

INFLUENCE OF IMMUNOENHANCEMENT BY DIETARY VITAMIN E
SUPPLEMENTATION ON THE DEVELOPMENT OF *LISTERIA MONOCYTOGENES*
INFECTION IN AGED AND YOUNG GUINEA PIGS

By

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ABSTRACT OF THE THESIS

Influence of Immunoenhancement by Dietary Vitamin E Supplementation
on the Development of *Listeria monocytogenes* Infection in Aged and Young
Guinea Pigs

by Wen-Hsuan Wu

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Dr. Karl R. Matthews

L. monocytogenes is a facultative intracellular pathogen of humans and animals. Although *L. monocytogenes* is responsible for approximately 2500 cases of foodborne illnesses in the United States, it results in 88- 92 % of hospitalizations attributed to foodborne pathogens and causes an associated high mortality rate of 25- 40 %. Due to the natural decline of the immune system, infectious disease is one of the major causes of mortality in the elderly. Orthomolecular levels of vitamin E have been shown to promote T cell proliferation in healthy aged animals and human. We investigated the impact of immunomodulation on the development of listerial infection within an aged and young population after low dose challenge with *L. monocytogenes*. Animals were immunoenhanced with daily supplementation of vitamin E for a period of 21 days. An

untreated control group was included. Blood samples were immunostained, and changes in the level of CD8⁺ and CD3⁺ T cells were determined using flow cytometry analysis. Animals were orally challenged with 100 CFU of *L. monocytogenes*. Daily supplementation with vitamin E increased the level of total (CD3⁺) T cells and cytotoxic (CD8⁺) T cells significantly in young but not old animals. Results showed that 8 % and 15 % of young and aged control animals respectively, became infected; whereas, 0 % and 13 % of young and aged animals, respectively, receiving orthomolecular doses of vitamin E became infected. During post-challenge period, vitamin E treated aged animals showed a faster CD8⁺ T cell proliferation response than control aged animals. Results also showed that dietary vitamin E supplementation boosted a pool of T cells. In conclusion, experiments demonstrate the effect of vitamin E in mitigating listerial infection in young animals; and stimulating immune response in young and aged animals.

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Dedication

To my parents,
Ding-Jung Wu and Shen-Chih Chang

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Chapter 1

Introduction

Listeriosis is a serious infection and considered an important public health problem in the United States (Centers for Disease Control and Prevention, 1999). In the United States, infection associated with *Listeria monocytogenes* is associated with the highest mortality rate and a high number of hospitalizations in respect to other foodborne pathogens (Swartz, 2002). *L. monocytogenes*, a facultative intracellular bacterium, is able to evade the hosts' immune system and result in the disease listeriosis. Listeriosis occurs more frequently in immunocompromised people, such as cancer patients, pregnant women and the elderly. Moreover *L. monocytogenes* is able to survive at refrigeration temperatures and is ubiquitous in the environment (Rocourt and Cossart, 1997). Survey data suggests that people consume *L. monocytogenes* on a daily basis (Scientific Opinion of the Panel on Biological Hazards, 2007). Research directed at lowering the incidence and the severity of listeriosis is important and desperately needed.

Vitamin E is an important antioxidant in human plasma and tissue, and is necessary to maintain the function of the hosts' immune system (Sies and Murphy, 1991). However, the recommended daily allowance (RDA) of vitamin E is not sufficient to maintain optimum immune function; orthomolecular doses of vitamin E have been studied with respect to immunological and clinical implications. Orthomolecular doses of vitamin E have been shown to promote both the cellular and humoral immune responses in human and animals (Meydani *et al.* 1986, 1990). In this study, orthomolecular levels of

dietary vitamin E were evaluated as an immunoenhancer.

The elderly comprises 7% of the global human population; the number of elderly is expected to increase rapidly during the next five decades (U.S. Census Bureau, 2004). Immunosenescence is known to correlate with aging. The elderly are at greater risk of developing listeriosis because of immunosenescence, declining health status, and a litany of other factors. Aged and young guinea pigs were used in this study to compare the influence of vitamin E supplementation on listerial infection in an aged and young population.

In the present study, a diet containing an orthomolecular level of vitamin E was introduced to guinea pigs and followed by intragastric gavage with low numbers of *L. monocytogenes*. Levels of CD3⁺ and CD8⁺ T cells, and plasma vitamin E concentration were monitored during the pre-challenge and post-challenge period. Incidence and latency of infection were evaluated post-challenge.

Chapter 2

Literature Review

2. A. *Listeria monocytogenes*

L. monocytogenes, one of the six species of *Listeria*, is a Gram-positive small rod-shaped foodborne pathogen. It is ubiquitous in nature; found in association with animals, birds, insects, soil, waste water and vegetation. The bacterium may contaminate raw products such as dairy products, fruits and vegetables. Moreover, it is part of the normal flora in human and animal intestines; individuals colonized are often asymptomatic. People are likely exposed to *L. monocytogenes* in their diet on a daily basis (Ramaswamy *et al.*, 2007; Swaminathan and Gerner-Smidt, 2007).

L. monocytogenes is a concern due to its resistance to extreme conditions. It grows over a wide temperature range (2-45 °C), importantly, at refrigeration temperature (2-4 °C). The pathogen also survives and multiplies at low pH and high salt concentrations. Given this, the bacterium is able to survive and multiply under preservation and refrigeration conditions, increasing potential human health risks (Rocourt and Cossart, 1997; Gandhi and Chikindas, 2006).

2. A. 1. Human Listeriosis

Listeriosis is an important human illness in the United States; although rare (~2,500 cases/year), the illness is associated with a high hospitalization rate of 88-92 %, and the highest mortality rate (20-30 %) of the major foodborne pathogens (Mead *at al.*,

1999; Swaminathan and Gerner-Smidt, 2007). Listeriosis has been linked to both sporadic cases and large outbreaks of *L. monocytogenes*. Epidemiological data shows that immunocompromised individuals are more susceptible to listeriosis, such as cancer patients, infants, pregnant women, and the elderly (Smerdon *et al.*, 2001; Schlech 3rd, 2000). The infectious potential of the pathogen is associated with environment conditions (intrinsic factors of the food), the virulence of the bacterium and the susceptibility of the host (McLauchlin *et al.*, 2004).

Invasive listeriosis mainly occurs in immunocompromised individuals, such as infants whose immune system is not yet well-developed, the elderly that have an impaired immune system the result of immunosenescence, or those who are undergoing immunosuppressive therapy. The infectious dose may be low (Schlech 3rd *et al.*, 1983); since strains associated with cases of listeriosis have been shown to carry a full complement of the virulence genes. *L. monocytogenes* serotype 4b is commonly isolated from patients with invasive listeriosis. Invasive listeriosis causes systemic infections whereby the bacterium crosses the intestinal barrier and can reach the brain and attack the central nervous system (CNS). The clinical symptoms are meningitis, encephalitis, septicemia and bacteremia. *Listeria monocytogenes* infection in pregnant women may result in stillbirth, premature birth, and spontaneous abortion (Lecuit, 2007). Invasive listeriosis can be clinically silent, the result of long incubation times (5-70 days), in which case identifying the contaminated product is difficult. Non-invasive listeriosis is characterized as self-limiting gastroenteritis and with a short incubation period (18 hours - 15 days). Usually it is associated with the consumption of highly contaminated food

products by healthy individuals and accompanied by mild symptoms, such as diarrhea, fever and myalgia. According to the epidemiological data non-invasive listeriosis is associated with most frequently with *L. monocytogenes* serotype 1/2a and 1/2b (Swaminathan and Gerner-Smidt, 2007).

2. A. 2. Outbreaks

A range of contaminated food products have been linked to outbreaks in the United States and worldwide (Swaminathan and Gerner-Smidt, 2007; McLauchlin *et al.*, 2004). Food products may become contaminated post-processing or through cross-contamination. Raw milk and dietary products have been linked to cases of listeriosis. Currently, ready-to-eat (RTE) food products are of special concern as a source of human listerial infections. Low levels of *L. monocytogenes* contaminated RTE foods may increase during refrigeration and present a significant human health hazard since these products are usually consumed without cooking. This scenario renders RTE products a higher risk to cause listeriosis. A summary, by no means inclusive, of listeriosis outbreaks is provided (Table 1.1 and 1.2).

In the past 30 years, one large outbreak in United States occurred in Los Angeles, California in 1985. There were 142 cases of listeriosis reported and the identified vehicle of *Listeria* was Mexican style cheese. Individuals involved in the outbreak were mainly pregnant women or their offspring, and the immunocompromised. The epidemiological data suggest that the outbreak was caused by contaminated unpasteurized milk (Linnan *et al.*, 1988). The largest recorded outbreak of listeriosis occurred in 1992 in France; 279

cases were reported. Of the cases, 33 % were pregnant women. Pork tongue in jelly, a RTE food, was implicated as the vehicle of *L. monocytogenes*. Secondary contamination occurred during handling (Jacquet *et al.*, 1995). More recently, a listeriosis outbreak occurred in Massachusetts in 2007, *L. monocytogenes* was detected in milk products from a local manufacturer. Patients were predominantly the elderly and pregnant women. The State inspector concluded that contamination likely occurred during bottling. Collectively, serotype 4b was the predominant serotype associated with those severe outbreaks, and the cases were mainly pregnant women and people with suppressed immune systems.

Table 1.1. List of recent cases of non-invasive listeriosis.

Year	Location	No. of cases	Implicated vehicle	Serotype	Pathogen load (CFU/g or ml)
1993	Northern Italy	18	Rice salad	1/2b	$>10^3$
1994	Illinois, USA	44	Chocolate milk	1/2b	10^9
1997	Northern Italy	1566	Cold corn and tuna salad	4b	10^6
1998	Finland		Cold-smoked fish	1/2a	2×10^5
2000	New Zealand	32	Ready-to-eat meats	1/2	$> 2 \times 10^5$
2001	California	16	Delicatessen turkey ready-to-eat meat	1/2a	$> 10^9$
2001	Sweden	48	Raw milk cheese	1/2a	10^1 - 10^7
2001	Japan	38	Cheese	1/2b	Not known

(Swaminathan and Gerner-Smidt, 2007)

Table 1.2. List of recent outbreaks of invasive listeriosis.

Year	Location	No. of cases	Perinatal cases	No. of deaths	Suspect/ implicated	Vehicle Serotype
1981	Nova Scotia, Canada	41	34	18	Coleslaw	4b
1983	Massachusetts, USA	49	7	14	Pasteurized milk	4b
1985	California, USA	142	94	48	Mexican-style cheese	4b
1983- 1987	Switzerland	122	65	34	Vacherin Mont d'Or cheese	4b
1987- 1989	United Kingdom	366	?	?	Pate'	4bx
1989- 1990	Denmark	26	3	7	Blue mold cheese	4b
1992	France	279	0	85	Pork tongue in jelly	4b
1993	France	38	31	10	Rillettes	4b
1998- 1999	Multiple states, USA	108	?	14	Hot dogs	4b

Table 1.2. Continued.

1999	Finland	25	0	6	Butter	3a
1999- 2000	France	10	3	3	Rillettes	4b
1999- 2000	France	32	9	10	Pork tongue in aspic	4b
2000	Multiple states, USA	30	8	7	Delicatessen turkey ready-to-eat meats	1/2a
2000	North Carolina,	13	11	5	Home-made Mexican-style cheese	4b
2002	Multiple states, USA	54	12	8	Delicatessen turkey ready-to-eat meats	4b
2002	Quebec, Canada	17	3	0	Cheese made from raw milk	
2003	Texas, USA	12	?	?	Mexican-style cheese	4b

(Swaminathan and Gerner-Smidt, 2007)

2. A. 3. Infectious Process

L. monocytogenes is able to invade and multiply in non-phagocytic and phagocytic cells, including epithelial cells and phagocytes, respectively. This renders the microbe more difficult to eliminate by the immune system. Following the ingestion of contaminated food products, *L. monocytogenes* traverses the stomach and eventually reaches the small intestine where the microbe is able to cross the intestinal epithelial barrier and disseminate via the systemic system. The translocation involves two pathways for crossing the intestinal epithelial layer: Peyer's Patch (PP)-dependent or PP-independent in which *L. monocytogenes* infiltrates M cells or phagocytes and then enter lymphatic tissues (Jensen *et al.*, 1998; Havell *et al.*, 1999). After crossing the intestinal barrier, *L. monocytogenes* can disseminate to the brain or the placenta (Lecuit, 2005, 2007).

The infectious process involves five major events; each of them is mediated by the regulation of genes and proteins (Fig 1.1) (Vazquez-Boland *et al.*, 2002). First, adhesion and internalization: *L. monocytogenes* are engulfed by macrophages, neutrophils or dendritic cells via phagocytosis; in addition, it can also induce its own internalization in other non-phagocytic cells. Phagocytosis of *L. monocytogenes* by non-phagocytic cells occurs by bacterium-cell membrane interaction (Mengaud *et al.*, 1996). Internalization is mediated by the virulence protein, internalin A (InlA), which interacts with E-cadherin of guinea pigs and humans.

Second, lysis of the primary vacuole: after entry, *L. monocytogenes* lyses the

vacuole through the action of listeriolysin O (LLO), a pore forming toxin, in synergy with two phospholipase C (PLCs); phosphatidylinositol (PI) phospholipase (PLC-A), and phosphatidylcholine phospholipase C (PLC-B), to hydrolyze a wide variety of phospholipids. *L. monocytogenes* can then escape from the primary intracellular vacuoles (Gedde *et al.*, 2000). Third, intracellular growth: Once in the host cell cytosol, *L. monocytogenes* can now multiply. Although little is known about the nutritional requirements, it has been reported that the process relies on the hexose phosphate transporter (Hpt) (Geotz *et al.*, 2001).

Fourth, intracellular movement and cell-to-cell spread: cytosolic *L. monocytogenes* accomplish intracellular movement by inducing the polymerization of the cellular actin by the ActA protein. The bacterium forms an actin tail that propels it in random directions, and occasionally the bacterium encounters a peripheral membrane. A protrusion forms and allows the bacterium to spread to an adjacent cell without contacting the extracellular milieu. The protrusion invaginates into the adjacent cell and generates a double membrane vacuole (Tilney and Portnoy, 1989; Kocks *et al.*, 1992). Fifth, lysis of the secondary vacuole; lysis of the double membrane requires both LLO and PLC-B, and ultimately releases *L. monocytogenes* to the neighboring cell (Camilli *et al.*, 1993; Grundling *et al.*, 2003).

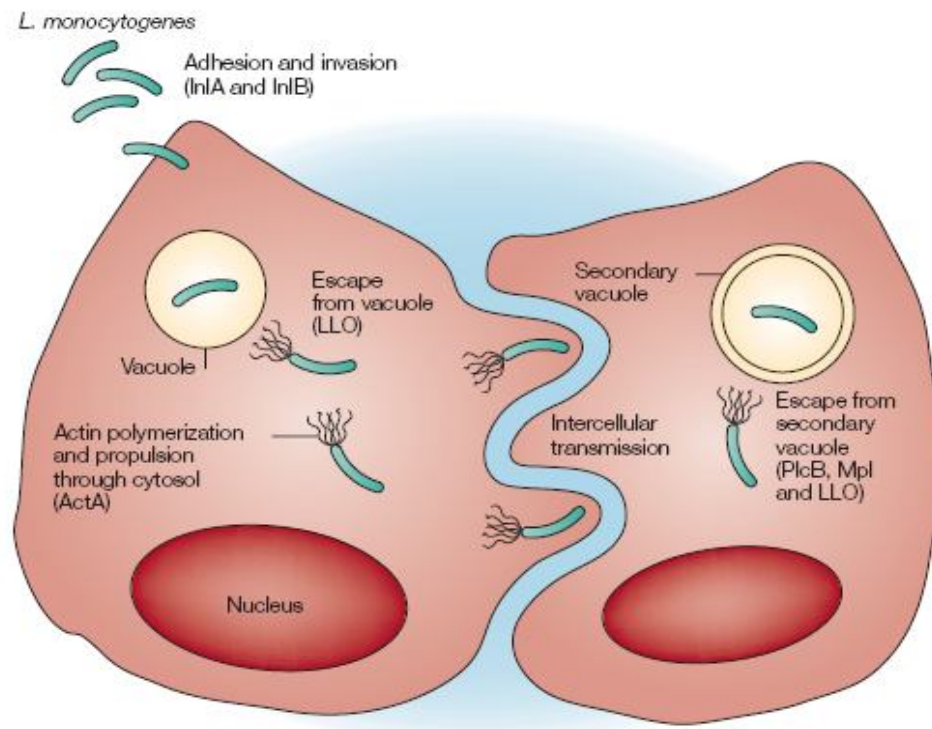


Figure 1.1. Intracellular infectious process of *L. monocytogenes* in host cells.

This scheme illustrates the five infectious processes of *L. monocytogenes*: 1) adhesion and internalization. 2) lysis of the primary vacuole. 3) intracellular growth. 4) intracellular movement and cell-to-cell spread. 5) lysis of secondary vacuole. InlA: internalin A. InlB: internalin B. LLO: listeriolysin O. PlcB: phosphatidylcholine phospholipase C (Pamer, 2004).

2. B. Immune Response to *L. monocytogenes*

For decades, *L. monocytogenes* has been used as a model to study the hosts' immune response against intracellular pathogens. A sub-lethal dose of *L. monocytogenes* is able to induce a strong immune response that leads to clearance of the pathogen (Zenewicz and Shen, 2007). The immune system is a defense system that protects the

host against foreign substances, including pathogens, virus, and allergens. The immune response can generally be classified as innate and adaptive. The innate immune response is antigen non-specific, rapid triggered, and essential for initially controlling the foreign entity (e.g., *Listeria*). The adaptive immune response is antigen specific, delayed response, and critical for clearance of *L. monocytogenes*.

During the early stage of an infection, the innate immune response is critical for controlling the expansion of a bacterial population, and for secretion of cytokines. Neutrophils play a key role at the site of infection by engulfing bacteria resulting in the generation of reactive nitrogen and oxygen intermediates. Mice depleted of neutrophils succumbed to an early and lethal listerial infection (Edelson and Unanue, 2000). Macrophages and natural killer (NK) cells are also important in the early stages of listeriosis; macrophages produce tumor-necrosis factor- α (TNF- α) and interleukin-12 (IL-12). These two cytokines induce NK cells to produce interferon- γ (IFN- γ), a major cytokine that activates macrophages (Tripp *et al.*, 1993). Activated macrophages become listeriocidal through the production of free radicals.

The adaptive immune response controls the latter stage of infection and the clearance of microbe. *L. monocytogenes* mainly induces a cell-mediated immune response, since the majority of *L. monocytogenes* remain intracellular during infection. Because of the intracellular location of the pathogen the humoral-mediated immune response is not a major component in fighting a listerial infection. In short, antibodies are not critical in the control of intracellular pathogens (Zenewicz and Shen, 2007). In the

cell-mediated immune response, T lymphocytes play the major role since *L. monocytogenes* induce a strong T cell response that is essential for clearance of the bacteria (McGregor *et al.*, 1970). During this stage, dendritic cells (DCs) connect innate and adaptive immune responses in that DCs process and present bacteria in order to prime naïve T cells into functional effector T cells (Jung *et al.*, 2002). Cytotoxic T cells or CD8⁺ T cells lyse infected cells by perforin and granzymes to expose intracellular bacteria for killing by macrophages, and secrete IFN- γ to activate macrophages (Harty and Badovinac, 2002). The mechanism of helper T cells, or CD4⁺ T cells, is less understood, however, it is known that *L. monocytogenes* induce a strong Th1 response. Th1 CD4⁺ T cells produce IFN- γ , as do CD8⁺ T cells, which activate macrophages (Daugelat *et al.*, 1994).

2. C. Immunosenescence in the Elderly

The age-related dysfunction of the immune system is called immunosenescence which increases the elderly's susceptibility to disease and infection (Ginaldi *et al.*, 2001). Based on epidemiological data, listeriosis is more common in children (100 cases per million) and elderly (14 cases per million) compared with the general population (7 cases per million) (Southwick and Purich, 1996).

The accumulative random damage of free radicals, produced by somatic cells and other tissues in the elderly, results in impaired biochemical, physiological, and immunological functions. Cell-mediated immunity is more severely affected than humoral-mediated immunity in the elderly; a result of the marked involution of the

thymus where T cells develop and mature (Aspinall and Andrew, 2000). T cell function is altered directly and indirectly with age. As humans age involution of the thymus occurs resulting in lower output of new T cells. This contributes to the change of the T cell repertoire to a population low in naïve T cells and high in memory T cells, compared with young counterparts (Miller, 1996). A shift in levels of interleukin-2 (IL-2), a cytokine that is a T cell growth factor that mediates T cell proliferation also occurs. Interleukin-2 is produced preferentially by naïve T cells, not memory T cells; therefore, IL-2 production and IL-2 receptor (IL-2R) expression are diminished (Adolfsson *et al.*, 2001). The process contributes to reduce T cell proliferation.

T cell function is affected indirectly by elevated production of prostaglandin E₂ (PGE₂), which is a metabolite of arachidonic acid (AA) from the membrane of the macrophage. Studies have demonstrated greater synthesis of PGE₂ in old mice or the elderly compared to young counterparts (Meydani *et al.*, 1986; Hayek *et al.*, 1994). An inhibitory mechanism of PGE₂ is associated with the alteration of the early stage of signaling transduction in T cell activation (Choudhry *et al.*, 1999). Therefore, PGE₂ causes impaired T cell proliferation.

2. D. Vitamin E as an Immunoenhancer

Vitamin E is a fat soluble chain-breaking antioxidant and one of the primary scavengers of peroxy radicals that prevent lipid peroxidation in membrane phospholipids or plasma lipoproteins (Burton *et al.*, 1983). There are eight naturally occurring forms: α -, β -, γ -, δ -tocopherols or tocotrienols, as showed in Figure 1.2 (Traber, 1999); only

α -tocopherols are maintained in plasma and are inter-convertible. Edible vegetable oils such as wheat germ oil, soybean oil and walnut oil are an abundant source of vitamin E, (Sheppard *et al.*, 1993). Some vegetables, fruits, and meats, especially the fatty portion, contain vitamin E as well.

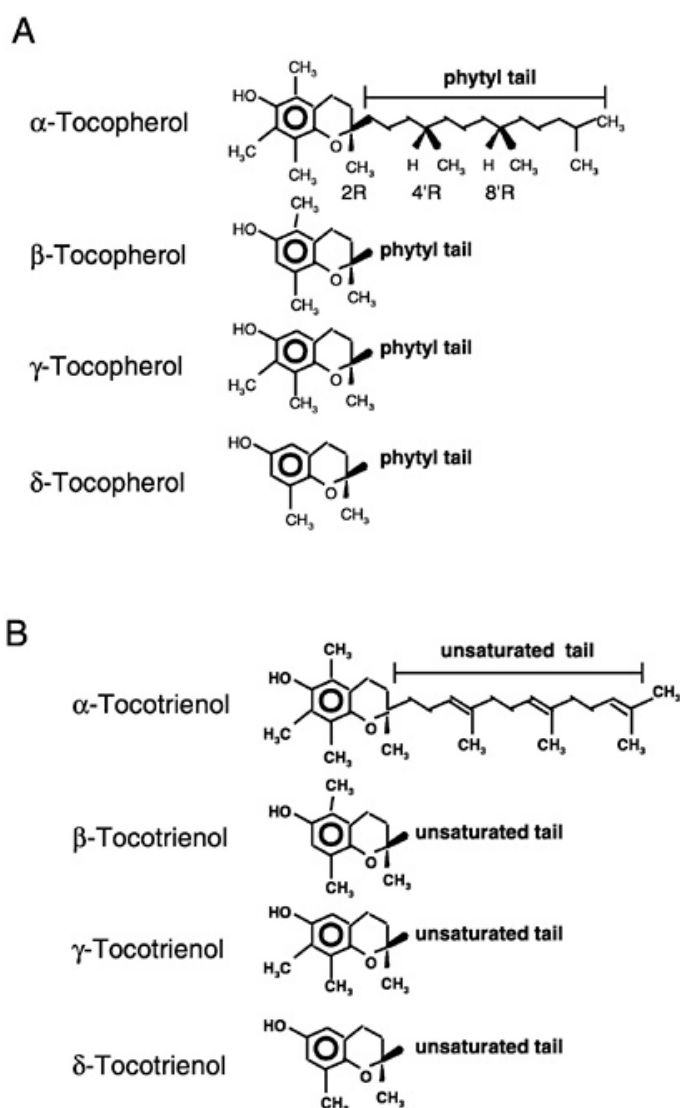


Figure 1.2. Structures of tocopherols (A) and tocotrienols (B). All tocopherols are in the *RRR*-form (Traber, 1999).

Vitamin E supplementation above recommended daily requirements has been shown to influence the immune response. During infection immune responses such as phagocytosis result in the production of reactive oxygen species, which are needed for macrophage activation, will depress T cell responses (Metzger *et al.*, 1980; Otsuji *et al.*, 1996). However, vitamin E supplementation enhances cell-mediated immunity by scavenging those free radicals, resulting in promotion of T cell proliferation. In addition, immune cells have a high content of polyunsaturated fatty acids, and are susceptible to oxidative damage; thus vitamin E plays a protective role for immune cells (Coquette *et al.*, 1986; Hatman and Kayden, 1979). In general, vitamin E increases T cell function and proliferation through at least two mechanisms (Fig 1.3). It prevents macrophages from producing PGE₂ by reducing its activator, peroxynitrite. It also directly promotes IL-2 production (Adolfsson *et al.*, 2001; Meydani *et al.*, 2005). Therefore, vitamin E is considered an immunoenhancer.

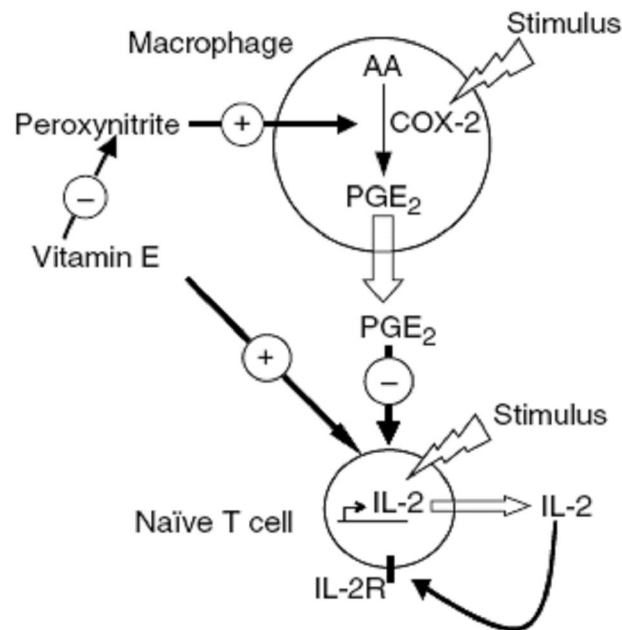


Figure 1.3. Two mechanisms for the influence of vitamin E on T cell proliferation.

This scheme illustrates the two mechanisms of vitamin E promotion on T cell proliferation. Peroxynitrite, produced by macrophages, contribute indirectly to the inhibitory effect on T cell proliferation. Vitamin E is able to reduce peroxynitrite formation. Vitamin E also directly induces IL-2 production and IL-2 receptor expression and resulting in the promotion of T cell proliferation. PGE₂: prostaglandin E₂, a metabolite of membrane phospholipid. COX-2: cyclooxygenase-2, an inducible form of the enzyme. IL-2: interleukin-2, a growth factor of T cell proliferation. IL-2R: interleukin-2 receptor, the receptor of IL-2 (Meydani *et al.*, 2005).

The absorption of vitamin E occurs in the intestinal lumen by enterocytes and requires chylomicron secretion. Similar apparent efficiencies of intestinal absorption occur for all isoforms. However, absorbed vitamin E is transported to the liver through

the hepatic α -tocopherol transfer protein (α -TTP), and only α -tocopherol has the highest affinity to α -TTP (Traber and Arai, 1999). The absorbed vitamin E in the liver disseminates to other tissues by very low density lipoprotein (VLDL). The scheme of absorption and transportation of vitamin E is shown in Figure 1.4. The whole process of vitamin E transport involves several lipoproteins (Traber *et al.*, 1994, 1988; Traber, 1996).

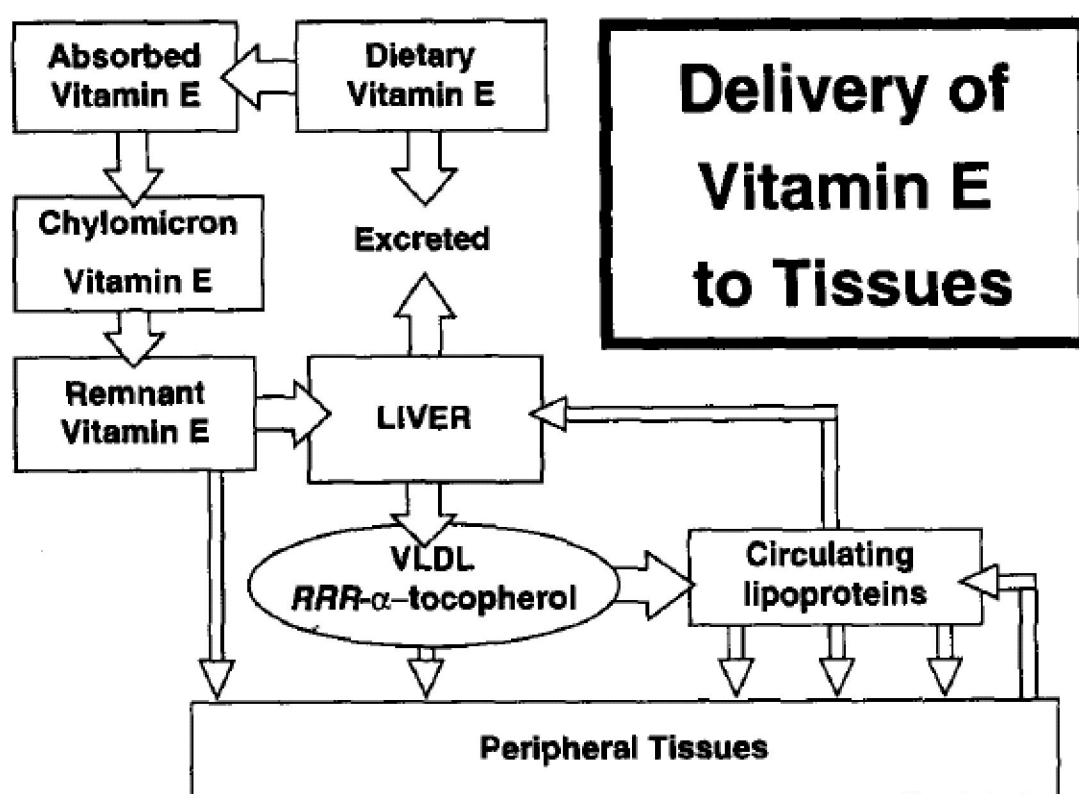


Figure 1.4. The biokinetic pathway of vitamin E.

This schematic illustrates the deliver of vitamin to various tissues. Vitamin E is absorbed from the intestine through digestion. Vitamin E is then secreted in chylomicrons, and transferred to the liver through the circulatory system. The liver then secretes newly absorbed vitamin E to various tissues using very low density lipoprotein (VLDL) (Traber,

1996).

, Supplemental vitamin E is predominantly in the ester form of α -tocopherol; the esterification of the hydroxyl group on the chromanol ring forms α -tocopherol acetate, which has an extended shelf life and retains the bioactivity of α -tocopherol (Cheeseman *et al.*, 1995). Therefore, the correlation between α -tocopherol and α -tocopherol acetate is evaluated to obtain the level of vitamin E consumption.

Researchers have proven that intake of elevated levels of vitamin E improves human health, and is safe. The RDA (recommended daily allowance) for adult men and women is 15mg (22.5IU) / day of α -tocopherol, and the UL (tolerable upper intake limit) is 1000mg (1500IU)/ day of α -tocopherol. The toxicity of vitamin E is low; therefore it's considered safe up to 1000 mg/day. An orthomolecular dose, approximately 10 \times the RDA of vitamin E, is required in order to stimulate the immune system.

2. E. Guinea Pig Model

In this study, guinea pigs were used as the animal model to investigate the development of listerial infection. An appropriate animal model should reflect human response physiologically and perhaps genetically. It had been shown that listeriosis is most often associated with the oral ingestion of contaminated foods (Schlech 3rd *et al.*, 1983), so in an ideal animal model listerial infection should be capable of occurring via the oral route. Infection via the oral route in mice and rats is not reproducible, and does not become systemic. Marco *et al.* (1992) demonstrated that murine listerial infection by

the oral route is subclinical. The bacteria were maintained in the Payer's Patches, and the number of bacteria found in organs, such as the liver, spleen, and mesenteric lymph nodes, were low. Other researchers showed that mice and rats cannot induce a reproducible lethal infection following oral challenge with *L. monocytogenes*, even using extremely high inocula levels (Huleatt *et al.*, 2001; Marco *et al.*, 1997). However, a lethal listerial infection via the oral route can be induced in guinea pigs, because *L. monocytogenes* can cross the intestinal barrier and disseminate throughout the body. In studies addressing the natural route of listerial infection the guinea pig is the preferred animal model compared to the mouse or rat model.

Once the pathogen enters the intestine, it must interact with the hosts' cells to induce infection. The entry and dissemination of *L. monocytogenes* in the animal model should be the same as in humans. It has been shown that the internalization of *L. monocytogenes* in humans is specific, relying on the interaction between human E-cadherin and Internal A (InlA) of *L. monocytogenes* (Mengaud *et al.*, 1996). E-cadherin is a transmembrane glycoprotein which is expressed in epithelial cells, and mediates calcium-dependent cell-cell adhesion (Geiger and Ayalon, 1992). The ligand-receptor interaction is species specific based on a single amino acid in E-cadherin; humans and guinea pigs have the same crucial amino acid, proline at the 16th position. In contrast, mice and rats have glutamic acid at the 16th position which does not fit the conformation of InlA (Lecuit *et al.*, 1999). Subsequently, for a systemic infection the bacteria should be able to cross the epithelial cell barrier. *L. monocytogenes* is able to cross the intestinal barrier by internalin-E-cadherin interaction in the guinea pig.

The incidence of listeriosis is low relative to disease caused by other foodborne pathogens. The severity of the disease compels researchers to prevent contamination of food with *L. monocytogenes* and develop strategies to mitigate or prevent listeriosis from occurring. Novel approaches that will aid the most susceptible population, the immune suppressed, must be developed. A promising strategy is the use of orthomolecular levels of vitamin E to boost the immune response in aged humans and animals.

Chapter 3

Hypothesis and Objectives

Results of a previous study conducted in our laboratory suggest that dietary supplementation with 400 IU/day vitamin E influenced development of listerial infection in aged guinea pigs (Pang *et al.*, 2007). In the present study, we used dietary vitamin E supplementation and two groups of subjects, young and aged guinea pigs, to evaluate the influence of dietary vitamin E supplementation on the development of listerial infection.

We hypothesize that Vitamin E supplementation will have a greater influence on immunoenhancement and subsequent development of listerial infection in aged compared to young guinea pigs.

The objectives are:

1. To determine the influence of vitamin E on the development of listerial infection.
2. To determine the influence of an orthomolecular dose of vitamin E on immunomodulation biomarkers.

Chapter 4

Materials and Methods

4. A. Bacterial Strains

The *L. monocytogenes* cocktail used contained three serotype 4b strains: J1-110 (food epidemic), N1-225 (human epidemic), MMS97-1 (raw beef) (Pang and Matthews 2006, Pang *et al.*, 2007). Each isolate was cultured individually in 10 mL brain heart infusion (BHI) broth (Difco, MI) and incubated at 37 °C with agitation for 18 h. Cells were harvested by centrifugation (5,500 g, 4 °C, 10 min) and stored -70 °C in BHI: glycerol (1:1). Prior to use isolates were cultured as described above, cells pelleted by centrifugation (5,500 g, 4 °C, 10 min), and resuspended in 10mL phosphate buffered saline (PBS; Sigma, St Louis, MO). The cocktail was made by mixing equal volumes of each strain and then serially diluting in PBS to achieve 100 CFU/ mL. Cell numbers were confirmed by plating on BHI agar.

4. B. Animals

Outbred female Dunkin-Hartley guinea pigs weighing *c.* 250-300g and retired breeders weighing *c.* 1000 were designated as young and aged animal models, respectively. Animals were purchased from Charles River Lab. Inc. (Willington, MA). All animals were housed under standard conditions (50% humidity and 12h-dark-12h-light cycle) at the Rutgers animal facility, and each animal was housed in individual cages; fresh water and food was available *ad lib*. Upon arrival, all animals were weighted and assigned to each group randomly (control or vitamin E). The four

groups were vitamin E young, control young, vitamin E aged, and control aged. A total of 76 animals were used, 19 animals in each group, and control was included; the experiment was conducted in duplicate. All animal experiments were conducted in accordance with federal guidelines and were approved by Rutgers University animal care and facilities committee.

4. C. Dietary Vitamin E Supplementation

Supplemental vitamin E was incorporated into the diet to investigate the influence of vitamin E supplementation on immunoenhancement. Purina guinea pig chow #5025 was supplemented with 5,000 IU/Kg vitamin E [DL- α -tocopherol acetate] (Research Diets Inc., New Brunswick, NJ). The feed was stored at room temperature and under dry conditions. Animals from the control groups received general Purina guinea pig chow #5025. Animals were maintained on the respective diets for the duration of the study.

4. D. Blood and Plasma Samples Collection

Blood samples were collected once a week during the pre-challenge period and on 0, 3, 6, 9, 12, 16 and 20 days post-challenge. During the pre-challenge period at 7 d intervals five animals were randomly selected from each group (young [control and treated]; aged [control and treated]) were anesthetized with ketamine/xylazine at 80mgkg⁻¹/ 20mgkg⁻¹. A 0.5mL (young) and 1mL (aged) blood sample was collected from the peripheral jugular vein of each animal. Blood samples were kept with 5% K₂EDTA, and used within 4 h for direct immunostaining. Blood plasma samples were collected by centrifugation of the whole blood at 1,600g, 4 °C for 10 min. Plasma samples were

frozen immediately at -80 °C until required for further analysis. Blood samples were collected from three animals from each group on each post-challenge day.

4. E. Direct Immunostaining

Blood samples were collected for immunostaining in order to determine the levels of target T cells (CD3⁺, CD4⁺ and CD8⁺). A 100 µL whole blood sample was incubated with 100 µL of IQ lyse (IQ Products, The Netherlands) for 10 min at room temperature. Cells were washed with PBS containing fetal bovine serum (FBS, 2% v/v). Monoclonal antibodies to CD4⁺, CD8⁺, and CD3⁺ conjugated to PE, FITC and APC (AbD Serotec Inc., Raleigh, NC), respectively, were added to a plasma sample preparation in series. The sample was incubated for 10 min at room temperature in the dark, and cells were washed between each incubation. Working concentrations of antibodies were used according to the manufacturer's recommendations. Coulter Flow Count (100µL) (Beckman Coulter Inc., Hialeah, FL) and PBS + 2 % FBS (900µL) were added to cells immediately prior to flow cytometry analysis. Samples incubated with a single antibody preparation served as negative controls. Samples were analyzed in a Coulter Cytomic FC500 Flow Cytometer (Becton-Dickinson, North Carolina, USA).

4. F. HPLC Analysis of α -tocopherol Concentration in the Plasma

Plasma samples stored at -80 °C from the guinea pigs receiving the vitamin E diet were thawed at room temperature, and mixed thoroughly. A 150µL plasma sample was transferred to a new centrifuge tube and 150µL internal standard (0.1mgmL⁻¹ α -tocophoerol acetate / acetonitrile) and 700 µL hexane were added. Samples were

agitated for 20s and then centrifuged (14,000g, 6 min). The α -tocopherol extracts (hexane fraction) was transferred to a new centrifuge tube and vacuum dried for 30 min. The extracted samples were reconstituted with 150 μ L methanol and then subjected to centrifugation (14,000g, 6 min). The final sample (120 μ L) was transferred to a vial for HPLC analysis. Each extracted sample (20 μ L) was injected into a Luna C18 column (150 x 4.6 mm, 3 μ m; Phenomenex, Torrance, CA) by an autosampler. Samples were analyzed using a DIONEX Ultimate 3000 HPLC (California, USA) under the following conditions: mobile phase: methanol, flow rate: 0.85mL/min, UV detector: 295nm, running time: 18 min.

4. G. Intragastric *Listeria monocytogenes* Challenge

Food was withheld for 12 h prior to intragastric challenge in order to prevent regurgitation during challenge (Pang and Matthews, 2006). Each animal received *c.* 1.5×10^2 CFU of the *L. monocytogenes* serotype 4b cocktail using a 38-cm nasogastric feeding tube (Jorgensen Laboratories Inc., Loveland, CO) fitted onto a 3 mL syringe.

4. H. Organ Collection and Microbiology

Guinea pigs were euthanized on day 3, 6, 9, 12, 16 and 20 post-challenge using sodium pentobarbital at 100mg/kg, liver and spleen were collected aseptically *in toto* (Lecuit *et al.*, 1999; Pang and Matthews, 2006). Organ samples were weighed and placed into sterile plastic tubes to which a volume equal to the weight of the sample of cold PBS containing 0.001% triton X was added. The samples were then homogenized using a tissue homogenizer (Polytrons, Kinematica, Switzerland). A 100 μ L volume was spread

plated onto Rapid L'mono plate (Bio-Rad Laboratories, Inc., Richmond, CA) and plates incubated at 37 °C for 24-48 h. *L. monocytogenes* colonies were counted and numbers expressed as mean Log CFU/g of organ. When no *L. monocytogenes* was detected based on colony formation, organ samples were subjected to microbiological enrichment procedures. Remaining organ samples were stored at 4 °C.

4. I. Enrichment

All organ samples were subjected to enrichment procedures. An amount of 2X BHI broth (1 ml for spleen samples, 10 ml for liver samples) were added to the organ samples and samples incubated at 37 °C with agitation for 24-48 h. A (100 µL) aliquot was plated onto Rapid L'mono plate or onto PALCAM agar plates in duplicate, and the plates were incubated at 37 °C for 24-72 h to detect the presence of *L. monocytogenes* (Leclercq, 2004).

4. J. Statistical Analysis

All data were analyzed using Microsoft Excel or SAS. Means and standard deviations were calculated by STDEV function, and the significant differences between the treatment means were determined by SAS Duncan's multiple range test, one-way ANOVA procedure. Data were presented as means \pm standard deviation. The level of statistical relevance for all comparisons was set at $P < 0.05$.

Chapter 5

Results

5. A. Change in Plasma α -tocopherol Concentration during Dietary Vitamin E Supplementation

The concentration of plasma α -tocopherol was analyzed by HPLC and was converted according to the concentration of α -tocopherol acetate. Plasma α -tocopherol concentration was determined prior to (wk 0) and during the vitamin E-supplementation period (wk 1, 2 and 3 pre-challenge); data are shown in Table 5.1. The plasma α -tocopherol concentration at week 0 was considered as the baseline level; and the aged animals showed a higher concentration (10.33 μ M) compared to the young animals (4.96 μ M). The sharp increase of plasma α -tocopherol in young animals at wk 1 (15.48 μ M) was highly significant ($P < 0.01$), and was followed by a slight increase from wk 1 to wk 2 (16.03 μ M). By week 3 (8.23 μ M) the plasma α -tocopherol level decreased, however, it remained 2-fold greater ($P < 0.01$) than at wk 0. In aged animals, the concentration of plasma α -tocopherol slightly changed from the baseline level (10.33 μ M) to 12.71 μ M, 10.01 μ M, 12.70 μ M by weeks 1, 2, and 3, respectively ($P > 0.05$).

Table 5.1. Plasma α -tocopherol concentration of young and aged guinea pigs during dietary vitamin E supplementation (pre-challenge period).

Week	Average \pm SEM μ M	
	Vitamin E young	Vitamin E aged
0	4.96 \pm 0.17 ^{‡c}	10.33 \pm 2.43
1	15.48 \pm 1.88 ^a	12.71 \pm 0.18
2	16.03 \pm 0.71 ^a	10.01 \pm 3.54
3	8.23 \pm 1.19 ^b	12.70 \pm 3.74

[‡]Blood samples were obtained from two independent experiments before introducing vitamin E supplementation and at weeks 1, 2, 3 post-supplementation. Wk 0 is considered the baseline. Plasma vitamin E concentration was expressed as mean \pm SEM μ M.

^{a-c}Significant differences were analyzed by ANOVA Duncan's multiple range test. Column within a table with different letters are significantly different ($P < 0.01$, $n = 4$).

5. B. The Influence of Dietary Vitamin E Supplementation on the Levels of CD3⁺ T Cells

CD3⁺ T cell populations were determined so that changes in total T lymphocytes populations could be evaluated during the pre-challenge period. The CD3⁺ T cell levels are presented as the absolute number (cells/ μ L) of cells. Levels of CD3⁺ T cells of young animals in the control group fluctuated, but rose significantly by week 3. During this same period a gradual increase in CD3⁺ T cells in young animals in the vitamin E group. The increase was significance ($P < 0.05$) at week 2 and week 3 (Fig. 5.1) compared to week 0. However, there were no significant changes in CD3⁺ T cells during the pre-challenge period in both control and vitamin E treated aged guinea pigs (Fig. 5.2).

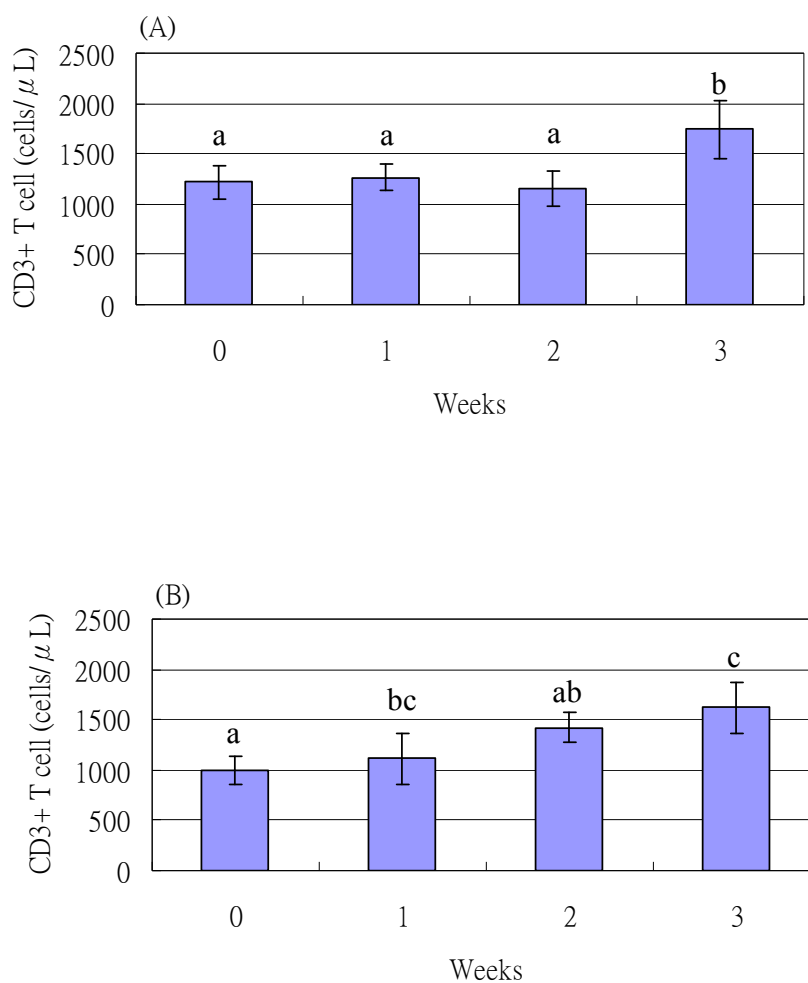


Figure 5.1. Changes in the levels of total (CD3⁺) T lymphocytes of young control (A) and treated (B) guinea pigs during the pre-challenge dietary vitamin E supplementation. Data of CD3⁺ T cell were presented in the absolute number of cells per micro liter of plasma (cells/ μ L). Blood samples were obtained from two independent experiments before introducing vitamin E supplementation and weeks 1, 2, 3 post-supplementation. Wk 0 is considered the baseline.

^{a-c} Bars within a graph with different letters are significantly different ($P < 0.05$, $n = 4$).

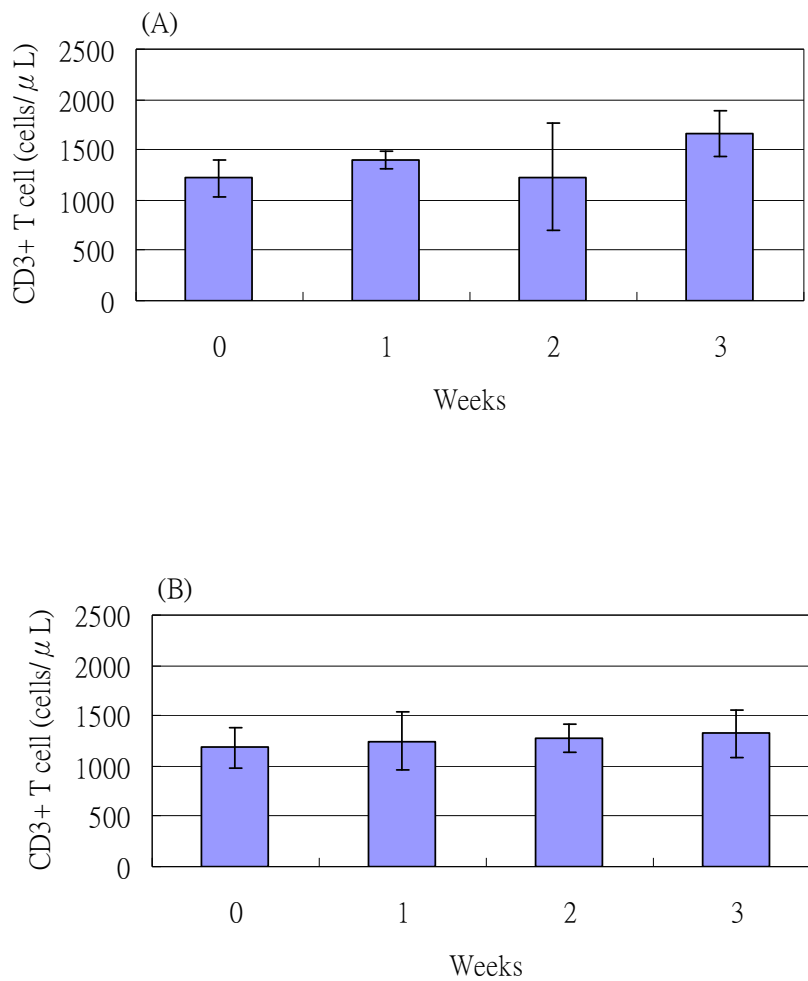


Figure 5.2. Changes in the levels of total (CD3⁺) T lymphocytes of aged control (A) and treated (B) guinea pigs during the pre-challenge dietary vitamin E supplementation. Data of CD3⁺ T cell were presented in the absolute number of cells per micro liter of plasma (cells/ μL). Blood samples were obtained from two independent experiments before introducing vitamin E supplementation and weeks 1, 2, and 3 post-supplementation. Wk 0 is considered the baseline. (n = 4).

5. C. The Influence of Dietary Vitamin E Supplementation on the Levels of CD8⁺ T Cells

To evaluate the influence of dietary vitamin E supplementation on changes in the cytotoxic T cell population, the levels of CD8⁺ T cells were monitored before oral challenge. Data for CD8⁺ T cells are given as the percent CD8⁺ T cells per total (CD3⁺) T cells. Guinea pigs were fed daily a diet supplemented with vitamin E at 5,000 IU/kg feed. During the 21-day pre-challenge period (Fig. 5.3 and 5.4), levels of CD8⁺ T cells remained constant in both young and aged control groups. In the vitamin E treated groups, there was a significant ($P < 0.05$) increase in percent CD8⁺ T cells in young guinea pigs at week 1, 2 and 3 compared to the baseline. The percentage of CD8⁺ T cells increased from an initial level 17.4% to 24.0%, 20.7%, and 21.0% by weeks 1, 2, and 3, respectively. However, there was no significant change in levels of CD8⁺ T cell observed in aged animals ($P > 0.05$).

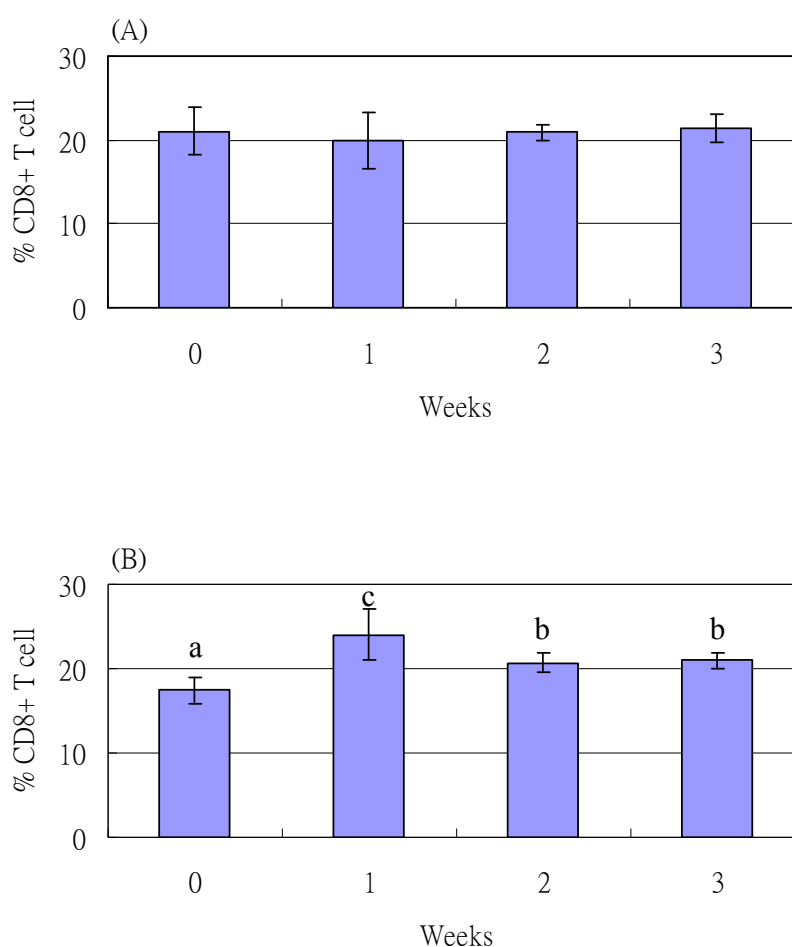


Figure 5.3. Changes in the levels of cytotoxic (CD8⁺) T lymphocytes of young control (A) and treated (B) guinea pigs during the pre-challenge dietary vitamin E supplementation period. Percent CD8⁺ T cells were calculated by dividing the number of CD8⁺ T cells by the number of CD3⁺ (total) T lymphocytes and multiplying by 100. Blood samples were obtained from two independent experiments before introducing vitamin E supplementation and at weeks 1, 2, 3 post-supplementation. Wk 0 is considered the baseline.

^{a-c} Bars within a graph with different letters are significantly different ($P < 0.05$, $n = 4$).

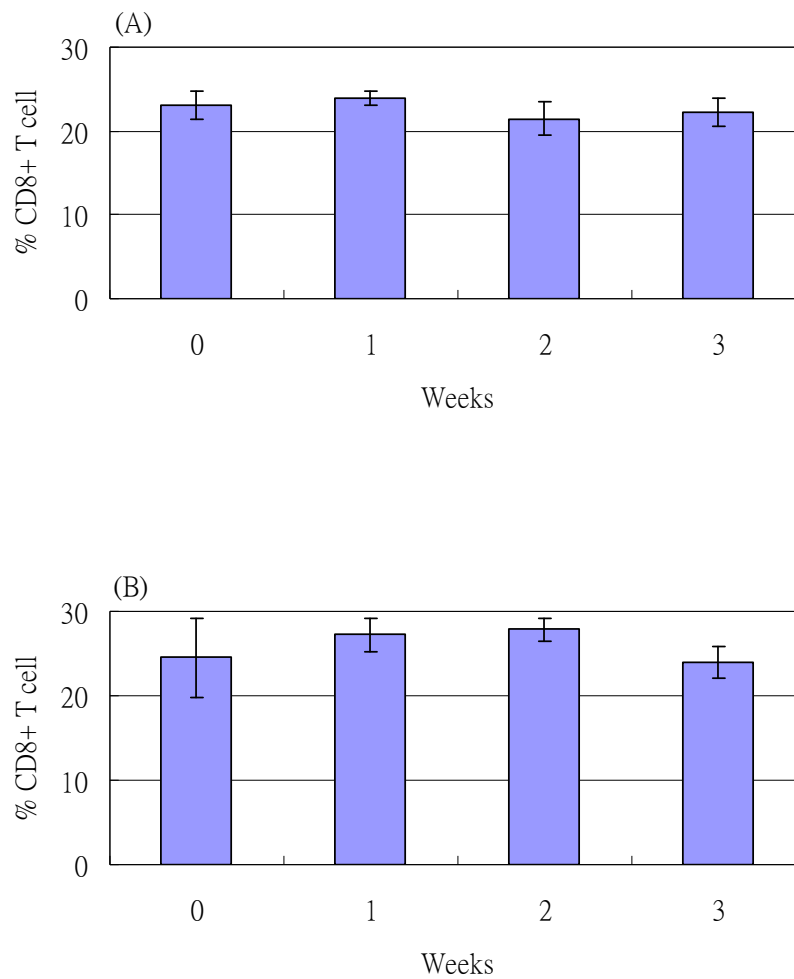


Figure 5.4. Changes in the levels of cytotoxic (CD8⁺) T lymphocytes of aged control (A) and treated (B) guinea pigs during the pre-challenge dietary vitamin E supplementation period. Percent CD8⁺ T cells were calculated by dividing number of CD8⁺ T cell by number of CD3⁺ (total) T lymphocyte and multiplying by 100. Blood samples were obtained from two independent experiments before introducing vitamin E supplementation and at weeks 1, 2, 3 post-supplementation. Wk 0 is considered the baseline. (n = 4).

5. D. Listerial Infection Following Oral Challenge with 100 CFU *L. monocytogenes*

All animals were intragastrically challenged with 100 CFU *L. monocytogenes* and infection was determined based on microbiological culture of liver and spleen samples. Few animals became infected and diarrhea observed only sporadically; no deaths occurred. The results of microbiological analysis of liver and spleen samples showed that all animals were negative for listerial infection after direct plating. All organ samples were processed for microbiological enrichment (Table 5.2). When comparing the percentage of total animals infected, vitamin E treated groups had a lower percentage of infected animals compared to the control group; difference were not significant ($P>0.05$). None of the young guinea pigs receiving vitamin E developed infection, in contrast to untreated young guinea pigs where 8 % of animals became infected. Infections were not evident young animals (control and vitamin E groups) beyond day 3 post-challenge. For aged animals, 13 % of vitamin E treated and 15 % of control animals became infected. Infections developed by day 3, 6 and 9 post-challenge, no animals were infected beyond day 12 (Table 5.2). Overall, a higher percentage of the aged animals became infection compared to young animals.

Table 5.2. Enrichment of spleen and liver samples following oral challenge with 100 CFU *L. monocytogenes*.

Day post-challenge	Organs	<u>Young</u>		<u>Aged</u>	
		Vit E	Control	Vit E	Control
Day 3	Liver [‡]	0/6	3/6	2/6	2/6
	Spleen [‡]	0/6	2/6	0/6	3/6
Day 6	Liver	0/6	0/6	2/6	3/6
	Spleen	0/6	0/6	1/6	0/6
Day 9	Liver	0/6	0/6	1/6	0/6
	Spleen	0/6	0/6	0/6	0/6
Day 12-20	Liver	0/20	0/19	0/20	0/21
	Spleen	0/20	0/19	0/20	0/21
Total no. of animals infected		0/38	3/37	5/38	6/39
Percentage of animals infected		0%	8%	13%	15%

[‡]Liver and spleen samples were obtained from two independent experiments before oral gavage with *L. monocytogenes* and on day 3, 6, 9, 12, 16 and 20 post-challenge. All organ samples were negative for *L. monocytogenes* by direct plating. Total number of animals infected was determined by the presence of *L. monocytogenes* in the liver or spleen. (n = 4).

5. E. Changes of Plasma α -tocopherol concentration during Post-challenge Period

Plasma vitamin E concentrations were also analyzed post-challenge to determinate the influence of listerial infection on systemic levels of vitamin E. The concentration at day 0 (week 3 pre-challenge) was consider as the basal level (Fig. 5.5). The starting vitamin E concentration was 12.7 μM and increased significantly ($P < 0.01$) to 32.05 μM and 43.42 μM by D3 and D6, respectively, in aged animals receiving vitamin E. A subsequent significant dramatic decline to 7.86 μM at D9 and 3.85 μM at D12 occurred ($P < 0.01$). Initially a similar scenario occurred in young animals receiving vitamin E; plasma vitamin E levels rose from 8.23 μM at D0 to 32.45 μM at D3, and declined (6.97 μM) at D9.

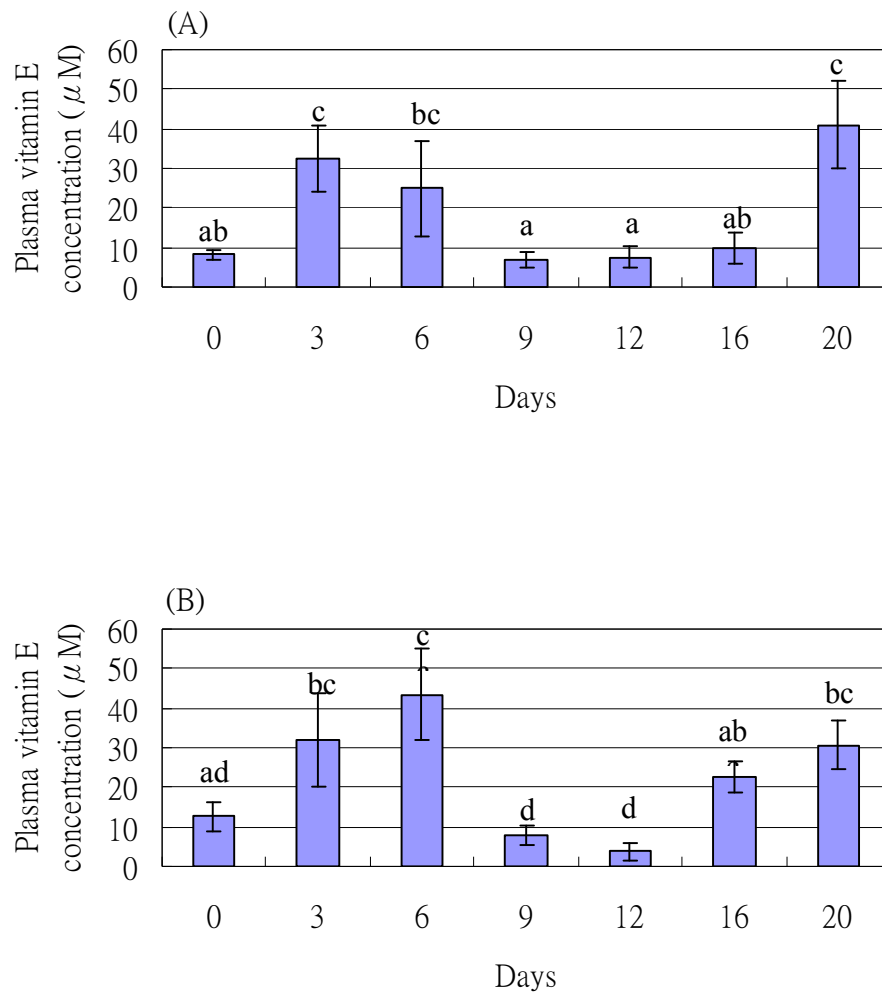


Figure 5.5. Plasma α -tocopherol concentration of young and aged guinea pigs during the infection period. (A) vitamin E young (B) vitamin E aged. Plasma samples were obtained from two independent experiments before oral gavage with *L. monocytogenes* and on day 3, 6, 9, 12, 16 and 20 post-challenge. D0 is the day of challenge and is considered the baseline.

^{a-d} Bars within a graph with different letters are significantly different ($P < 0.01$, $n = 4$).

5. G. Levels of CD3⁺ T Cells during Infection Period

Levels of CD3⁺ T cells population were also determined to evaluate the influence of listerial infection on overall T cell response. The CD3⁺ T cells population on D0 was set as the basal level prior to oral challenge. Levels of CD3⁺ T cells fluctuated over the post-challenge period (Fig 5.6 and 5.7). On day 3 post-challenge only the vitamin E treated aged group showed a substantial (38.7 %) increase in levels of CD3⁺ T cells; however, CD3⁺ T cells levels declined in the other groups. In the control young group, there was a significant decrease ($P < 0.05$) in numbers of CD3⁺ T cell on D6 compared to D0. In control aged animals, a significant decline in numbers of CD3⁺ T cells occurred at D9 ($P < 0.05$). The absolute numbers of CD3⁺ T cells stayed below the base line in both control groups; however, in both vitamin E treated group elevated numbers of CD3⁺ T cells were observed periodically during the post-challenge period.

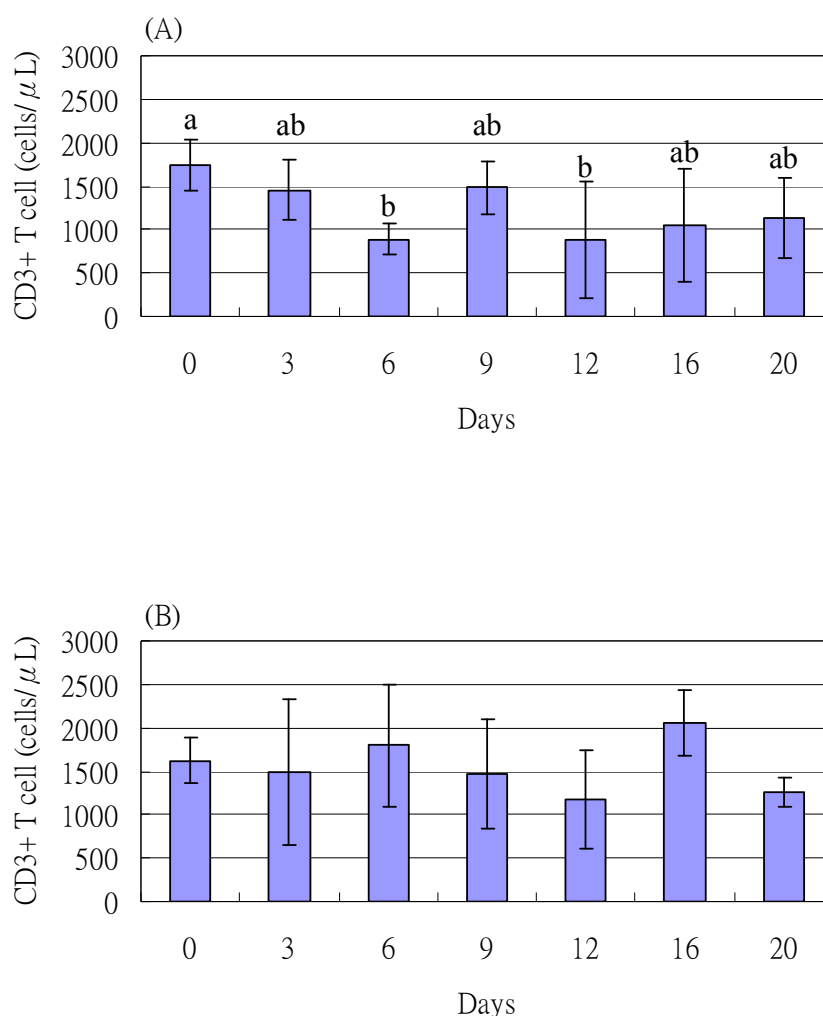


Figure 5.6. Changes in the levels of total (CD3⁺) T lymphocytes of young control (A) and treated (B) guinea pigs during the infection period. Data of CD3⁺ T cell were presented in the absolute number of cells per micro liter of plasma (cells/ μL). Blood samples were obtained from two independent experiments before oral gavage with *L. monocytogenes* and day 3, 6, 9, 12, 16 and 20 post-challenge. D0 is the day of challenge and is considered the baseline.

^{a-b} Bars within a graph with different letters are significantly different ($P < 0.05$, $n = 4$).

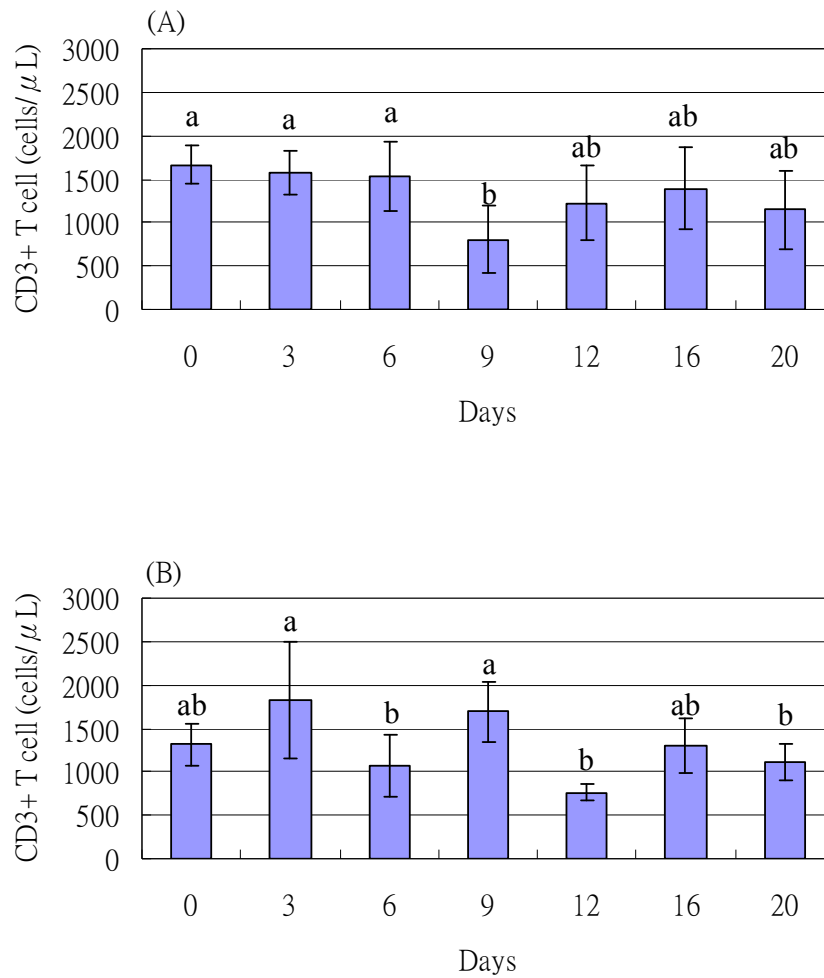


Figure 5.7. Changes in the levels of total (CD3⁺) T lymphocytes of aged control (A) and treated (B) guinea pigs during the infection period. Data of CD3⁺ T cell were presented in the absolute number of cells per micro liter of plasma (cells/ μL). Blood samples were obtained from two independent experiments before oral gavage with *L. monocytogenes* and day 3, 6, 9, 12, 16 and 20 post-challenge. D0 is the day of challenge and is considered the baseline.

^aBars within a graph with different letters are significantly different ($P < 0.05$, $n = 4$).

5. F. Levels of CD8⁺ T Cells during Post-challenge Period

Changes in percent cytotoxic (CD8⁺) T cells were determined to evaluate the influence of dietary vitamin E on the development of cell-mediated immune response against listerial infection. Percent CD8⁺ T cells started to significantly change on different days for each group: D3 in vitamin E aged, D6 in control aged, D9 in control young and D12 in vitamin E young, corresponding to a 59 %, 36 %, 58 % and 75 % increase from D0, respectively (Fig 5.8 and 5.9). In all groups CD8⁺ T cell populations varied during post-challenge, gradually increasing and then dropping back to the basal level.

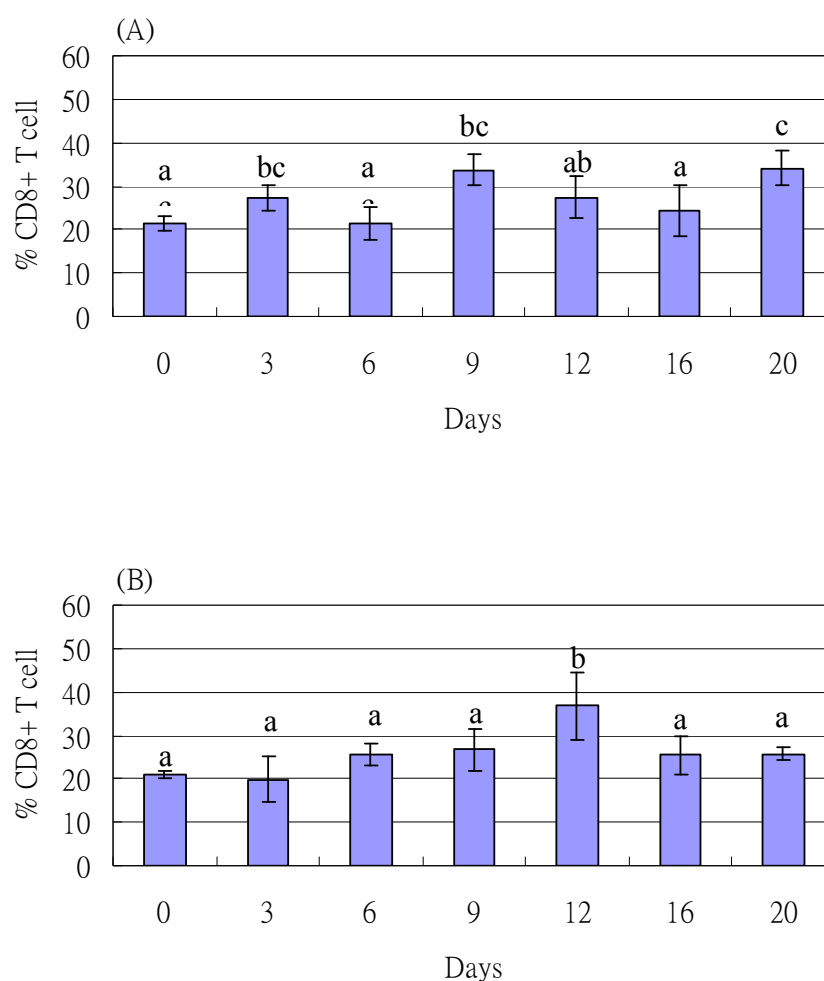


Figure 5.8. Changes in the levels of cytotoxic (CD8⁺) T lymphocytes of young control (A) and treated (B) guinea pigs during the infection period. Percent CD8⁺ T cells were calculated by dividing the number of CD8⁺ T cell by number of CD3⁺ (total) T lymphocytes and multiplying by 100. Blood samples were obtained from two independent experiments before oral gavage with *L. monocytogenes* and on day 3, 6, 9, 12, 16 and 20 post-challenge. D0 is the day of challenge and is considered the baseline.

^{a-c} Bars within a graph with different letters are significantly different ($P < 0.05$, $n = 4$).

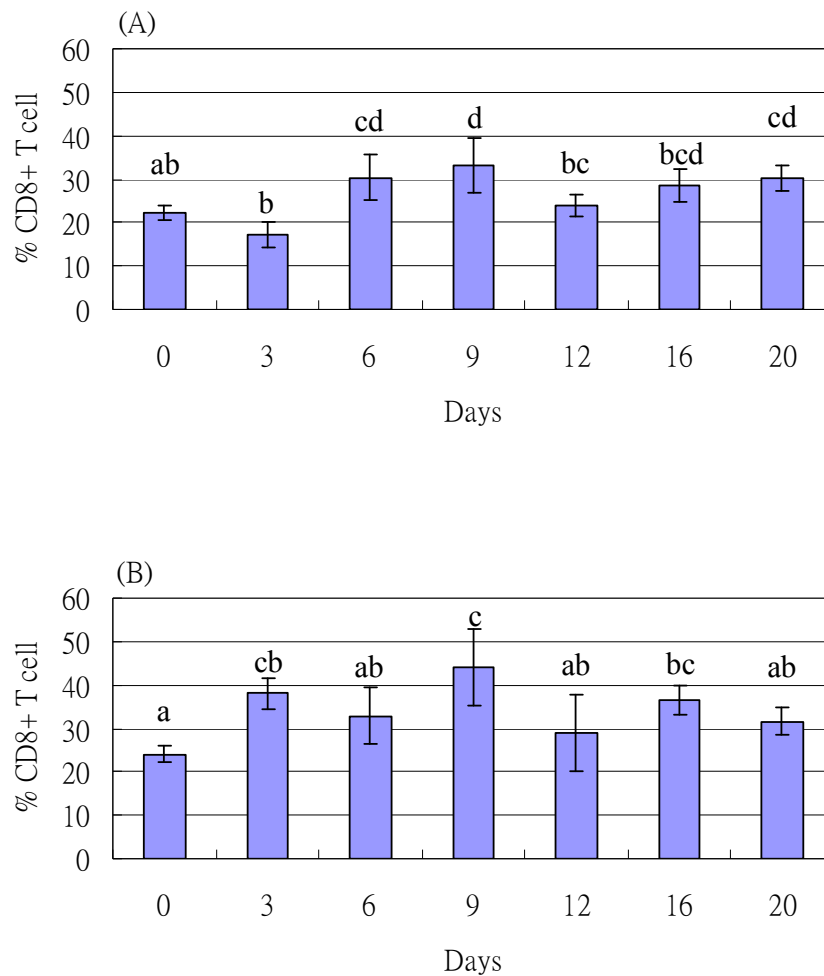


Figure 5.9. Changes in the levels of cytotoxic (CD8⁺) T lymphocytes of aged control (A) and treated (B) guinea pigs during the infection period. Percent CD8⁺ T cells were calculated by dividing number of CD8⁺ T cells by the number of CD3⁺ (total) T lymphocytes and multiplying by 100. Blood samples were obtained from two independent experiments before oral gavage with *L. monocytogenes* and day 3, 6, 9, 12, 16 and 20 post-challenge. D0 is the day of challenge and is considered the baseline.

^{a-d} Bars within a graph with different letters are significantly different ($P < 0.05$, $n = 4$).

Chapter 6

Discussion

The purpose of the present study was to determine the influence of dietary vitamin E supplementation on the development of listerial infection in young and aged guinea pigs. Young and aged guinea pigs were fed a diet supplemented with vitamin E at 5,000 IU/ kg feed for 21 days and then intragastrically challenged with a low dose of *L. monocytogenes* (100 CFU). Aged guinea pigs (*c.* 2 years of age) represented an elderly human population and young guinea pigs (*c.* 6 months of age) represented a young adult of human population. Epidemiological evidence suggested that the elderly and immunocompromised are at greater risk for listerial infection compared to young healthy adults. The function of immune system of humans declines with age. Researchers demonstrated that vitamin E is a potent immunoenhancer specifically stimulating T cell proliferation and differentiation. Oral intake of vitamin E at levels approximately 10-fold greater than the recommended daily allowance (22.5 IU per day) exhibits a positive immunostimulatory effect.

The change in plasma α -tocopherol levels indicates the absorption of vitamin E from the intestine (Traber *et al.*, 1988). During the initial 21-day period of vitamin E supplementation, the plasma α -tocopherol concentration significantly increased in vitamin E treated young guinea pigs (Table 5.1). The change in plasma α -tocopherol levels in the vitamin E treated aged group were not significant. Research demonstrated that plasma α -tocopherol concentrations are limited and levels can only increase 2-3 folds,

regardless of the supplementation regime (Baker *et al.*, 1980; Brim *et al.*, 1989; London *et al.*, 1984; Dimitrov *et al.*, 1991), which results from the rapid turnover rate of α -tocopherol in plasma. In addition, Hollander and Dadufalza (1989) concluded that the total amount of vitamin E absorbed increases with aging due to the high demand for antioxidants in the elderly. The study showed higher total absorption of vitamin E in aged rats than in young rats. In the present study, higher basal levels of plasma vitamin E were observed in the vitamin E aged group than in vitamin E young group, a result of high demand for antioxidants. Levels of plasma α -tocopherol concentration remained constant in aged guinea pigs during this period. The result may indicate the possible saturation of vitamin E absorption which as the consequence of the saturation of α -TTP. Once the animals reached maximum absorption the plasma vitamin E remained constant.

Short-term daily supplementation with vitamin E resulted in a positive immunoenhancing effect on young guinea pigs, based on an increase in the CD8⁺ T cell population (Fig. 5.3). The result showed a significant ($P < 0.05$) increase in the level of CD8⁺ T cells. In the previous research conducted by Matthews group showed that the increased levels of CD8⁺ T cells indicate the maturation of CD8⁺ T cells during vitamin E supplementation (Pang *et al.*, 2007). In the present study, CD8⁺ T cell populations of aged animals receiving vitamin E increased only slightly ($P > 0.05$). Those results correspond with the plasma α -tocopherol concentration during the same period; significantly ($P < 0.01$) higher levels occurred in young animals receiving vitamin E. During the initial 21-day period of vitamin E supplementation, the levels of CD3⁺ T cells showed a similar result as CD8⁺ T cells. In both control groups, levels of CD3⁺ T cells increased only

slightly (Figs. 5.1, 5.2), although, by week 3 a significant increase occurred in young animals; the normal maturation of the immune system may account for these results. For both vitamin E treated groups, CD3⁺ T cells significantly increased on week 2 and 3 in young guinea pigs, but not significantly changed in aged guinea pigs. This corresponds to the results of the plasma vitamin E concentration which showed the same trend. Given this, dietary vitamin E supplementation results in promotion of total (CD3⁺) T cells and cytotoxic (CD8⁺) T cell proliferation, and the impact of dietary vitamin E supplementation is greater in young animals than in aged animals.

All guinea pigs in the treated groups received daily *c.* 176 IU vitamin E which is approximately 8-fold greater than the recommended daily allowance (RDA). The RDA for a 70 kg human is 22.5 IU vitamin E/ day, and the upper limit (UL) is 1500 IU vitamin E/ day. Many animal and clinical studies have shown that there are no important adverse effects of high dose vitamin E supplementation; although a few clinical cases have been reported that vitamin E supplementation has adverse results (Hathcock *et al.*, 2005; Bendich and Machlin, 1988; Meydani *et al.*, 1997; Weber *et al.*, 1997). In the present study, no adverse effects were observed. In the previous study conducted by Matthews (Pang *et al.*, 2007), aged guinea pigs received 400 IU/ day vitamin E for five weeks. Significant increases in levels of CD8⁺ T cells and plasma α -tocopherol concentration were reported. Meydani *et al.* (1986) fed aged mice a diet containing 500 mg vitamin E/ kg diet for 6 weeks and reported improve immunity based on decreased prostaglandin synthesis. Han *et al.* (2006) reported that vitamin E supplementation (500 mg/ kg diet for 4 weeks) improves T cell proliferation in old mice by altering gene expression. In each of

those studies higher levels of vitamin E or longer treatment period time compared to the present study was used. In the present study, the failure of CD8⁺ T cells and total (CD3⁺) T cells proliferation in aged guinea pigs may be attributed to an insufficiently high level of vitamin E in the diet and period of vitamin E supplementation.

Dietary vitamin E supplementation at orthomolecular levels does not always result in desired effects. Wakikawa *et al.* (1999) provided young and old mice with 500 IU vitamin E for 9 weeks. Results of the study showed that lymphocyte proliferation was promoted in young mice but not old mice, since old mice were already in a depressed immunological state. A clinical trial conducted by De Warrt *et al.* (1997) included 74 subjects over 65 years old that received 100 mg/ day vitamin E for three months. Results of the study showed there was no positive effect on lymphocyte proliferation. Another clinical study showed 100 mg/ day for 6 months failed to promote IL-2 production in elderly subjects (Pallast *et al.*, 1999). Results of the present study are not unfounded based on the outcome of laboratory and clinical studies using orthomolecular levels of vitamin E (De Warrt *et al.* 1997; Pallast *et al.*, 1999; Wakikawa *et al.*, 1999).

Epidemiological reports and dose-response studies on animals showed that the infection dose can be low. The previous study conducted by Matthews (Pang *et al.*, 2007) showed that the challenge of guinea pigs with 100 CFU *L. monocytogenes* resulted in several guinea pigs having quantifiable levels of the pathogen in organ samples, demonstrating that low levels of the pathogen are adequate to cause listerial infection. In the present study, regardless of the treatment the greatest numbers of animals were

positive for listerial infection on day 3 post-challenge, the trend was also observed by other researchers (Pang *et al.*, 2007; Badovinac, 2002). After day 12 post-challenge none of the guinea pigs were positive for *L. monocytogenes*; demonstrating that the latency of listerial infection was not seen under the conditions evaluated. Fewer animals developed listerial infections in vitamin E treated groups than in control groups, and this result is more evident in young animals than aged animals; no differences were significant. None of the vitamin E treated young guinea pigs became infected during the post-challenge period. The immune enhancing affects of vitamin E may have provided protection against infection. Immune T cell response and infection status suggest that dietary vitamin E supplementation was more beneficial in young guinea pigs than in aged guinea pigs.

Plasma vitamin E concentrations were an average of 2.2-fold greater on 3- and 6-days post-challenge in vitamin E treated guinea pigs, although levels dramatically dropped by day 9. The initial significant increase may have been due to the acute demand for antioxidants during an immune response to combat the listerial challenge. Macrophage activation and various immune responses produce free radicals, and the host requires antioxidants immediately to eliminate those radicals. The mechanism of the mobilization is not yet known. The body likely responds with the release of tocopherol from liver under conditions of stress. The liver plays an important function in regulation of vitamin E disposition, metabolism, and excretion through α -TTP (Traber, 2005). The plasma tocopherol levels rose significantly in subjects during intensive exercise which also caused the production of oxidative radicals (Pincemail *et al.*, 1988). The mobilization of tocopherol could help to prevent lipoperoxidation phenomena occurring

in skeletal muscle during exercising. The rapid turnover rate of tocopherol in plasma may result in the marked decline of plasma tocopherol concentration.

The influence of vitamin E supplementation on CD3⁺ T cell population may not be readily apparent, even when shifts in specific T cell populations occur. The total T cell population is comprised of helper (CD4⁺) and cytotoxic (CD8⁺) T cells, and each consisting of subsets of naïve, memory or regulatory T cells. Although levels of CD3⁺ T cells fluctuated over the post-challenge period, trends were observed. For both control groups, levels of CD3⁺ T cells declined during the first 6-9 days post-challenge and then maintained a constant level (Figs. 5.6, 5.7). In animals receiving vitamin E, the CD3⁺ T cell population increased and then decreased before stabilizing during the final third of the experimental period. Results suggest that dietary vitamin E supplementation establishes a pool of T lymphocytes.

During the initial stage of an infection the increase of CD8⁺ T cells indicates the triggering of an immune response to eliminate intracellular pathogens. Notable changes in the CD8⁺ T cell response requires 4-5 days, and peaks at 7-9 days post-infection (Busch *et al.*, 1998). In aged animals the percent of CD8⁺ T cell significantly increased by D3, and remained significantly higher in vitamin E treated guinea pigs (Fig. 5.9). However, in control animals the levels of CD8⁺ T cells declined by D3, but expanded significantly after D6 (Fig. 5.9). These results demonstrate that dietary vitamin E supplementation promoted CD8⁺ T cell response in aged guinea pigs. Studies conducted by Adolfsson *et al.* using T cells from vitamin E supplemented animals showed that

vitamin E supplementation increases cell-dividing capacity, IL-2-production capacity, and high affinity IL-2R of the naïve T cells in aged mice (Adolfsson *et al.*, 2001). In the present study, the CD8⁺ T cell response in vitamin E treated aged guinea pigs was faster than in control aged guinea pigs (Fig. 5.9). In contrast, the T cell response of control aged guinea pigs was limited to CD8⁺ T cells. The CD8⁺ T cell levels declined in control aged guinea pigs immediately post-challenge, the result of the lower sensitivity to the pathogen in aged animals. For aged animals the vitamin E regime in the present study did not result in significant T cell proliferation; however vitamin E supplementation appeared to promote the proliferation capability. Upon listerial invasion, the host rapidly initiates a high affinity cell-mediated immune response to ward off the pathogen. After the initial immediate clearance of the pathogen, the antigen-specific memory T cell response was mounted for secondary infection, the result of constant levels of CD8⁺ T cells persisting into the latter part of the post-challenge period. Moreover, it was shown that the immune system in aged subjects is composed of a higher number of antigen-specific CD8⁺ T cells (Miller, 1996, Jackola *et al.*, 1994). Therefore, control aged guinea pigs were able to combat *Listeria* through mounting an adaptive immune response.

In vitamin E treated young guinea pigs, the level of CD8⁺ T cells remained constant in the early state of infection (Fig. 5.8). During this period, none of the young animals receiving vitamin E supplementation were positive for listerial infection which indicates that CD8⁺ T cell response were not induced significantly to combat the bacteria. The level of CD8⁺ T cells significantly increased (approximately 75%) by day 12 post-challenge. The significant increase may be related to the immunoenhancing effects

of vitamin E which resulted in increased levels of high affinity cytotoxic T cells available to kill off the *Listeria*, since animals were receiving vitamin E supplementation throughout the experiments. However once the host encounters antigen following innate and adaptive immune responses, T cells differentiate into antigen-specific memory $CD8^+$ T cells that can be used to combat secondary infections. Moreover, young guinea pigs would have a healthy robust immune system capable of promoting $CD8^+$ T cell proliferation. Indeed, $CD8^+$ T cell proliferation continued post-challenge.

Results of the present study, suggest that ingestion of low numbers of *L. monocytogenes* caused mild infection that the host was able to overcome. Only a few guinea pigs experienced mild diarrhea and lethargy, no animals died. Vitamin E supplementation did stimulate T cell proliferation and the establishment of a pool of T cells. The increase in $CD8^+$ T cell numbers would enhance the ability of the animals to ward-off infection by intracellular pathogens. In the present study, the infectious dose was 100 CFU, a level that based on statistical probabilities would be expected to result a limited number of infections. The European Safety Authority, estimated that the expected annual number of listeriosis cases from ingesting under 100 CFU/g *L. monocytogenes* contaminated food is 5.7 cases per year. However, people consuming low numbers of *L. monocytogenes* on a daily base may be at risk, particularly those individuals ≥ 60 years of age. A recent report suggested that in European countries the incidence of listeriosis among person ≥ 60 years of age is increasing (Goulet *et al.* 2008). The authors suggest that the increase may be associated with the consumption food contaminated with low-levels of *Listeria*.

Chapter 7

Conclusion

In the present study, short-term daily dietary vitamin E supplementation was more beneficial in promoting the immune system in young guinea pigs than aged guinea pigs under conditions evaluated. Immunomodulation occurred during the pre-challenge period in young animals receiving vitamin E; based on the increase in the CD8⁺ T cell population. Young and aged animals became infected following challenge with low levels of *L. monocytogenes* (100 CFU). A higher percent of animals in the control groups became infected; and latency of infection was not apparent. Microbiological analysis of target organs showed that infection occurred within nine days of challenge. No animals were positive for *L. monocytogenes* beyond day 12 post-challenge. Dietary vitamin E supplementation promoted CD8⁺ T cell proliferation and CD8⁺ T cell response especially in aged animals that became infected post-challenge. A significant cell mediated response was not seen in young animals. The robust nature of the immune system of young animals was capable of combating exposure to the low number of *L. monocytogenes* encountered without having to mount a significant immune response.

Chapter 8

Future Work

In the present study, we demonstrated that short-term daily dietary vitamin E supplementation was beneficial in mitigation of listerial infection. A more pronounced affect may have been demonstrated if the level of vitamin E supplementation was greater. In future studies, the level of vitamin E (500mg/ day) in the diet will be increased and *L. monocytogenes* (10^4 CFU) challenge dose will be increased and administered on several consecutive days. A more detailed understanding of the proliferation of T cells could be obtained by investigating different T cell phenotypes: CD45RA⁺ (naïve cell) and CD45RO⁺ (memory activated cells) permitting discrimination of naïve T cells from memory T cells subsets. Malmberg *et al.* (2002) evaluated the capacity of a short-term and high dose of vitamin E supplementation on the change of the T cell population by analysis of different T cell subsets. Moreover, IL-2 is the critical T cell proliferation promoter, and the immunostaining biomarkers of IL-2 could be used to evaluate the levels of IL-2 production affected by vitamin E. PGE₂ production by macrophages could also be assayed to reveal the influence of vitamin E on its production (Adolfsson *et al.*, 2001).

In general, dietary orthomolecular vitamin E supplementation could be applied to our daily life through fortified food products. Due to the increase in consumer demand for health promoting products, various fortified food products are now marketed and generate robust sales. For example, vitamin D and calcium fortified milk, and vitamin C

fortified orange juice to name a few. Vitamin E supplementation could also have relevance to animal nutrition; the formulation of diets with supplements for health promotion.

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