Research Notes

Influence of Dietary Vitamin E on Phagocytic Functions of Macrophages in Broilers

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ABSTRACT Vitamin E (VE) is known for its antioxidant properties and has been shown to modulate immune system functions in various species. This study examined the influence of different levels of dietary VE (α-tocopherol acetate) on phagocytic functions of macrophages (abdominal exudate cells) in broiler chickens at 3, 5, and 7 wk. Birds were fed commercial diets containing 16 (control), 110, or 220 mg of VE/kg of feed. Macrophages were elicited into the abdominal cavity by injecting a 3% Sephadex solution prepared in PBS (G50-50, 1 mL/100 g of BW) 42 h prior to harvest. The percentage of phagocytically active macrophages and the number of SRBC phagocytosed per macrophage for unopsonized and antibody-opsonized SRBC were determined. These aspects of macrophage function were assessed based on 900 macrophages per sample. When unopsonized SRBC were used, dietary VE supplementation above control level did not affect phagocytic function of macrophages at wk 3, 5, or 7. With antibody-opsonized SRBC, the percentage of phagocytically active macrophages and the number of SRBC phagocytosed per macrophage were higher (P = 0.08 and P = 0.01, respectively) in 3-wk-old birds fed 110 and 220 mg of VE/kg of feed compared with age-matched controls. This enhancing effect of VE supplementation on macrophage function was not observed in 5- and 7-wk-old broilers. It appears from this study that supplemental VE enhances Fc-receptor-mediated macrophage phagocytic activity at early stages of broiler growth.

(*Key words*: broiler, immune function, macrophage, phagocytosis, vitamin E)

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INTRODUCTION

Crucial involvement of monocytes and macrophages in humoral and cell-mediated immunity makes them indispensable factors in host defense. Macrophages by way of phagocytosis eliminate necrotic cells, circulating immune complexes, tissue debris, and invading microorganisms (Qureshi, 1998). Additional important functions of macrophages include antigen presentation and secretion of a number of cytokines, prostaglandins, and free radicals. Increased presence of free radicals can cause oxidation of biomolecules, leading to cell death and tissue injury involved in the pathogenesis of several disease states (Kehrer, 1993). Toxic effects of free radicals may be reduced by the presence of antioxidants, such as vitamin E (VE). Evidence gathered until now shows that VE is a highly lipophilic free radical scavenger that is preferentially distributed in cell membranes where it breaks lipid peroxidation chain reactions (Yu, 1994). The antioxidant properties of VE have been credited for the enhancement of humoral and cell-mediated immune functions that are observed in avian and mammalian species (Tengerdy and Brown, 1977; Moriguchi et al., 1990; Meydani and Beharka, 1998). Yet, the mechanisms of VE action on the cells of the immune system and the most optimal route and program of VE administration in broilers have not been completely elucidated.

Early research examining the effect of dietary VE (300 mg of VE/kg) on the immune system of broilers has shown that VE-supplemented broilers exhibit a 3- to 8-fold reduction in mortality caused by *Escherichia coli* infection (Tengerdy and Nockels, 1975; Tengerdy and Brown, 1977). Although macrophage phagocytic functions were not examined in those studies, the increased clearance of *E. coli* in VE supplemented broilers points to enhanced macrophage phagocytosis. Administration of 10 mg of VE to broiler and poult embryos in ovo (d 18) improved humoral and cellular effector components of the immune system (Gore and Qureshi, 1997). In ovo VE-treated birds that were challenged with SRBC at 7 d of age exhibited increased antibody response to SRBC at d 14 and 21. Additionally, at 4 wk of age, abdominal exudate macrophages of in ovo VE-treated birds showed increased ability to phagocytose SRBC and to produce nitric oxide in response to LPS stimulation in vitro (Gore and Qureshi, 1997).

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Abbreviation Key: AEC = abdominal exudate cells; VE = vitamin E.

Evidence that has been generated over the last few decades highlights the importance of nutrients (including VE) for the optimal functioning of the immune system. Thus, immune functions should be one of the criteria upon which nutrient requirements are based (Bendich, 1995). The recognition of the importance of VE for broiler health and production has resulted in practices of supplementing broiler diets with levels of VE exceeding those recommended by the NRC (BASF, 1994). Established NRC requirements (NRC, 1994) may be optimal for growth but not necessarily for best health and disease resistance. Therefore, it is of particular interest to further examine the role that VE plays on specific functions of the immune system of poultry. The objective of this study was to determine the effect of supplemental dietary VE (above NRC levels) provided throughout a 7 wk growing period on macrophage functions in broilers. Specifically, the effect of supplemental dietary VE on the in vitro adherence ability of abdominal exudate cells and the ability of abdominal exudate macrophages to phagocytose unopsonized or antibody-opsonized SRBC were examined in broilers at 3, 5, and 7 wk of age.

MATERIALS AND METHODS

Birds and Diet

One hundred 1-d-old male broiler chicks (Cobb 500)[2] were randomly assigned to 1 of 3 dietary VE treatments and placed in floor pens in 2 environmentally controlled chambers on wood shavings litter. Average chick weight used for the study was approximately 42 g. Chicks were vaccinated for Marek's disease in ovo (d 18) and for Newcastle disease and infectious bronchitis at hatch. Broilers were maintained on a 23L:1D lighting schedule and were provided feed and water ad libitum. The chicks received either a basal corn-soybean diet, formulated to meet or exceed NRC requirements (NRC, 1994), or the basal diet with additional 110 and 220 mg of VE/kg feed in a form of DL-α-tocopherol acetate.[3] For the first 3 wk, chicks were fed a broiler starter diet (CP, 25%; ME, 3,200 kcal/kg), and for the remaining 4 wk they were fed a grower diet (CP, 18%; ME, 3,200 kcal/kg).

Elicitation of Abdominal Macrophages

The abdominal exudate cells (AEC) were harvested as described by Qureshi et al. (1986). Briefly, at 3, 5, and 7 wk, 15 randomly selected birds from each treatment were weighed and given an intra-abdominal injection of a 3% Sephadex G-50[4] in sterile PBS solution (1 mL/100 g of BW). After 42 h, Sephadex-injected chicks were euthanized by i.v. injection of 6.5% pentobarbital solution.[4] The AEC were harvested by flushing the abdominal cavity with 20 mL of heparinized (0.5 U/L) Dulbecco's PBS[4] solution. The collected AEC suspensions obtained from 10 birds per treatment were placed into siliconized glass tubes and allowed to sit on ice for 10 min. Supernatant fluid that contained the AEC was transferred into a new tube, and AEC were pelleted by centrifuging the sample at 150 $\times$ *g* for 10 min at 4°C. The supernatant fluid was discarded, and the pelleted AEC were resuspended in 4 mL of LM Hahn medium (Qureshi et al., 1986).

Lymphoid Organ Weights

After AEC were harvested, thymus, spleen, and bursa were collected from each chick. Adherent fat was removed from these tissues, and the tissues were weighed. The data were presented as a percentage of tissue weight relative to the BW (relative organ weight).

AEC Adherence Assay

From each bird, 1 mL of the AEC suspension was added to a 35-mm culture dish that contained 4 glass cover slips. To allow cells to adhere, culture dishes were incubated at 37°C in a humidified chamber with 5% CO_2 for 1 h. Cover slips were then washed with cold PBS to remove nonadherent cells. After drying, cells were fixed and stained with Leukostat[5] and mounted on microscope slides, 3 coverslips per slide. Proportions of macrophages and other nonerythroid cells in the AEC suspension were determined by morphological classification (Lucas and Jamroz, 1961). The number of adherent cells per microscope viewing field (at 1,000×) was determined by counting all leukocytes until at least 300 abdominal exudate macrophages per cover slip were examined while keeping track of the number of microscope viewing fields and the numbers of macrophages observed. In addition, the total number of adherent leukocytes and macrophages identified was recorded, and the percentage of adherent leukocytes that were macrophages was calculated.

SRBC Phagocytosis Assay

To determine the in vitro phagocytic potential of macrophages, the AEC suspension was adjusted to contain 1×10^6 viable macrophages per milliliter. To establish macrophage monolayers, 1 mL of this suspension from each chick was pipetted into a 35-mm tissue culture dish that contained 4 glass cover slips. The culture was then incubated for 1 h at 37° C to allow cells to adhere. Macrophage monolayers were then incubated for 1 h with 1 mL of a 3% SRBC solution in a humidified chamber with 5% CO_2 at 37°C. The SRBC used were either unopsonized or opsonized with a nonagglutinating concentration of quail anti-SRBC antibodies. After the incubation, cover slips were washed with PBS, dried, fixed, and stained with Leukostat[5] and mounted on microscope slides, 3 coverslips per slide. A total of 300 macrophages per cover slip (900 macrophages per slide) were examined by bright field

[2]Randal Road Hatchery, Tyson Foods, Inc. Springdale, AR.
[3]Roche Vitamins, Inc. Nutley, NJ.
[4]Sigma Chemical Co. St. Louis, MO.
[5]Fisher, Pittsburgh, PA.

microscopy at 1,000× to determine the proportion of phagocytically active macrophages and the number of SRBC phagocytosed per phagocytically active macrophage.

Statistical Analysis

Data were analyzed separately for each sampling point (wk 3, 5, and 7) using the GLM procedures of SAS software package (SAS Institute Inc., 1997). Treatment means for all measurements were separated by LS means procedure and declared significantly different at $P \leq 0.05$.

RESULTS AND DISCUSSION

There is a small amount of information regarding the importance of dietary VE for the functioning of macrophages in broilers. The environmental conditions under which broilers are raised subject them to a number of stressful agents, including the constant exposure to various microorganisms. Macrophages phagocytose and kill ingested microbes by generating large amounts of highly reactive molecules such as reactive oxygen intermediates (ROI) (Qureshi, 1998; 2003). Therefore, the autooxidative damage of macrophages as well as tissue injury due to overproduction of ROI is an unavoidable consequence of macrophage activation (Splettstoesser and Schuff-Werner, 2002). Thus, it is important that macrophages have a number of antioxidant systems available to cope with oxidative stress. VE has been recognized as a very important hydrophobic antioxidant that can arrest or entirely prevent lipid peroxidation of the cell membrane (Tappel, 1970). This function of VE is particularly crucial for maintaining cell membrane integrity and enabling macrophages to efficiently phagocytose, contain, and kill ingested microbes (Tappel, 1970; Sakamoto et al., 1990).

Based on the above-mentioned information, this study was designed to examine the effect of dietary VE (above NRC requirements) on the in vitro phagocytic potential of abdominal exudate macrophages of broilers when VE was supplemented from day of hatch.

Body Weights and Relative Organ Weights

There were no differences in the BW of broilers fed different VE levels at wk 3, 5, or 7 (data not shown). No differences among treatments were observed in relative weights of lymphoid organs (data not shown) with the exception of the relative spleen weight at 7 wk, which was higher in chicks fed 110 and 220 mg of VE/kg compared with chicks fed the control diet (0.14 ± 0.01, 0.18 ± 0.01 and 0.10 ± 0.02, respectively; $P \leq 0.05$). Previously, several authors have reported that VE deficiency depletes leukocytes in the spleen (Scott et al., 1955; Dietert et al., 1983), whereas VE supplementation increases splenocyte proliferation (Sakai and Moriguchi, 1997). Hence, the increase in spleen weight with supplemental VE observed here is likely to represent an increase in the number of lymphocytes.

TABLE 1. Influence of dietary vitamin E (VE) on numbers of adherent abdominal exudate cells (AEC) and macrophages per microscope field and the percentage of adherent macrophages in broilers at wk 3, 5 and 7[1]

VE (mg/kg)	Week	Adherent AEC[2]	Adherent macrophages[2]	Macrophage (%)[3]
16	3	60 ± 6[b]	36 ± 4[b]	67.6 ± 3.2
110		77 ± 6[a]	53 ± 4[a]	69.9 ± 3.9
220		66 ± 5[b]	48 ± 3[ab]	72.4 ± 1.4
16	5	50 ± 7[b]	34 ± 5[b]	65.8 ± 2.8
110		80 ± 4[a]	49 ± 4[a]	63.2 ± 4.5
220		51 ± 7[b]	34 ± 5[b]	71.4 ± 3.7
16	7	73 ± 13[ab]	44 ± 9[ab]	59.1 ± 4.5
110		90 ± 8[a]	57 ± 7[a]	61.1 ± 2.7
220		55 ± 8[b]	31 ± 4[b]	58.8 ± 2.6

[a,b]Means within an age group and column with different superscripts are significantly different ($P \leq 0.05$).

[1]Cobb 500 broilers were fed diets supplemented with 16, 110, or 220 mg VE (α-tocopherol)/kg of feed from day of hatch up to 7 wk.

[2]At wk 3, 5 and 7, 1 mL of elicited AEC suspension was incubated on glass coverslips for 1 h. All adherent AEC were scored at 1,000× magnification until a count of 900 macrophages per bird was reached. Data was expressed as the number of adherent cells per microscope field.

[3]The percentage of macrophages (Mϕ) was calculated by the formula Mϕ % = (Mϕ number / total AEC number) × 100. Data were expressed as mean ± SEM based on 10 broilers.

Yield of AEC and Incidence of Adherent AEC and Macrophages

An increase in the yield of Sephadex-elicited AEC has been reported for broilers injected with VE (Gore and Qureshi, 1997). However, we did not observe differences in the numbers of AEC harvested from broilers fed different VE diets (data not shown). At 3 and 5 wk, the number of adherent AEC and macrophages per microscope field were highest in broilers fed 110 mg of VE/kg ($P = 0.05$, Table 1). At 7 wk, broilers fed 16 and 110 mg of VE/kg had higher numbers of adherent AEC and macrophages per microscope field compared with broilers fed 220 mg of VE/kg ($P \leq 0.05$, Table 1). It appears that higher numbers of adherent AEC were due to increased numbers of adherent macrophages, but this trend was not apparent when the percentage of macrophages within adherent AEC were determined (Table 1). Hence, in this study additional VE supplementation did not influence the yield of adherent AEC and macrophages harvested.

Phagocytic Potential of Abdominal Exudate Macrophages

The percentage of phagocytically active macrophages incubated with unopsonized SRBC and the number of unopsonized SRBC phagocytosed per individual macrophage were not affected by dietary VE at any age examined (Figure 1). The percentage of phagocytically active macrophages incubated with antibody-opsonized SRBC for 100 and 200 mg of VE/kgand the number of antibody-opsonized SRBC phagocytosed per individual macrophage were higher in 3-wk-old broilers fed supplemental VE ($P = 0.08$ and $P = 0.05$, for 100 and 200 mg of VE/kg,

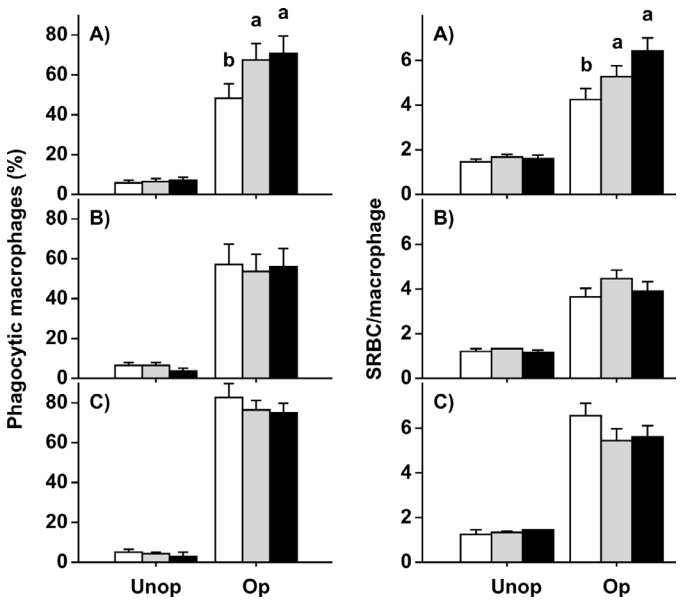

FIGURE 1. Influence of dietary vitamin E (VE; (-tocopherol)) on the proportions of phagocytically active macrophages and the numbers SRBC phagocytosed per macrophage in broilers at wk 3 (A), 5 (B), and 7 (C). Cobb 500 broilers were fed diets supplemented with 16 (white bars), 110 (gray bars), or 220 mg (black bars) of VE/kg from day of hatch up to 7 wk. At 3, 5, and 7 wk, Sephadex-elicited abdominal exudate macrophages were obtained from 10 broilers per treatment, and macrophage monolayers were incubated for 1 h with unopsonized (Unop) or antibody-opsonized (Op) SRBC. To determine the number of phagocytically active macrophages and the number of SRBC phagocytosed per macrophage, we examined 900 macrophages per slide (300 per cover slip) at 1,000x magnification. Data were expressed as the mean ± SEM. [a,b]Means without a common letter are different ($P = 0.08$ or less).

respectively, Figure 1). No treatment differences in the percentage of phagocytic macrophages incubated with antibody-opsonized SRBC or the numbers of antibody-opsonized SRBC phagocytosed per macrophage were observed at wk 5 and 7 (Figure 1).

Macrophages have receptors on their plasma membranes for the Fc portion of immunoglobulins. These receptors mediate and enhance phagocytosis and associated killing mechanisms (e.g., production of reactive oxygen intermediates) of antibody-opsonized antigens by macrophages (Yamamoto and Johnson, 1984). Hence, the observed increase in phagocytosis of opsonized SRBC by macrophages of the VE-fed broilers appears to be due to increased expression of these receptors on the macrophage membrane. The increased ability of macrophages from broilers receiving supplemental dietary VE to phagocytose antibody-opsonized SRBC may be of particular benefit at this young age. At 3 wk, broiler chicks are not fully immunocompetent and rely substantially on maternal antibodies and innate immune mechanisms for defense against environmental pathogens. Thus, the observed increase in Fc-receptor-mediated phagocytosis by macrophages from 3-wk-old broilers supplemented with VE suggests that dietary VE supplementation at levels above NRC recommendation may optimize the effectiveness of maternal antibodies and greatly benefit the immune defense of very young chicks.

Conclusions

Under the experimental conditions used in this study (i.e., environmental chambers) the standard VE supplementation (16 mg VE/kg) was optimal for most aspects of macrophage functions examined. However, supplemental dietary VE increased the receptor-mediated phagocytic potential of elicited abdominal exudate macrophages in 3-wk-old broilers and increased their ability to make effective use of maternal antibodies. When translated into large-scale broiler production, this seemingly modest enhancement of immune system function on aspects of innate immunity early in the life of broilers together with previous reports on beneficial effects of VE on the development and function of adaptive immunity in 5- to 7-wk-old broilers (Erf and Bottje, 1996; Erf et al., 1998) suggest that additional VE supplementation of broiler diets may have a substantial positive impact with respect to disease resistance and improved broiler health.

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REFERENCES

BASF. 1994. Vitamin supplementation rates for U.S. commercial poultry, swine and dairy cattle. Pages 14–169 in BASF Keeping Current. BASF Corporation, Parsippany, NJ.

Bendich, A. 1995. Immunology functions to assess nutrient requirements. J. Nutr. Immunol. 3:47–56.

Dietert, R. R., J. A. Marsh, and G. F. Combs, Jr. 1983. Influence of dietary selenium and vitamin E on the activity of chicken blood phagocytes. Poult. Sci. 62:1412–1418.

Erf, G. F., and W. G. Bottje. 1996. Nutrition and immune function in chickens: Benefits of dietary vitamin E supplementation. Pages 113–130 in Proceedings of the Arkansas Nutrition Conference, Fayetteville, AR.

Erf, G. F., W. G. Bottje, T. K. Bersi, M. D. Headrick, and C. A. Fritts. 1998. Effects of dietary vitamin E supplementation on the immune system in broilers: Altered proportions of CD4 T cells in thymus and spleen. Poult. Sci. 77:529–537.

Gore, A. B., and M. A. Qureshi. 1997. Enhancement of humoral and cellular immunity by vitamin E after embryonic exposure. Poult. Sci. 76:984–991.

Kehrer, J. P. 1993. Free radicals as mediators of tissue injury and disease. Crit. Rev. Toxicol. 23:21–48.

Lukas, A. M., and C. V. Jamroz. 1961. Atlas of avian hematology. USDA Agriculture Monograph Gr 25. USDA, Washington, DC.

Meydani, S. N., and A. A. Beharka. 1998. Recent developments in vitamin E and immune response. Nutr. Rev. 56:S49–58.

Moriguchi, S., Kobayashi, N., and Y. Kishino. 1990. High dietary intakes of vitamin E and cellular immune functions in rats. J. Nutr. 120:1096–1102.

National Research Council. 1994. Nutrient Requirements of Poultry. 9th rev. ed. National Academy Press, Washington, DC.

Qureshi, M. A., R. R. Dietert, and L. D. Bacon. 1986. Genetic variation in the recruitment and activation of chicken peritoneal macrophages. Proc. Soc. Exp. Biol. Med. 141:560–568.

Qureshi, M. A. 1998. Role of macrophages in avian health and disease. Poult. Sci. 77:978–982.
Qureshi, M. A. 2003. Avian macrophage and immune response: an overview. Poult. Sci. 82:691–698.
Sakai, S., and S. Moriguchi. 1997. Long-term feeding of high vitamin E diet improves the decreased mitogen response of rat splenic lymphocytes with aging. J. Nutr. Sci. Vitaminol. 43:113–122.
Sakamoto, W, K. Fujie, H. Handa, T. Ogihara, and M. Mino, 1990. In vivo inhibition of superoxide production and protein kinase C activity in macrophages from vitamin E-treated rats. Int. J. Vitam. Nutr. Res. 60:338–342.
SAS Institute. 2000. Users Guide to SAS. JMP version. 4 ed. SAS Institute Inc., Cary, NC.
Scott, M. L., F. W. Hill, L. C. Norris, D. C. Dobson, and T. S. Nelson. 1955. Studies on vitamin E in poultry nutrition. J. Nutr. 56:387–402.
Splettstoesser, W. D., and P. Schuff-Werner. 2002. Oxidative stress in phagocytes—The enemy within. Microsc. Res. Technol. 57:441–455.
Tappel, A. L. 1970. Biological antioxidant protection against lipid peroxide damage. Am. J. Clin. Nutr. 23:1137–1139.
Tengerdy, R. P., and J. C. Brown. 1977. Effect of vitamin E and A on humoral immunity and phagocytosis of *Escherichia coli* infected chickens. Poult. Sci. 56:957–963.
Tengerdy, R. P., and C. F. Nockels. 1975. Vitamin E or vitamin A protects chickens against *Escherichia coli* infection. Poult. Sci. 54:1292–1296.
Yamamoto, K., and R. B. Johnston Jr. 1984. Dissociation of phagocytosis from stimulation of the oxidative metabolic burst in macrophages. J. Exp. Med. 159:405–416.
Yu, B. P. 1994. Cellular defense against damage from reactive oxygen species. Physiol. Rev. 74:139–162.