken cytokines exhibit relatively low amino acid sequence homology to mammalian cytokines due to large phylogenetic distance between Aves and mammals. Avian cytokines may exhibit unique functions that are not present in their mammalian counterparts. Several sequenced chicken cytokines, including chicken myelomonocytic growth factor (cMGF) and interleukin-2 (IL-2), do not have closely related mammalian counterparts and may exemplify either avian cytokines so far not identified in mammals or phylogenetic progenitors of mammalian cytokines (Leutz et al., 1989; Sundick and Gill-Dixon, 1997; Kaiser and Mariani, 1999).

Chicken MGF, first identified in lectin-stimulated spleen cultures and lipopolysaccharide (LPS) stimulated macrophages, is structurally related to mammalian IL-6 and granulocyte-colony stimulating factor. Recombinant chicken MGF acts like IL-6 in induction of acute response genes (Leutz et al., 1989), but it also manifests activities related to granulocyte/macrophage-colony stimulating factor (GM-CSF) and macrophage-colony stimulating factor (Sterneck et al., 1992; York et al., 1996; Siatskas and Boyd, 2000). Chicken IL-2 is produced by LPS or concanavalin A-activated tissues, as well as by activated T cells and exhibits structural characteristics of IL-2 (Kaiser and Mariani, 1999). However, the sequence analysis shows homology with mammalian IL-15 (Sundick and Gill-Dixon, 1997). In mammals IL-2 stimulates the proliferation of activated NK cells, B lymphocytes, cytotoxic effector T lymphocytes, CD4, CD8, and $\gamma\delta$ T lymphocytes, and antibody production. The uniqueness of chicken MGF and IL-2 suggests a possibility of broader function of these cytokines than their mammalian homologues.

Among other cytokines that have been sequenced in chickens are IL-1β, IL-6, transforming growth factor-β_2 (TGFβ), and interferon-γ (IFNγ), which possess biological activity similar to that of mammals (Sterneck et al., 1992; Digby and Lowenthal, 1995; York et al., 1996; Kaiser and Mariani, 1999). Chicken IL-1β functions like its mammalian counterpart in mediating an inflammatory response

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[1]To whom correspondence should be addressed: kcklasing@ucdavis.edu.

Abbreviation Key: IL = interleukin; IFN = interferon; LPS = lipopolysaccharide; MGF = myelomonocytic growth factor; RT = reverse transcription; TGF = transforming growth factor; TNF = tumor necrosis factor; VE = vitamin E.

and increasing antibody production (Leutz et al., 1989; Sterneck et al., 1992). Interferon-γ exhibits pleiotropic effects on leukocytes and induces secretion of nitric oxide in primary monocyte-derived chicken macrophages (Lowenthal et al., 1995; Schultz et al., 1995). Transforming growth factor-β possesses multifunctional characteristics being either immunostimulatory or immunosuppressive, depending on the cell target and other stimuli (Rath et al., 1995).

In mammals, a variety of hormonal and nutritional factors modulate the immune response through their effects on cytokine release. Of dietary factors, vitamin E (VE) is among the most potent. Supplementation of dietary VE well above that needed to minimize pathology cause marked changes in immune responses (Meydani et al., 1998). In vitro studies show that the ratio of reactive oxygen species to antioxidants in a cell modulates the activation states of the transcription factors NFκB and AP-1 (Grimble, 1994; Sen and Packer, 1996). Tocopherols and its derivatives inhibit activation of NFκB, but augment activation of AP-1 (Packer and Suzuki, 1993; Sen and Packer, 1996). A variety of immunoregulatory genes carry responsive elements for NFκB and AP-1 transcription factors, including genes for cytokines, acute phase proteins, and adhesion molecules (Baeuerle and Henkel, 1994). In vitro and in vivo studies have demonstrated the effect of VE on levels of these gene products. For example in mammals, VE deficiency causes an increase in IL-6 level, whereas high levels of VE supplementation decreases IL-6 (Amarakoon et al., 1995), IL-2, and tumor necrosis factor (TNF) (Mendez et al., 1995).

The multiple mechanisms by which VE affects lymphocytes might explain the complex and sometimes conflicting relationship between dietary VE level and indices of immunocompetence. Previous studies with young chicks in our laboratory (Leshchinsky and Klasing, 2001) demonstrated that VE at moderate levels (2.5 to 5 times minimal requirement) elicits higher antibody responses to certain antigens than high levels of VE (10 to 20 times the requirement). In addition, the level of dietary VE influenced lectin-induced proliferation of peripheral blood lymphocytes and influx of heterophils into the blood during inflammation. In mammals, the degree and type of the immune response is determined by the profile of cytokines secreted by macrophages and lymphocytes. Therefore, we hypothesize that different supplemental VE levels may have dissimilar effects on cytokine production, which, in turn, may lead to divergent immune responses.

In this study, we analyzed the expression of chicken splenic IL-1β, MGF, IL-2, IFNγ, and TGFβ after LPS administration in chicks fed three different concentrations of VE (0, 30, and 200 IU/kg diet) supplemented to a diet that was marginally adequate in VE (10 IU/kg).

MATERIALS AND METHODS

Animals and Diets

One-day-old broiler chicks (average weight 42.6 g) of Avian × Avian line[2] were fed a corn-soybean diet prepared according to NRC requirements (NRC, 1994) for young broilers with the exception of VE, which was supplemented in a form of α-tocopheryl acetate[3] at either 0, 30, or 200 IU/kg diet. By analysis, the basal diet (0 IU added VE) was found to contain 10.2 IU/kg VE from the feed ingredients, and this level is similar to the minimal dietary level of 10 IU that is recommended by NRC (NRC, 1994). Each dietary treatment was fed to six pens of nine chicks. Chicks were housed in battery brooders kept in a room with controlled light (18L:6D), temperature, and ventilation. Brooder temperatures were set to 36.7°C for the first week and were decreased by 3°C each consecutive week until they reached 23°C. Feed and water were supplied ad libitum. All experimental procedures were approved by the University of California, Davis, Animal Care and Use Committee.

Heparinized venous blood was collected from five birds per dietary treatment and spleens from six birds per dietary treatment were collected at 4 wk of age for VE analysis. Spleens were frozen in liquid nitrogen and stored at –70°C until analysis. Plasma was isolated from heparinized blood and stored at –70°C until analysis. Vitamin E in plasma, spleen, and diets were analyzed by HPLC.[3]

Flow Cytometry

Heparinized blood was collected from six 4-wk-old chicks per dietary treatment and total lymphocytes were enumerated using a hemacytometer. Peripheral blood mononuclear cells were purified by gradient centrifugation in Histopaque–1077,[3] according to manufacturer's instructions, washed two times in PBS, and resuspended in PBS containing 2% of BSA[4] and 0.001% of sodium azide.[4] Cells were labeled with $CD4^+$-FITC, $CD8^+$-RPE, or both $CD4^+$-FITC and $CD8^+$-RPE[5] according to the manufacturer's technical bulletin. Labeled cells were analyzed using FACScan flow cytometer[6] with a single argon laser at 488 nm with detectors for fluorescein isothicyanate and R-phycoerythrin. For each sample, data from 10^4 cells were obtained and analyzed using CellQuest software.[6] A gate was created for each sample based on the distribution of the majority of cells. Dot plots and statistics for $CD4^+CD8^-$, $CD4^-CD8^+$ $CD4^+CD8^+$, and $CD4^-CD8^-$ were performed within the selected gate. The $CD4^+/CD8^+$ ratios were calculated by dividing the percentage of $CD4^+CD8^-$ cells by the percentage of $CD4^-CD8^+$ cells.

RNA Extraction

Forty 4-wk-old chicks per diet were injected i.v. with 0.5 μg of LPS (*Salmonella typhimurium*[4]) in 1 mL PBS.

[2]Peninsula Hatchery, Santa Cruz, CA.
[3]CEPS Central Analytical Laboratory, University of Arkansas, Poultry Science Center L-209, Fayetteville, AR.
[4]Sigma Chemical Company, St. Louis, MO.
[5]Southern Biotechnology Associates, Inc., Birmingham, AL.
[6]Becton Dickenson, San Jose, CA.

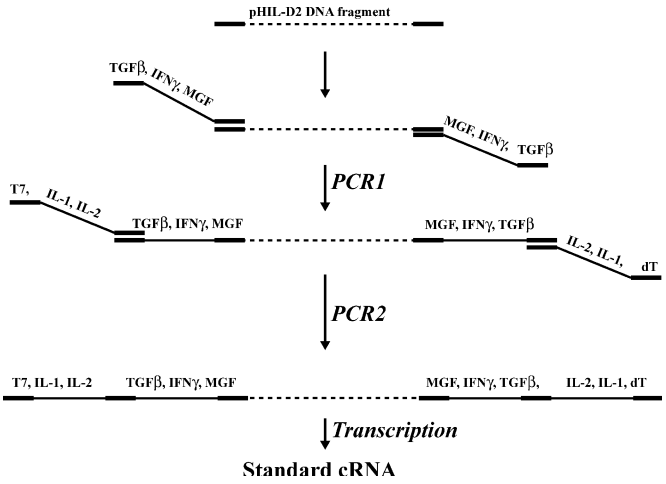

FIGURE 1. Strategy for the synthesis of the cytokine cRNA standard. IFN = interferon; IL = interleukin; MGF = myelomonocytic growth factor; TGF = transforming growth factor.

At 0, 1, and 3 h after LPS administration, spleens were removed from 10 chicks per diet, flash-frozen between two aluminum plates immersed in liquid nitrogen, and stored at –70°C until RNA extraction. RNA extraction was performed using RNAgents Total RNA Isolation System[7]. Analysis of ribosomal RNA bands on a formaldehyde denaturing gel electrophoresis validated RNA quality. The RNA concentration was measured by absorbance at 260 nm.

Synthesis of Chicken Cytokine Standard

The scheme of cRNA synthesis is illustrated in Figure 1. All molecular biology reagents, unless otherwise specified, were purchased from Promega.[7] Cytokine cRNA standard was synthesized by extension PCR followed by in vitro transcription directly from PCR product. After each reaction, the size of the PCR product was verified on a 1.2% agarose gel, and the product was purified using QIAEX II DNA Purification kit[8]. Plasmid pHIL-D2[9] was digested with restriction enzymes Not I and Sac I[9] and was used as a filling DNA. The size of the enzyme-digested products was verified by 1% agarose gel, and 213 bp product was purified using QIAEX II DNA Purification kit.[8] Extension PCR was conducted using 213-bp plasmid fragment primer sets 1, 2, and 3:[10]

Primer set 1:

Forward primer 5′-TGA GGC TGA CGG TGG ACC TAT TAT TGA GGT GTG CTT CAC TCA GGC CGC GAT CTA ACA TCC-3′ (60 mer)

Reverse primer 5′-GCG TGG ATT CTC AAG TCG TTC ATC GCT GCT GCT GGA TGT TGG AGC TCC AAT CAA GCC CAA-3′ (60 mer)

Primer set 2:

Forward primer 5′-GTG CTG CTT TGG CTG TAT TTC GGT AGC TGG ACT GCT ATC TCC TGA GGC TGA CGG TGG ACC-3′ (60 mer)

Reverse primer 5′- CTG AAG TTG GTC AGT TCA TGG AGA AAT TTT GTA AAC TTC TTT GCG TGG ATT CTC AAG TCG-3′ (60 mer)

Primer set 3:

Forward primer 5′-TAA TAC GAC TCA CTA TAG GAG ATG GCG TTC GTT CCC GAC CTG GAC GTG CTG CTT TGG CTG TAT-3′ (63 mer)

Reverse primer 5′-TTT TTT TTT TTT TTT ACT TAG CTT GTA GGT GGC GAT GTT GAC CTG AAG TTG GTC AGT TCA-3′ (60 mer)

The scheme of synthesis is illustrated in Figure 1. First PCR (PCR1) was run with filling DNA received by restriction enzyme digest of pHIL-D2 plasmid and primer set 1, which introduced complementary sequences for MGF and IFN specific primers. A second PCR (PCR2) was conducted with product of PCR1 and Primer set 2, which introduced complementary sequences for TGF and IL-2 specific primers. A third PCR (PCR3) was performed with product of PCR2 and Primer set 3, which introduced complementary sequences for IL-2, and IL-1 specific primers, as well as T7 promoter on the forward primer and polyA tail on the reverse primer.

The cRNA was synthesized from the final PCR product (PCR3) using T7 Megashortscript[11]. The concentration of the standard cRNA was measured by an absorbance at 260 nm. To verify the structure of the produced standard cRNA, 0.5 μg of cRNA was reverse transcribed with oligo$(dT)_{15}$ primers and amplified with specific cytokine primers by PCR; the size of the cytokine PCR products was verified by 2% agarose gel electrophoresis.

Reverse Transcription and PCR

Oligonucleotide primers were designed based on published chicken cytokine sequences (Sterneck et al., 1992; Digby and Lowenthal, 1995; York et al., 1996; Kaiser and Mariani, 1999) and were synthesized by a commercial laboratory[10]. Reverse transcription (RT) and PCR were performed in separate tubes in a PTC-100 Programmable Thermal Controller[12]. First, 1 μg of RNA was reverse transcribed into cDNA in a 30 μL RT reaction containing 5x reaction buffer [50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 3 m*M* MgCl2, 10 m*M* DTT], 0.5 m*M* dNTP, 750 μg of oligo$(dT)_{15}$, 30 U of ribonuclease inhibitor (Rnasin), and 300 U of M-MLV reverse transcriptase at 25°C for 15 min, 42°C for 50 min, and 95°C for 5 min. Then, PCR was performed in a 25 μL reaction containing 1 μL of cDNA from the RT reaction, 10x reaction buffer [50 m*M* KCl, 10 m*M* Tris-HCl (pH 9.0), and 0.1% Triton X-100], 2.0 m*M* $MgCl_2$, 10 pmol of each forward and reverse specific cytokine primer, 0.2 m*M* of dNTP, and 1.5 units of *Taq* DNA polymerase.

[7]Promega, Madison, WI.
[8]QIAGEN Inc., Valencia, CA.
[9]Invitrogen, Carlsbad, CA.
[10]OPERON Technologies, Inc., Valencia, CA.
[11]Ambion, Austin, TX.
[12]MJ Research, Inc., South San Francisco, CA.

Primer sequences:

IL-1:	primer 1 5′ - ATG GCG TTC GTT CCC GAC CTG GAC GTG CTG - 3′
	primer 2 5′ - ACT TAG CTT GTA GGT GGC GAT GTT GAC CTG - 3′
MGF:	primer 1 5′ - GAG GTG IGC TTC ACT CAG - 3′
	primer 2 5′ - CTG CTG CTG GAT GTT GGA - 3′
IL-2:	primer 1 5′ - CTT TGG CTG TAT TTC GGT AGC - 3′
	primer 2 5′ - AAG TTG GTC AGT TCA TGG AGA A - 3′
IFNγ:	primer 1 5′ - GCT GAC GGT GGA CCT ATT ATT - 3′
	primer 2 5′ - TGG ATT CTC AAG TCG TTC ATC G - 3′
TGFβ_2:	primer 1 5′ - TGC ACT GCT ATC TCC TGA G - 3′
	primer 2 5′ - ATT TTG TAA ACT TCT TTG GCG - 3′

Quantitative PCR cycle conditions were established for each gene in a series of preliminary experiments. In general, the PCR Thermal Controller was programmed for 94°C for 2 min, 30 cycles of (94°C for 30 s, T-annealing for 1 min, 68°C for 1 min), 68°C for 7 min, and 15°C until removed. T-annealing was 60°C for IL-1 and IFNγ; 58°C for MGF and TGFβ; and 63°C for IL-2. The PCR products were extracted from agarose gel using a QIAEX II Gel Extraction Kit[8] and analyzed by restriction enzyme digest using Alw 26 I for MGF, Sin I for TGF β_2, Eco I CR I for IFNγ, Eco I CR I and Alw 26 I for IL-2. Restriction digests of each PCR product produced bands of expected size, confirming the specificity of the primers.

Cytokine standard of known concentration was reverse transcribed with each set of samples. Next, PCR were performed for cytokine standard in duplicate and each sample in triplicate. The PCR products were resolved by 2% agarose gel electrophoresis, visualized by ethidium bromide staining, and photographed. The absorbance values of PCR products on photograph negatives were determined by a laser scanner and fluorImager.[12] To compare PCR runs and gels, the absorbence values of genes were adjusted to the cytokine standard present in each PCR run and gel. The ratio of the absorbance of the cytokine gene to the standard was used as a measurement of cytokine expression.

Statistical Analysis

Data were analyzed by JMP software.[13] Data for VE concentrations in tissues, lymphocyte concentrations in the peripheral blood, and percentage of $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$, $CD4^-CD8^-$, and $CD4^+/CD8^+$ ratios of the mononuclear leukocytes in the peripheral blood were analyzed by one-way ANOVA using dietary VE level as the independent variable. Data for cytokine expression were analyzed as a two-way factorial arrangement of treatments with three dietary levels of VE and three times of measurement. Means were compared by preplanned orthogonal contrasts. The main effect was considered significant when $P < 0.05$. Data are presented as treatment means ± SD.

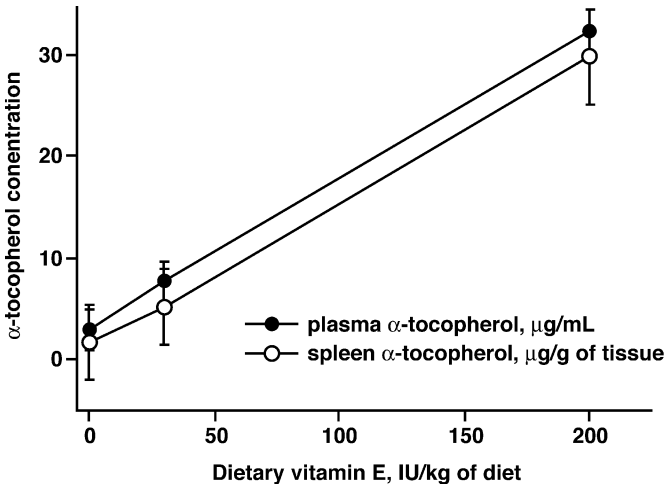

FIGURE 2. The α-tocopherol concentration in plasma and spleen (on a fresh tissue basis) of 4-wk-old chicks supplemented with 0, 30, or 200 IU of vitamin E / kg of diet. Data are means ± SD (n = 5 for plasma and n = 6 for spleen).

RESULTS

Analysis of α-Tocopherol Content in the Plasma and Spleen

The effect of dietary VE on plasma and spleen α-tocopherol content are shown in Figure 2. Chicks fed the basal diet had 2.96 ± 2.04 μg/mL of α-tocopherol in plasma and 1.61 ± 3.67 μg/g in spleen. Alpha-tocopherol content in plasma and spleen was increased significantly ($P < 0.01$) and linearly ($P < 0.01$, $R^2 = 0.91$ and $P < 0.01$, $R^2 = 0.70$) with increasing dietary VE content, reaching 32.36 ± 2.04 μg/mL in plasma and 29.86 ± 4.69 μg/g in spleen when chicks were fed 200 IU/kg VE. These results indicate that α-tocopherol deposition in tissue does not reach saturation with dietary VE supplementation up to 200 IU/kg. Level of α-tocopherol was slightly lower in spleen than in plasma, but plasma VE concentration was a good indicator of splenic VE.

The δ- and γ-tocopherol contents of plasma (Table 1) were affected by dietary VE ($P = 0.01$). The δ-tocopherol content of plasma increased as dietary VE increased, whereas γ-tocopherol levels decreased.

Lymphocyte Concentrations and $CD4^+/CD8^+$ Ratio in Peripheral Blood

Lymphocyte concentrations in peripheral blood were not affected ($P = 0.17$) by dietary VE and averaged 60.7

[13]SAS Institute, Cary, NC.

TABLE 1. Effect of dietary vitamin E level on the δ- and γ-tocopherol contents of plasma and spleen of 4-wk-old chicks[1]

Dietary vitamin E (IU/kg)	Tissue	δ-Tocopherol concentration (μg/mL)	γ-Tocopherol concentration (μg/g)
0		0.026 ± 0.003[a]	2.280 ± 0.147[b]
30	Plasma	0.020 ± 0.003[a]	1.600 ± 0.147[a]
200		0.040 ± 0.003[b]	1.560 ± 0.147[a]
P-value		0.006	0.014
0		0.235 ± 0.048	2.407 ± 0.306
30	Spleen	0.120 ± 0.048	1.677 ± 0.310
200		0.141 ± 0.061	1.816 ± 0.392
P-value		0.247	0.257

[a,b]Means with different superscripts in a column within a tissue are significantly different ($P < 0.05$). *P*-values are from one-way ANOVA.

[1]Values are presented as mean ± SE (n = 5 for plasma and n = 6 for spleen).

$\times 10^6$ cells/mL across the dietary treatments. The percentages of $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^-CD8^-$, and $CD4^+CD8^+$ lymphocytes were not affected by dietary VE (Figure 3). However, due to a tendency for increased $CD4^-CD8^+$ lymphocytes in chicks fed 30 IU/kg VE ($P = 0.07$), the ratio of $CD4^+/CD8^-$ to $CD4^-/CD8^+$ was significantly lower in chicks fed 30 IU/kg VE than in those receiving 0 IU/kg VE ($P = 0.04$).

Cytokine Expression

The effects of dietary VE and time after LPS administration on cytokine expression are presented in Figure 4, and the results of statistical analysis of this data are shown in Table 2. In general, the level of LPS-induced cytokine expression was highest for MGF and IFNγ followed by IL-1β. Expression of cytokines in the absence of LPS was highest for TGFβ, whose levels were close to levels of MGF and IFNγ at their highest level of expression. The IL-2 mRNA expression was the lowest among the analyzed cytokines.

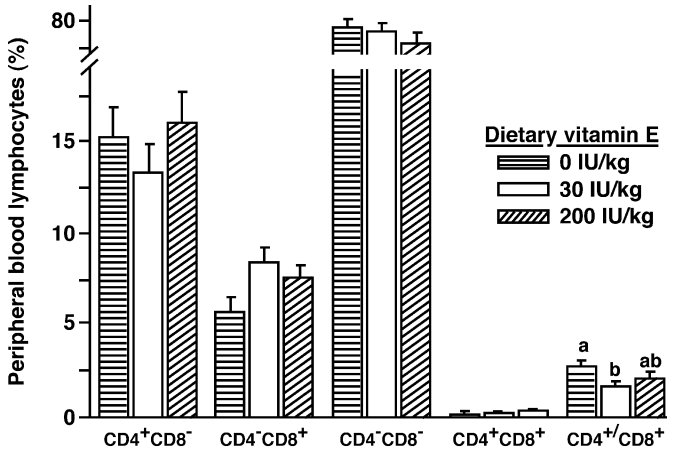

FIGURE 3. The effect of dietary vitamin E on numbers of peripheral blood $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^+/CD8^+$, $CD4^+CD8^+$, and $CD4^-CD8^-$ lymphocytes expressed as percentages of the mononuclear leukocytes. Data are means ± SD (n = 6). [a,b]Bars with different letters are significantly different.

IL-1β

Lipopolysaccharide administration significantly increased IL-1β mRNA at all dietary levels of VE ($P < 0.01$). The increase in IL-1β mRNA expression from 0 to 1 h was approximately fourfold, and this level of induction was maintained at 3 h.

MGF

The LPS administration significantly increased the MGF mRNA expression at all dietary VE concentrations ($P < 0.01$). The MGF mRNA levels increased sixfold from 0 to 1 h and remained elevated at 3 h. The MGF levels decreased with increasing dietary VE levels ($P = 0.04$). The decrease in MGF expression due to VE occurred earlier in chicks fed 200 IU/kg VE compared with those fed 30 IU/kg.

IL-2

The overall effects of LPS and VE on IL-2 mRNA expression were not significant.

IFNγ

The LPS administration caused an increase in IFNγ mRNA expression ($P < 0.01$). The IFNγ mRNA levels increased threefold at 1 h after LPS injection ($P = 0.01$) and slightly decreased by 3 h. The overall effect of dietary VE on IFNγ mRNA expression was not significant.

TGFβ

The overall effect of LPS and VE level on TGFβ mRNA expression was not significant. However, a VE × time interaction ($P < 0.01$) indicates that constitutive expression of TGFβ mRNA (time 0) was decreased by high VE (200 IU/kg), while LPS induced TGFβ mRNA expression at the highest level of VE (200 IU/kg) but not at 0 IU/kg or 30 IU/kg. This result is supported by comparison of TGFβ mRNA expression levels at time 0 h by orthogonal contrasts, which indicated a difference in TGFβ mRNA expression between chicks fed 0 IU/kg vs. 200 IU/kg VE ($P < 0.01$).

DISCUSSION

Bacterial LPS is often used in experiments to mimic bacterial infection and induce inflammation, which is characterized in mammals by the production of proinflammatory cytokines (IL-1, IL-6, and TNF) and inflammatory mediators like inducible cyclooxygenase and nitric oxide synthase (Endo et al., 1998). Many effects of LPS on leukocytes are mediated by activation of transcription factor NFκB. Examples of mammalian immunoregulatory genes responsive to NFκB activation include cytokines (IL-1, IL-2, IL-6, TNF, IFNγ, M-CSF, G-CSF, and GM-CSF), adhesion molecules, and acute phase proteins

TABLE 2. Effect of dietary vitamin E supplementation and time after lipopolysaccharide (LPS) administration on the mRNA expression of cytokines (*P*-values) in 4-wk-old chicks[1,2]

Variable	IL-1β	MGF	IL-2	IFNγ	TGFβ
Vitamin E	0.026	0.009	0.056	0.747	0.152
Time	<0.001	<0.001	0.302	<0.001	0.145
Vitamin E × time	0.537	0.034	0.095	0.028	<0.001

[1]*P*-alues from analysis of cytokine data by two-way ANOVA for the main effects of vitamin E concentration and time after LPS administration (n = 10 chicks per vitamin E level at each time point).

[2]IL = interleukin; MGF = myelomonocytic growth factor; IFN = interferon; TGF = transforming growth factor.

(Baeuerle and Henkel, 1994). Thus, in this experiment the increased expression of the proinflammatory cytokines IL-1β, MGF, and IFNγ mRNA expression after LPS administration was expected. The increase in biologically active chicken IL-1β and MGF after LPS was reported previously (Leutz et al., 1989; Nicolas-Bolnet et al., 1995; Siatskas and Boyd, 2000). We presume that an increase in IL-1 and MGF mRNA levels correlated with an increase in biologically active IL-1 and MGF in this experiment. IL-2 is also an NFκB-responsive (LPS-sensitive) gene in mammals, yet its levels were unaffected by LPS in chickens. We hypothesize that the lack of change in chicken IL-2 mRNA expression after LPS administration observed in our experiment could be an artifact of the relatively late time point selected for our first observation (1 h). It is possible that the IL-2 mRNA transcripts may have already been degraded by the time of our first observation at 1 h poststimulation.

The effect of VE on the expression of mammalian cytokines and other immunoregulatory molecules has been measured in vivo and in vitro under normal and pathological conditions. Vitamin E and its derivatives decrease expression of proinflammatory cytokines as well as other proinflammatory mediators, such as prostaglandins, nitric oxide synthase, and inducible cyclooxygenase in healthy animals (Wang et al., 1993; Mendez et al., 1995) and normalize cytokine expression in malnourished, aged, and virus-infected mammals (Wang et al., 1994; Meydani et al., 1998). It has been suggested that VE and other antioxidants modulate gene expression by quenching free radicals, which results in decreased activation of NFκB transcription factor and leads to decreased expression of NFκB responsive genes (Grimble, 1994; Sen and Packer, 1996). For example, VE inhibits NFκB translocation, and consequently, TNFα release by LPS-activated rat Kuppfer cells (Bellezzo et al., 1998). In our experiment, it is possible that the decrease in MGF mRNA after LPS administration in chicks fed moderate and high levels of VE was related to the VE effect on NFκB. Interestingly, higher levels of dietary VE (and presumably higher degree of inhibition of NFκB activation) resulted in faster inhibition of MGF expression.

Even though the expression of IL-1β and IFNγ is directly regulated by NFκB (Aronica et al., 1999), we did

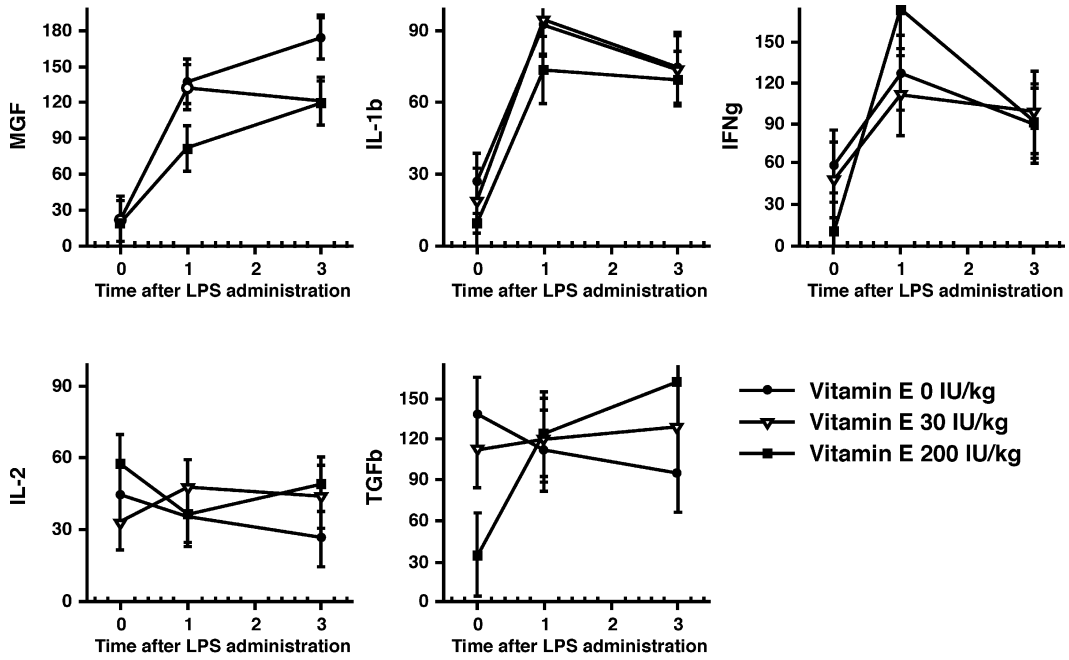

FIGURE 4. Effect of vitamin E on cytokine mRNA expression in spleens after lipopolysaccharide (LPS) administration. Data are means ± SD of optical density values ($\times 10^{-3}$) of 10 chicks for each vitamin E level at each time point. IFN = interferon; IL = interleukin; MGF = myelomonocytic growth factor; TGF = transforming growth factor.

not observe an inhibition of expression of these cytokines by VE supplementation. It is known that the stimulation of IFNγ expression is mediated by macrophage-derived cytokines IL-1β, TNFα, and IL-12 and is autostimulated by IFNγ itself (Boehm et al., 1997). It is possible that the amount of LPS used in our experiment was sufficient to maximally stimulate the induction of these cytokines and override the more subtle effects of VE.

We did not observe an increase in TGFβ mRNA expression after LPS administration. However, the amount of biologically active TGFβ released from cells also depends on posttranslational regulation (Burt and Paton, 1991), and others have reported an increase in TGFβ activity following inflammation (Rath et al., 1995). We were surprised that TGFβ mRNA expression was decreased by dietary VE level before LPS administration (0 h). Both monocytes and neutrophils constitutively produce TGFβ (Parekh et al., 1994), and our data indicate that high levels of VE decrease this constitutive production. Qureshi and Gore (1997) observed a similar effect of VE on constitutive release of inflammatory mediators by a chicken macrophage cell line. In their experiment, VE inhibited basal, but not LPS induced, release of prostaglandin E_2 and thromboxane B_2. Considering immunosuppressive effect of TGFβ on activated lymphocytes, monocytes, and macrophages (McCartney-Francis et al., 1998), it is possible that low initial TGFβ activity at 200 IU/kg VE may permit greater or faster activation of leukocytes. However, TGFβ is critical for immune homeostasis in the gut associated lymphoid tissue and the prevention of inflammation and immunopathology in the gastrointestinal tract.

Dose dependent effects of VE on murine cytokine production in vitro were reported previously by Yu (Yu et al., 1996). Very low concentrations of VE succinate (0.1 μg/mL), a VE analog used in cell culture research, enhances EL-4 cell proliferation due to increase in IL-2 production, whereas high concentration of VE succinate (10 to 20 μg/mL) inhibits cell proliferation due to increases in biologically active TGFβ. In our experiments, the VE concentration in chicken plasma increased linearly from 2.96 ± 2.04 μg/mL at 0 IU/kg dietary VE to 32.36 ± 2.04 μg/mL at 200 IU/kg dietary VE. The VE concentration in spleen was slightly lower than in plasma. The linear increase in plasma and tissue content of α-tocopherol and decrease in γ-tocopherol agrees with previous reports of tissue response to dietary VE (Hassan and Hakkarainen, 1990). The dose dependent effect of VE on the expression of MGF and TGFβ mRNA may help to explain its differential effect at low, moderate, and high levels on indices of immunocompetence that we have previously observed (Leshchinsky and Klasing, 2001). Different dietary levels of VE could create dissimilar ratios of cellular antioxidants to free radicals and lead to various degrees of activation of NFκB, or other transcription factors, or both (Baldwin, 1996; Nakamura et al., 1997).

We observed a decrease in $CD4^+/CD8^+$ ratio at a dietary VE level of 30 IU/kg due partly to elevated $CD4^-CD8^+$ percentages. An effect of dietary VE on $CD4^+/CD8^+$ ratio was reported previously in chicks (Erf et al., 1998) and mammals (Gu et al., 1999). Erf et al. (1998) reported that dietary VE (87 mg/kg diet) increases the percentage of $CD4^+CD8^-$ cells in spleen of adult birds. The percentage of $CD4^+CD8^-$, $CD4^-CD8^+$ cells, and their relative ratio depended on the age of the bird and the dietary VE level. However, Gu et al. (1999) showed that VE significantly decreased the percentage of $CD4^+CD8^-$ cells and $CD4^+/CD8^+$ ratio in spleen and mesenteric lymph node of rats. We assume that the discrepancy in results of these studies and our study may be due to the difference in animal age, the relative levels of dietary VE, and the pool of lymphocytes examined (peripheral vs. tissue). Many factors determine the $CD4^+/CD8^+$ ratio in different pools, including specific antigen stimulation, cytokines, and expression of adhesion molecules, which determine homing of lymphocytes. For example, $CD4^-CD8^+$ cell proliferation (memory phenotype) is enhanced by IL-15 and IFN (Sprent et al., 1999); therefore, it is possible that modulation of cytokine and adhesion molecule expression is one of the mechanisms by which different dietary levels of VE affect $CD4^+/CD8^+$ ratio.

In a summary, dietary VE decreased mRNA expression of the proinflammatory cytokine MGF after LPS administration, which might explain the anti-inflammatory effect of VE. High VE concentrations decreased constitutive TGFβ mRNA expression, which suggests that TGFβ may be at least partially responsible for the divergence of the immune responses at different dietary levels of VE. However, it would be desirable to analyze the in vivo biological activity of cytokines at different levels of VE. The difference in $CD4^+/CD8^+$ ratios in peripheral blood lymphocytes suggests that moderate VE levels may have different effects on lymphocyte population dynamics compared with higher VE levels.

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