The Role of Dietary Vitamin E in Experimental *Listeria monocytogenes* Infections in Turkeys[1]

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ABSTRACT The current study was designed to determine if dietary vitamin E influenced either the gut clearance or levels of peripheral blood $CD4^+$ and $CD8^+$ T lymphocytes in adult turkeys experimentally infected with *Listeria monocytogenes*. Turkeys were fed vitamin E (0, 100, or 200 IU) from day of hatch to time of necropsy. After 6 wk on the experimental diet, turkeys were orally inoculated with *L. monocytogenes* (~ 10^9 cfu). To monitor infection status, cloacal swabs were taken on selected days post-inoculation (DPI). At necropsy, samples of viscera, including liver, spleen, cecum, duodenum, ileum, and colon were collected and cultured for *L. monocytogenes*. In experiments 1 and 2, recovery of *L. monocytogenes* from cloacal swabs, tissues, and intestines from turkeys fed vitamin E was generally lower than that from turkeys fed the control diet, although these differences were not statistically significant. When data from both trials were combined, *L. monocytogenes* was cultured less frequently from cloacal swabs of the vitamin E-treated group (200 IU) on 2 and 3 DPI, when compared to controls (0 IU, $P < 0.01$). There were no changes in virulence characteristics of *L. monocytogenes* cells, as measured by in vitro killing of Ped-2E9 cells, recovered from cloacal swabs or tissues of experimentally infected turkeys fed the control or a vitamin E treatment diet. Flow cytometric analysis indicated that $CD4^+$ and $CD8^+$ peripheral blood T lymphocytes were elevated at 6 and 8 DPI in infected turkeys given 200 IU vitamin E.

(*Key words*: $CD4^+$ and $CD8^+$, immune response, *Listeria*, turkey, vitamin E)

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INTRODUCTION

Listeria monocytogenes is a major human bacterial foodborne pathogen that annually accounts for ~2,500 cases (meningitis, encephalitis, sepsis, fetal death, prematurity) and 504 deaths (Mead et al., 1999). Sporadic human cases of listeriosis have been epidemiologically linked to the consumption of undercooked poultry products (Schwartz et al., 1988). Analysis of risk factors associated with sporadic human listeriosis in the U.S. indicated that cancer patients and immunocompromised patients, in whom 69% of listeriosis cases occur, were more likely than controls to have eaten undercooked poultry (odds ratio = 3.3; Schuchat et al., 1992). A 1998 multistate outbreak of human listeriosis, ascribed to serotype 4 (101 cases, resulting in 22 deaths), was linked to delicatessen meats, including turkey (MMWR, 1998). A 2002 outbreak involving 46 cases, seven deaths, and three stillbirths was linked to contaminated delicatessen turkey meats (MMWR, 2002). The recall of 26 million pounds of turkey meat in 2002 indicates the economic consequences of ready-to-eat meats contaminated with *L. monocytogenes* (U.S. Department of Health and Human Services, 2002).

Adult turkeys may be transiently colonized by consuming contaminated feed or water (Husu et al., 1990). Thus, *L. monocytogenes* may enter the packing plant at low levels in the intestine of recently infected birds, survive in biofilms, and ultimately contribute to both environmental and ready-to-eat product contamination (Genigeorgis et al., 1990; Ojeniyi et al., 1996). In the United States, *L. monocytogenes* was found on 5.9% of turkey carcass rinses and in 31% of ground turkey meat examined in the Nationwide Young Turkey Microbiological Baseline Data Collection Program (U.S. Department of Agriculture, 1998).

Vitamin E is required for normal development and function of the immune system in poultry (Boa-Am-

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Abbreviation Key: DPI = days post-inoculation.

ponsem et al., 2000; Leshchinsky and Klasing, 2000, 2001). In chickens, vitamin E supplement increased the number of lymphocytes in the bursa and the thymus gland and stimulated the proliferation and differentiation of T cells (Chang et al., 1994). In broilers, vitamin E selectively increased the percentage of mature $CD4^+$ T helper cells in the thymus and spleen but did not alter the percentage of thymic and splenic B cells and macrophages in total immune cell (Gore and Qureshi, 1997; Erf et al., 1998). Vitamin E enhanced immunity of birds to *Escherichia coli* infection, coccidiosis, infectious bursal disease, and Newcastle disease and altered cytokine expression in broilers (Tengerdy and Brown, 1977; Colnago et al., 1984; Erf et al., 1998; Leshchinsky and Klasing, 2000). However, vitamin E, although increasing serum levels of α-tocopherol, did not reduce the severity of *Eimeria maxima* infections in broilers (Allen and Fretterer, 2002).

Besides stimulating immune parameters, dietary vitamin E also enhances meat quality. Dietary vitamin E contributes to oxidative stability, extends shelf life and prevents oxidative off-odor of poultry meat, thus preserves the sensory quality of both frozen and refrigerated turkey breast meat (Ahn et al., 1997, 1998; Sheldon et al., 1997).

As part of the immune response during acute listeriosis, the host marshalls neutrophils, macrophages, natural killer cells, and T lymphocytes, especially $CD4^+$ and $CD8^+$ (Unanue, 2002). In vitro transfer experiments have shown that $CD4^+$ and $CD8^+$ cells are required to eliminate infection with wild-type strains of *L. monocytogenes* in mice (Kaufmann, 1993).

The current study was designed primarily to assess the effectiveness of dietary vitamin E, using doses previously shown to enhance meat quality, in accelerating the gut clearance of *L. monocytogenes* in experimentally infected adult turkeys. Secondarily, we monitored $CD4^+$ and $CD8^+$ T lymphocytes to evaluate the role of vitamin E as an immune potentiator.

MATERIALS AND METHODS

Bacterial Inoculum

L. monocytogenes (ATCC 700301) was obtained from American Type Culture Collection.[3] The stock cultures were maintained (–70°C) in 50% glycerol. For experimental inoculations, cultures of *L. monocytogenes* were grown on brain heart infusion agar with 20% bovine blood and 0.5% yeast extract (30°C, 24 h, in 5% O_2, 10% CO_2, 85% N_2) and harvested in PBS buffer (5 mL/plate). After centrifugation (5,000 × *g*, 10 min, 4°C), the pellet was washed twice with PBS and resuspended in 20 mL PBS. A 1-mL aliquot of the suspension was serially diluted in PBS. *L. monocytogenes* (cfu) were enumerated after incubation of brain heart infusion agar plates seeded with 0.1 mL of each serial 10-fold dilution (30°C for 24 h, in 5% O_2, 10% CO_2, 85% N_2).

Dietary Vitamin E

DL-α-Tocopherol acetate (500 IU/g Rovimix E-50%),[4] was used in the corn-soybean meal-based diet formulation, as described by Nam et al. (2003).

Turkeys

Experiment 1. One-day-old mixed sex Large White turkeys (n = 90) were obtained from a local hatchery and allotted to six rooms. Two rooms each (30 turkeys total) were randomly assigned to one of the three dietary treatments containing 0, 100, or 200 IU vitamin E/kg feed. After 5 wk, cloacal swabs were taken with sterile cotton-tipped applicators[5] to ensure that birds were culture-negative for *Listeria*. Any turkeys positive for *Listeria* were eliminated prior to experimental inoculation. No attempt was made to select birds, which were innately resistant to *Listeria*. One week later, only *Listeria*-negative turkeys were orally challenged with 1 mL of *L. monocytogenes* (1×10^9 cfu/mL). To monitor infection status, cloacal swabs were taken at 1, 4, 5, and 6 d post-inoculation (DPI). Turkeys (4 to 5 per group) were necropsied at 5, 8, 11, 14, and 25 DPI. Liver, spleen, cecum, duodenum, ileum, and colon from each bird were collected and processed for *Listeria* isolation as described below.

Experiment 2. One-day-old mixed sex Large White turkeys (n = 70) were obtained from a local hatchery and allotted to 4 rooms. Two rooms (35 turkeys total) were randomly assigned to diets containing either 0 or 200 IU vitamin E/kg feed. Prior to infection at 5 wk, cloacal swabs were taken and cultured for *Listeria*. At 6 wk, 30 *Listeria*-negative turkeys in each diet group were orally challenged with 1 mL of *L. monocytogenes* (1×10^9 cfu/mL). The remaining five *Listeria*-negative turkeys in each diet group were moved to the clean pens and served as non-infected controls. To monitor infection status, cloacal swabs were taken at 1, 2, 3, 4, 6, 8, and 10 DPI. Turkeys (~ 5 per group) were necropsied at 2, 4, 6, 8, and 10 DPI. Liver, spleen, cecum, duodenum, ileum, and colon from each bird were collected and processed for *Listeria* isolation as described below.

Bacterial Isolation

To culture *Listeria*, cloacal swabs were placed in UVM I (10 mL)[6] and incubated (2 to 3 d, 30°C, in 5% O_2, 10% CO_2, and 85% N_2). After enrichment, 100-μL UVM I enrichment was transferred into 10-mL *Listeria* secondary enrichment broth (UVM II) and incubated (30°C, in 5% O_2, 10% CO_2, and 85% N_2). After ~48 h, 100 μL of UVM II was plated to PALCAM *L. monocytogenes* selective agar[6] (30°C, 5% O_2, 10% CO_2, and 85% N_2 for 48 h). At necropsy, the liver, spleen, cecum, duodenum, ileum, and colon from each bird were sampled, enriched in UVM I (10% wt/vol), and cultured as described above.

[3]American Type Culture Collection, Manassas, VA.
[4]Roche Vitamins, Inc., Ames, IA.
[5]Puritan Hardwood Products, Guilford, ME.
[6]Oxoid Ltd., Basingstoke, Hampshire, UK.

Two presumptive *Listeria* spp. colonies were recovered from each PALCAM agar plate and were verified as *L. monocytogenes* by a multiplex PCR assay as described (Wesley et al., 2002). A total of 245 isolates from cloacal swabs and viscera were stored (4°C) on tryptic soy agar slants supplemented with 0.6% yeast extract and assayed for virulence.

Virulence Assay for L. Monocytogenes Isolates

Single microcolonies from each of 245 *Listeria* isolates recovered from infected turkeys were tested in vitro for virulence, as described (Bhunia et al., 1994, 1995). The ratio of *L. monocytogenes* cells per each target hybridoma cell was approximately 1,000:1. Tissue culture plates were incubated (37°C and 7% CO_2) for 6 h prior to scoring Ped-2E9 cell death using a trypan blue[7] exclusion assay.

Percent hybridoma cell death at 6 h after microcolony challenge was calculated as follows: $[(L_{NC} - L_{LC}) / L_{NC}] \times 100$, where L_{NC} = number of total live Ped-2E9 cells observed in untreated or unchallenged control wells, and L_{LC} = number of live Ped-2E9 cells in wells challenged with *Listeria* strain. Isolates that killed >70 to 90% Ped-2E9 cells within 6 h of challenge, which was comparable to the reference strains of *L. monocytogenes*, were scored as highly virulent. Isolates that killed <10% of Ped-2E9 cells, which was comparable to the 8 to 10.6% cell death observed for *L. innocua* (negative control), were scored as avirulent.

Serum Vitamin E (α-Tocopherol) Analysis

Blood samples (10 mL) were collected in serum separation vacutainer tubes.[7] Serum vitamin E analyses were performed with a Hewlett Packard (HP) 6890 GC equipped with an on-column capillary injector and a FID detector.[8] Serum vitamin E was calculated using an internal standard, 5α-cholestane, and expressed as micrograms per milliliter (Du and Ahn, 2002).

Flow Cytometric Analysis of Lymphocyte Population

Five milliliters of blood was collected from the wing vein into a vacutainer tube containing sodium heparin.[7] The heparinized whole blood was transferred into a 15-mL conical centrifuge tube containing 5 mL of fluorescence buffer (FB is PBS containing 1% heat-inactivated fetal blood serum and 0.05% NaN_3). The contents were mixed and centrifuged (200 g for 15 min, 4°C). The buffy coat was collected, washed three times with FB, and resuspended in 0.5 mL FB, as described (Stabel et al., 2000).

[7]Becton Dickinson, Franklin Lakes, NJ.
[8]Hewlett Packard Co., Wilmington, DE.
[9]Southern Biotechnology Associates, Inc., Birmingham, AL.
[10]Becton Dickinson Co., Cockeysville, MD.
[11]SAS Institute, Cary, NC.

TABLE 1. Serum vitamin E levels (μg/mL) in turkeys fed 0, 100, 200 IU of vitamin E (experiments 1 and 2)

Days post-infection	0 IU	100 IU	200 IU
Experiment 1			
5	0.11 ± 0.29[c]	2.08 ± 0.33[b]	2.88 ± 0.29[a]
8	0.16 ± 0.20[c]	1.51 ± 0.20[b]	3.10 ± 0.20[a]
11	0.09 ± 0.22[c]	1.75 ± 0.22[b]	3.88 ± 0.22[a]
14	0.15 ± 0.21[c]	1.89 ± 0.21[b]	3.67 ± 0.22[a]
Experiment 2			
0	0.10 ± 0.09[b]		3.42 ± 0.24[a]
2	0.10 ± 0.08[b]		3.31 ± 0.39[a]
4	0.12 ± 0.04[b]		3.19 ± 0.29[a]
6	0.09 ± 0.06[b]		3.41 ± 0.35[a]

[a–c]Means data in same row with significant difference ($P \leq 0.05$). Four to five birds were analyzed at each sampling point for each dietary regimen.

Direct dual color immunofluorescence staining was performed as described previously (Stabel et al., 2000). Briefly, 50 μL of buffy coat (~1 × 10^6 viable cells) was incubated (20 min at room temperature) with 10 μL each of 1:20 diluted CT4-FITC (fluorescein isothiocyanate conjugate mouse anti-chicken CD4 monoclonal antibody; catalog number 8210-02)[9] and CT8-PE (phycoerythrin mouse anti-chicken CD8a antibody; catalog number 8405-09).[9] After fixation with 1.5% formalin, the fluorescence intensities were measured with a Becton-Dickson FACScan flow cytometer.[10] Cells incubated with the fluorescently labeled isotype served as controls.

Statistical Analysis

A completely randomized design with unequal numbers of subjects was used to examine the effects of *L. monocytogenes* recovery from cloacal swabs of infected turkeys on vitamin E supplemented (200 IU) and the control (0 IU) diets. Regression equations were fit for percentage of *L. monocytogenes* recovery as a function of DPI. A general linear models F-test for full and reduced models was used for comparison. If a significant test statistic was found, indicating unequal swab responses of the two diet groups, 95% confidence intervals were calculated for each group on each day to highlight where differences were occurring (Neter et al., 1990). The mean and standard deviations of serum vitamin E as well as immune cells were analyzed statistically by the general linear models procedure using SAS software.[11] Student-Newman-Keuls' multiple range test was used to compare differences among mean values ($P < 0.05$). Means and SEM are reported.

RESULTS

Serum a-Tocopherol (Vitamin E)

Dietary vitamin E resulted in an increase in serum α-tocopherol (vitamin E) for experiments 1 and 2 (Table 1). At the time of experimental inoculation with *L. monocytogenes* (wk 6), serum vitamin E levels in both the 100 IU and 200 IU vitamin E treatment groups were significantly

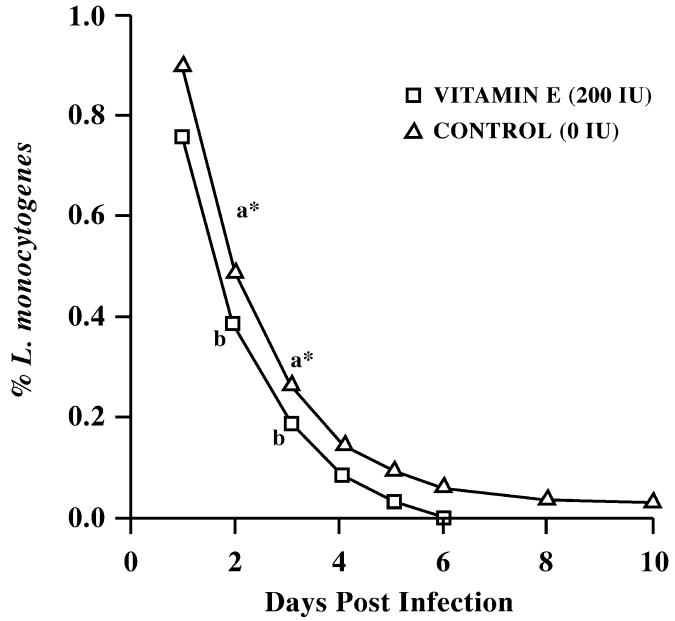

FIGURE 1. Comparison of the recovery of *Listeria moncotyogenes* from clocal swabs of turkeys fed control (0 IU) or a vitamin E (200 IU) diet. Significantly fewer turkeys harbored *L. monocytogenes* on d 2 and 3 post-infection (DPI) when fed vitamin E (200 IU). An asterisk (*) indicates a statistically significant difference between 0 IU (curve a) and 200 IU vitamin E (curve b) treatments at 2 and 3 DPI.

elevated when compared to birds on the control diet (0 IU). For both trials 1 and 2, serum vitamin E titers were significantly elevated in birds receiving 200 IU when compared to controls (0 IU). Because serum vitamin E levels, at 8, 11, and 14 DPI, were consistently higher in turkeys fed 200 IU than that in turkeys receiving 100 IU vitamin E, only the 200 IU diet was evaluated in experiment 2.

Recovery of L. monocytogenes in Cloacal Swabs

In experiments 1 and 2, pre-inoculation cloacal swabs were negative for *L. monocytogenes*. In experiment 1 at 1 DPI, *L. monocytogenes* was detected in cloacal swabs of turkeys (22/24, 91.7%) fed the control diet (0 IU vitamin E), as well as birds fed 100 IU (21/24, 87.5%) and 200 IU (17/25, 68%) vitamin E. At 4 DPI, *L. monocytogenes* was detected in swabs of 8.3% of control diet turkeys (2/24), whereas only one bird each was positive in the 100 IU (1/24, 4.2 %) and 200 IU (1/25, 4 %) treatment groups. On d 5 PI, three control birds (3/24, 12.5%) were positive, in contrast to none of the turkeys in the 100 IU and 200 IU groups. In experiment 2, at all days of post-infection sampling, *L. monocytogenes* was recovered more frequently from cloacal swabs of turkeys fed the control diet (0 IU) than in turkeys fed 200 IU vitamin E. When data from the two trials were combined (Figure 1), *L. monocytogenes* was cultured more frequently ($P < 0.01$) on d 2 and 3 in birds receiving 200 IU vitamin E when compared to control birds (0 IU).

Recovery of L. monocytogenes in Tissues

In experiment 1, *L. monocytogenes* was recovered more often in the ceca and ileum of control diet turkeys *versus* vitamin E-treated birds at 5 DPI. *L. monocytogenes* was not recovered from the intestine or tissue samples of turkeys on either control of vitamin E diets after 8 DPI (data not shown). For experiment 2, as summarized in Table 2, there were fewer tissue samples positive for *Listeria* in vitamin E-treated birds at 2, 4, or 6 DPI. At 8 DPI, *L. monocytogenes* was not recovered from any group (control and vitamin E).

Virulence Assays for L. monocytogenes Isolates

A total of 240 out of 245 isolates (98%) recovered from infected turkeys and submitted for virulence testing killed >70% to 90% of target hybridoma cells within 6 h of challenge. These were scored as highly virulent as were the inoculating strain (ATCC 700301) and the reference *L. monocytogenes* strains (positive controls). Of these 245 isolates, 156 were recovered from cloacal swabs and the remaining 89 were from intestinal tissues of turkeys. Four remaining isolates killed 3.8 to 12.8% of Ped-2E9 cells. This finding was comparable to the 8 to 10.6% cell death observed for *L. innocua* (negative control). These were scored as avirulent, and later were confirmed by PCR as isolates that were not *L. monocytogenes*.

TABLE 2. Recovery of *Listeria monocytogenes* from tissues of turkeys fed 0 and 200 IUof vitamin E (experiment 2)

				Small intestine		
Diet	Liver[1]	Spleen	Cecal	Duodenum	Ileum	Colon
Day 2						
0 IU	20%	0%	60%	40%	60%	40%
200 IU	40%	0%	60%	20%	20%	0%
Day 4						
0 IU	0%	0%	60%	20%	0%	0%
200 IU	0%	0%	60%	0%	0%	0%
Day 6						
0 IU	20%	0%	0%	0%	0%	40%
200 IU	0%	0%	0%	0%	0%	20%

[1]Percentage of infected birds sampled for each day for each group, n = 5.

Flow Cytometric Analysis of Lymphocytes

As summarized in Figure 2, for experimentally infected birds, $CD4^+$ populations of turkeys fed 200 IU vitamin E were increased ($P < 0.05$) at 6, 8, 10, and 31 DPI when compared to infected turkeys fed control diets (0 IU vitamin E) (Figure 2a). At 6 and 8 DPI, the $CD8^+$ T lymphocytes were higher ($P < 0.05$) in infected turkeys given 200 IU vitamin E than in infected turkeys on control diets (0 IU vitamin E) (Figure 2b). $CD4^+CD8^+$ double positive lymphocytes of experimentally infected turkeys on the 200 IU vitamin E diet were also markedly elevated ($P < 0.05$) at 6 and 8 DPI when compared to infected birds on the control (0 IU vitamin E) diet (Figure 2c).

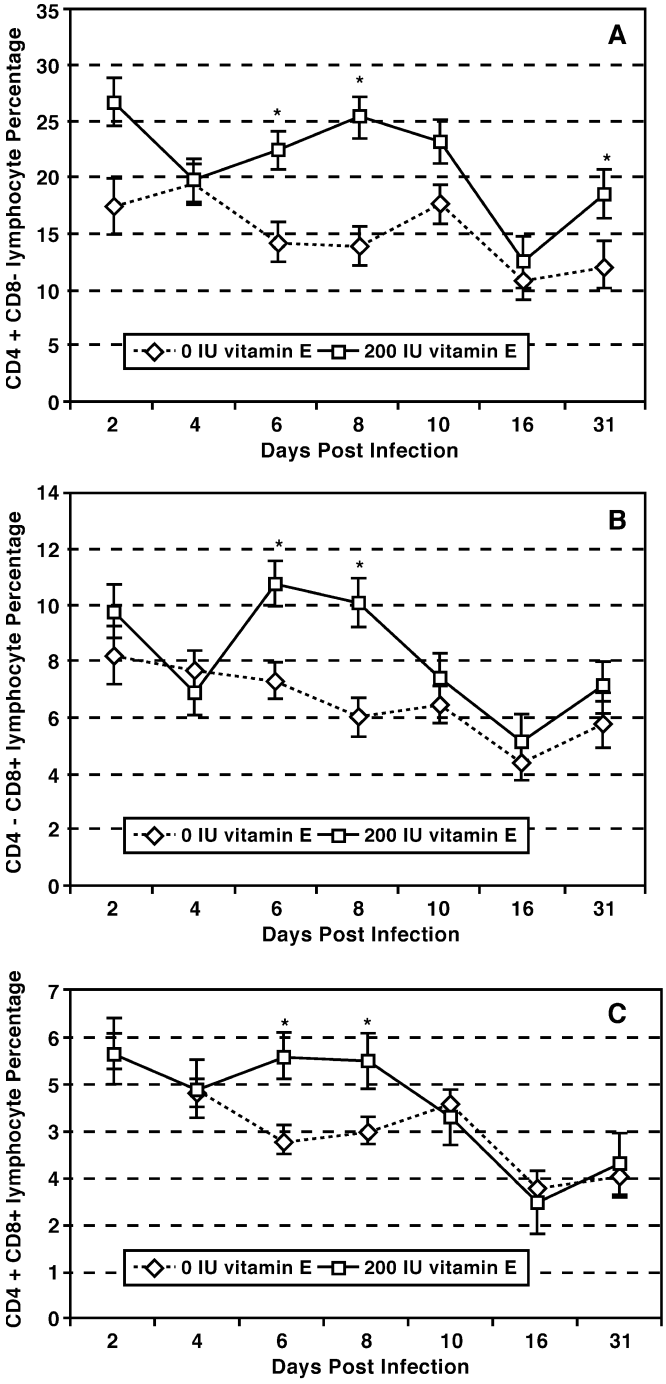

FIGURE 2. The response of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ populations, measured as percentage of lymphocytes, of turkeys fed 0 or 200 IU of vitamin E and experimentally infected with *Listeria monocytogenes*. The response was monitored for $CD4^+$ (a), $CD8^+$ (b), and $CD4^+CD8^+$ (c) lymphocytes. An asterisk (*) indicates a statistically significant difference between 0 IU and 200 IU vitamin E treatments. $CD4^+$ populations of turkeys fed 200 IU vitamin E were higher at 6, 8, 10, and 31 d post-infection (DPI) when compared to infected turkeys fed a control diet (0 IU vitamin E) (b). At 6 and 8 DPI, the $CD8^+$ lymphocytes were significantly higher in infected turkeys given 200 IU vitamin E than in infected turkeys on control diets (0 IU vitamin E). $CD4^+CD8^+$ double positive lymphocytes of experimentally infected turkeys on 200 IU vitamin E diet were also markedly elevated ($P < 0.05$) at 6 and 8 DPI when compared to infected birds on the control (0 IU vitamin E) diet.

DISCUSSION

The impact of dietary vitamin E on both gut colonization as well as on $CD4^+$ and $CD8^+$ T lymphocyte populations was evaluated in turkeys experimentally infected with *L. monocytogenes*. The vitamin E doses used were those previously shown to improve meat quality (Ahn et al., 1997, 1998). Serum vitamin E increased proportionately with dietary vitamin E content. This reflects effective gut absorption of vitamin E by the time of experimental challenge at the sixth week of dietary treatment. When cloacal swab data for the two trials were combined, *L. monocytogenes* was cultured more frequently ($P < 0.01$) on d 2 and 3 in birds receiving 200 IU vitamin E when compared to control birds (0 IU). Vitamin E supplement was previously reported to have increased the resistance of mice to influenza virus as well as of chickens to Newcastle disease virus, and increased antibody production and phagocytosis in chickens infected with *E. coli* (Tengerdy and Brown, 1977; Franchini et al., 1991; Han et al., 2000).

No virulence differences of *L. monocytogenes* recovered from in birds receiving vitamin E were detected using the PedE9 assay. The inoculating strains (ATCC 700301) and nearly 99% of the isolates recovered from cloacal swabs as well as from tissues, including spleen, liver, and intestine, were pathogenic for Ped-2E9 hybridoma cells after 6 h of incubation.

In order to determine the role of dietary vitamin E on immune parameters, the lymphocytes of infected turkeys were analyzed. In this current study, dietary vitamin E (200 IU) was associated with elevation of $CD4^+$ (6, 8, and 31 DPI), $CD8^+$, as well as $CD4^+CD8^+$ T lymphocytes (6 and 8 DPI) in *Listeria*-infected turkeys, when compared with infected turkeys on control diets. This observation was in concert with the requirement of $CD4^+$ and $CD8^+$ T cells to eliminate listeriosis (Unanue, 2002).

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