AN ABSTRACT OF THE THESIS OF

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Jean A. Hall and Rosemary C. Wander

We have previously shown that diets enriched with (n-3) fatty acids reduced the delayed-type hypersensitivity (DTH) skin reaction to keyhole limpet haemocyanin (KLH) in geriatric-Beagles. Although the amount of α-tocopheryl acetate in diets of the previous study exceeded requirements, plasma α-tocopherol concentration was significantly lower in dogs fed the high (n-3) fatty acid diets. There are several reasons that could explain the decreased DTH response. Some of these include decreased cytokine production, specifically, interleukin (IL) IL-1β, tumor necrosis factor (TNF) and IL-6 by mononuclear cells. Furthermore, the reduced DTH response could be attributed to increased levels of lipid peroxides or changes in plasma α-tocopherol levels. In this study we examined the effects of feeding 32 healthy, female, geriatric-Beagles diets containing (n-6) to (n-3) fatty acid ratios of 37:1 and 1.7:1, while varying the content of α-tocopheryl acetate, [high (447 ug/g), med (101 ug/g) and low (17 ug/g)] for 82 days on the DTH reaction. Consumption of the 1.7:1 fatty acid diets significantly increased the total content of (n-3) fatty acids in plasma compared to the 37:1 fatty acid diets (17.00 and 2.02 wt %, respectively). There was a significant interaction between the (n-6) and

(n-3) fatty acid ratio and the concentration of α-tocopheryl acetate in the diet on the plasma concentration of α-tocoopherol. The concentration of α-tocopheryl acetate in plasma of dogs fed the 1.7:1 fatty acid diets was 17.3, 25.4, and 35.4 ug/ml, respectively, for the low, med and high α-tocopheryl acetate containing diets, and in dogs fed the 37:1 fatty acids diets was 20.8, 34.9, 52.4 ug/ml, respectively. Consumption of the 1.7:1 fatty acid diets with either low or high α-tocopheryl acetate showed no differences in DTH response from each other or from dogs consuming the 37.1:1 fatty acid diets. When the dietary α-tocopheryl acetate concentration was moderate, a significant suppression of the DTH response occurred at 48, 72, and 96 hr in dogs consuming the 1.7:1 fatty acid diet. These data suggest that an interaction exits between dietary (n-3) fatty acid content and α-tocopheryl acetate on the immune response as measured by the DTH test.

Dietary (n-3) and (n-6) Fatty Acids and Vitamin E: Their Effects on the Immune Response of Healthy Geriatric Beagle Dogs

By

Katie A. Tooley

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Katie A. Tooley, Author

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DEDICATION

I would like to dedicate this thesis to my cat Crackers. Not only has he been my "little BUG" since he has come into my life, but he also made a great study/sleeping partner. My life will forever be changed and enriched by having him be a part of it.

Dietary (n-3) and (n-6) Fatty Acids and Vitamin E: Their Effects on the Immune Response of Healthy Geriatric Beagle Dogs

INTRODUCTION

It is well documented that dietary supplementation with polyunsaturated fatty acids (PUFAs), both (n-3) and (n-6) fatty acids, affects the immune response. Fatty acids affect cell membrane phospholipids, eicosanoid production, and signal transduction pathways used by cells. Consequently, receptors, enzymes, and cytokines are also altered, which directly affects cells of the immune system.

In dogs, dietary supplementation with high levels of (n-3) fatty acids suppress prostaglandin E_2 (PGE_2) production in stimulated monocytes (Chen et al. 1994). In Wander et al. (1997), 20 healthy, female, aged Beagles were fed diets of varying ratios of (n-6) to (n-3) fatty acids (high, medium, and low) for 8 to 12 wk, and various parameters of the immune response were studied. Compared to the diet with the high (n-6) to (n-3) ratio (31:1), the diets with the medium (5.4:1) and low (1.4:1) (n-6) to (n-3) fatty acid ratios greatly increased the amount of (n-3) fatty acids found in plasma. After consumption of the 1.4:1 diet, stimulated mononuclear cells produced 52% less PGE_2 than those from dogs fed the 31:1 diet. Wu et al. (1996) also demonstrated that diets rich in (n-3) fatty acids reduced the amount of PGE_2 produced by peripheral blood mononuclear cells of non-human primates.

However, other things such as aging also affect PGE_2 production. Meydani et al. (1990) found that peripheral blood mononuclear cells taken from healthy older

human subjects produced a greater amount of PGE_2 then those taken from younger subjects. Meydani et al. (1986) reported increased PGE_2 production from aged mice as well. Thus, both age and (n-3) fatty acid intake can have profound effects on PGE_2 production. In another study, it was shown that supplementing elderly subjects with (n-3) fatty acids not only decreased PGE_2 production but, in addition, the decrease was more pronounced with increasing age (Meydani et al. 1991b). Although the cause for this is not known, it could be due in part to increased levels of cyclooxygenase activity (Hayek et al. 1994).

In addition to the change in PGE_2 production, the delayed type hypersensitivity (DTH) response is reduced after ingestion of diets containing large amounts of (n-3) polyunsaturated fatty acids (PUFA). In the dog study by Wander et al. (1997), the T-cell mediated immune response (assessed by a DTH skin reaction) was depressed when the dogs were fed high levels of (n-3) PUFA. At both 24 h and 48 h the diameter of induration was less in those dogs fed the most (n-3) fatty acids in their diet. The results of the dog study are consistent with the results reported in other studies. Meydani et al. (1993) noted less induration when normolipidemic humans were fed a low fat, high fish [good source of (n-3) FAs] diet compared to those consuming a low fat, low fish diet. Yoshino and Ellis (1987) also reported that rats fed fish oil concentrate had lower DTH responses then rats fed water, oleic acid or safflower oil containing diets. Since a DTH response reflects the T-cell side of the immune system, it is unlikely that these results were caused by decreased ability to produce PGE_2. This is because, in general, an increase, not a decrease, in PGE_2 concentration suppresses T-cell mediated function

(Meydani 1995). A more reasonable mechanism for the reduced DTH skin test response noted in the dogs receiving high levels of dietary (n-3) fatty acids may be because of decreased cytokine production, specifically, interluekin (IL) IL-1β, tumor necrosis factor (TNF) and IL-6 by mononuclear cells. Meydani et al. (1993) showed that the production of these cytokines was decreased in stimulated mononuclear cells after humans had consumed low fat, high fish diets compared to humans consuming low fat, low fish diets. A decrease in cytokine production might contribute to a decrease in antigen-presenting cell activity, thereby resulting in a decreased DTH skin response.

Vitamin E deficiency has also been shown to suppress the immune response in a wide variety of subjects ranging from rodents to humans (Meydani 1995). Conversely, supplementation with vitamin E greatly enhanced the DTH response in both animals (Finch and Turner 1995) and humans (Kelley and Bendich 1996). Likewise, lymphocyte proliferation is quite sensitive to the level of vitamin E in the diet, for example, low levels of vitamin E have been shown to suppress lymphocyte proliferation (Eskew et al. 1985). In addition, vitamin E levels in the diet also affect cytokine production. In humans, the level of IL-2 produced in response to concanavalin A (Con A) was greater in those individuals supplemented with 800 IU/day of dl-α-tocopherol for 30 days versus those subjects not supplemented (Meydani et al. 1990). Other cytokines affected in a similar way include TNF-α and IL-6, both of which were increased in human subjects supplemented with vitamin E.

In Wander et al. (1997), it is possible to consider the decreased DTH response as a direct consequence of changes in plasma vitamin E. The amount of α-tocopherol in the plasma was 20% lower in the dogs fed the 1.4:1 diet compared to the dogs fed the 31:1 diet. Some studies (Alexander et al. 1995) have shown that high intakes of (n-3) PUFAs decrease the level of vitamin E in plasma. As a result, more α-tocopherol may need to be added to diets containing high amounts of (n-3) fatty acids. The amount of α-tocopherol in all of the diets of the Wander et al. (1997) study was similar and exceeded the calculated requirements described by Muggli (1989) for diets high in (n-3) fatty acids by six to eight times. However, plasma α-tocopherol concentration was still significantly lower in those dogs fed the high (n-3) fatty acid diet (as expressed on a molar basis) compared to those dogs fed the other two diets. Because decreased plasma concentrations of vitamin E and high levels of (n-3) PUFA can both effect the immune response in similar ways, it is uncertain which factor was responsible for the effects observed in Wander et al. (1997). Furthermore, the interaction of the two may have caused these results.

Another possible explanation for the results seen in Wander et al. (1997) could be that increased levels of lipid peroxides caused a decreased immune response. Plasma and urine lipid peroxide concentrations, as measured by thiobarbituric acid reactive substances (TBARS), increased in the dogs fed diets containing high levels of (n-3) fatty acids. Meydani et al. (1993) also reported a rise in lipid peroxide levels induced by feeding (n-3) fatty acids and concluded this may have been responsible for the decrease in DTH skin test responses noted in their study. Zoschke and Messner

(1984) also reported that human lymphocyte mitogenesis was suppressed by lipid peroxidation products. Therefore, a rise in lipid peroxide products after dietary (n-3) fatty acid supplementation may contribute to the decrease in cell-mediated immunity (DTH response) in the Wander et al. (1997) study.

The results of the Wander et al. (1997) study showed that diets high in (n-3) PUFA reduced the DTH skin response, changed the profile of the plasma lipids, decreased PGE_2 production and plasma concentrations of α-tocopherol, while increasing lipid peroxidation in geriatric dogs. We have also shown (Hall et al. 1999) that feeding a diet high in (n-3) fatty acids (ratio of (n-6) to (n-3) fatty acids was 1.4:1) had significant effects on CD4+ T-lymphocytes in healthy geriatric dogs. Consumption of a diet enriched in (n-3) fatty acids increased the total numbers of lymphocytes, reduced the number of CD4+ T-lymphocytes and decreased the CD4+/CD8+ ratio after vaccination with keyhole limpet haemocyanin (KLH) in geriatric dogs. This study demonstrated *in vivo* modulation of the immunophenotype profiles of T-lymphocytes by dietary (n-3) fatty acids. The same confounding variables described above were present in the latter study, namely decreased plasma concentration of vitamin E and increased plasma concentration of lipid peroxides.

Based on these results, we have designed a new study to determine a more appropriate level of vitamin E to be consumed when (n-3) fatty acid supplementation occurs. In this new study we will examine how changing the levels of vitamin E in the diet [high (446, 448 μg/g), medium (95.0, 106 μg/g) and low (16.6, 17.2 μg/g)], while keeping (n-6) to (n-3) ratio constant (approximately 40:1 or 1.4:1) affects the

following parameters: PGE_2 production by stimulated monocytes, DTH skin test, KLH antibody titer, phagocytosis by monocytes, CD4+, CD8+, and CD69+ surface markers, and IL-2R expression. Plasma vitamin E and lipid peroxide concentrations (TBARS) will also be monitored.

REVIEW OF THE LITERATURE

Nomenclature

A brief introduction to the nomenclature of fatty acids and vitamin E is presented.

Fatty Acid Nomenclature

A fatty acid is an organic compound comprised of a hydrocarbon chain that terminates with a carboxylic acid group. These chains may be as short as two or as long as 36 carbons. The ones of interest in nutrition usually have an even number of carbon atoms. Fatty acids may be saturated (SFA), monounsaturated (MUFA) or polyunsaturated (PUFA).

Saturated fatty acids have no double bonds. The two saturated fatty acids most abundant in foods are palmitic (16:0) and stearic acid (18:0). Common dietary sources of these fatty acids are coconut and palm oils, and animal fats.

There are numerous unsaturated fatty acids important to nutrition. Unsaturated fatty acids can have one double bond (MUFA) or two or more double bonds (PUFA). They are usually grouped into families: the (n-9), (n-6), and (n-3) families. The unique feature to each family is the location of the double bond. According to the International Union of Pure and Applied Chemists (IUPAC), the location of the first

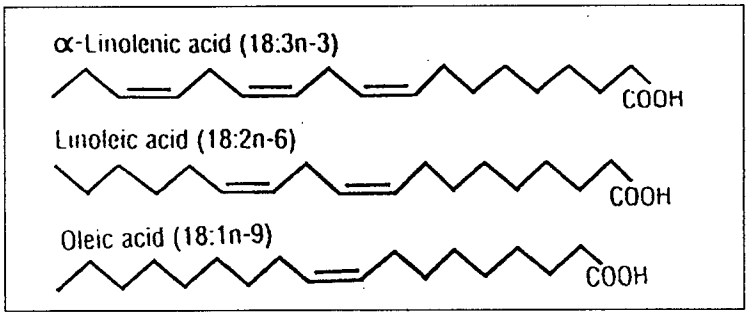


Figure 1. Examples of chemical structures of some common fatty acids.

double bond is determined by counting from the carboxyl end. According to lipid chemists, the location of the first double bond is determined by counting from the methyl end. Using the latter system, an (n-9) fatty acid has the first double bond nine carbons from the methyl end. See **Figure 1** for an example of the MUFA, oleic acid. The shorthand method for referring to oleic acid is 18:1 (n-9) or 18:1 (ω-9). The number 18 represents the total number of carbon atoms, the number following the colon refers to the number of double bonds, and the number after the "n" or "ω" refers to the location of the first double bond counting from the methyl end. Whenever a carbon-carbon double bond exits, there is opportunity for either *cis* or *trans* configuration. *Cis* iosmerization causes the molecule to fold back onto itself forming a "U" shape. The *trans* form extends the molecule into a more linear shape (Groff et al. 1995). The most abundant MUFA in foods is oleic acid. Olive oil and canola oil are rich sources of oleic acid.

Examples of PUFA include linoleic acid, a fatty acid that belongs to the (n-6) family. The (n-6) fatty acids contain a double bond between the sixth and seventh

carbon atoms counting from the methyl end. Linoleic acid is designated 18:2 (n-6) (**Figure 1**). Linoleic acid is commonly found in vegetable oils, e.g., corn, safflower, soybean, and cottonseed oils. Arachidonic acid, which contains 20 carbons, also belongs to the (n-6) family, and is designated 20:4 (n-6). It is found in small quantities in meats and can be synthesized by some mammals.

Linolenic acid is also a PUFA; however, it belongs to the (n-3) family of fatty acids. An (n-3) fatty acid has its first double bond located between the third and fourth carbon atoms counting from the methyl end. Linolenic acid is found in soybean, flax, and canola oils, and is designated 18:3 (n-3) (**Figure 1**). An (n-3) fatty acid of heightened interest is eicosapentaenoic acid (EPA). EPA is an (n-3) PUFA with 20 carbons, including five double bonds. It is designated by 20:5 (n-3) in the shorthand form. Mammals synthesize it *in vivo* from linolenic acid. Another important (n-3) fatty acid is docosahexaenoic acid (DHA). DHA contains 22 carbons and six double bonds and is designated 22:6 (n-3). DHA is a major component of membrane phospholipids in the retina of the eye and the cerebral cortex (gray matter) of the brain. Fish and fish oils are particularly rich in (n-3) fatty acids (Groff et al. 1995). It can be synthesized by most mammals from EPA.

A very important group of molecules that can be synthesized from the 20-carbon fatty acids are the eicosanoids. Included in this group are the physiologically potent families of substances called prostaglandins, thromboxanes and leukotrienes. These molecules are formed by two pathways (**Figure 2**): 1) the cyclooxygenase pathway, which produces both the prostaglandins and thromboxanes, and 2) the

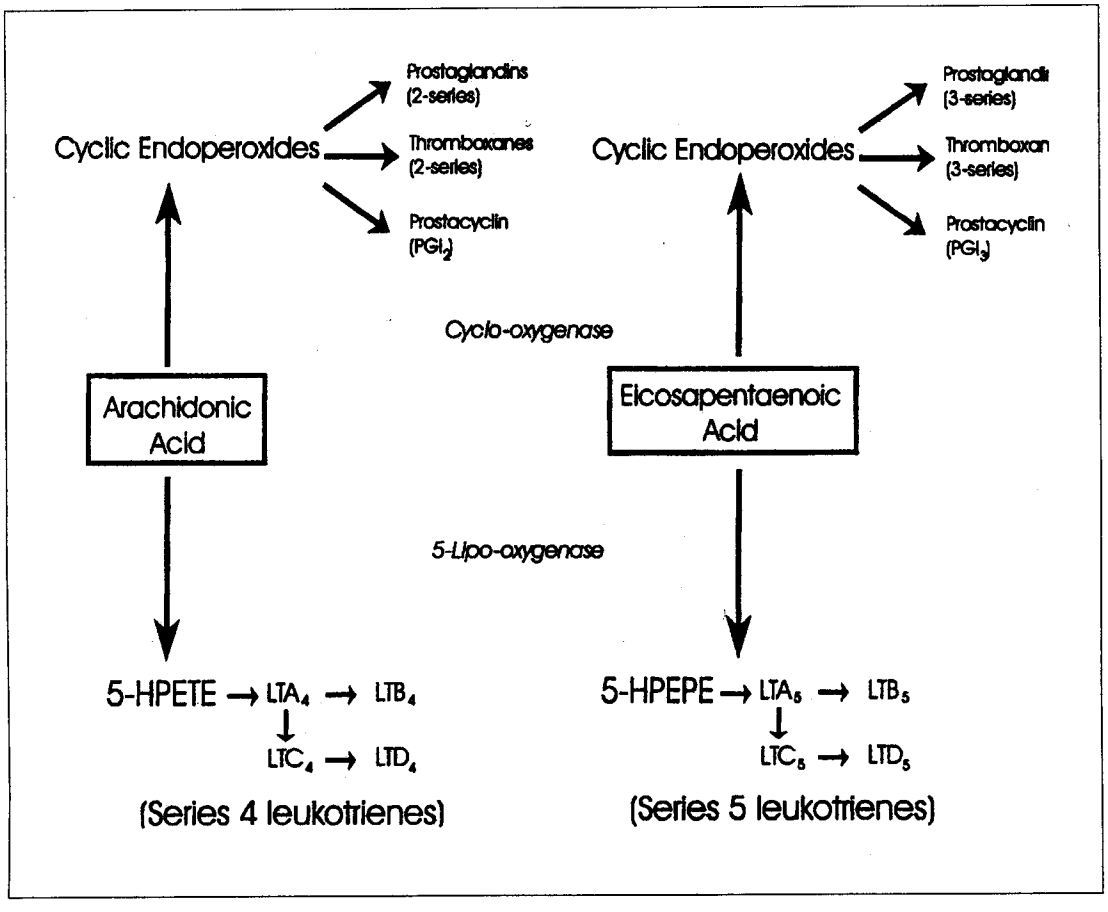


Figure 2. The arachidonic and eicosapentaenoic acid pathways and their end products.

lipoxygenase pathway, which forms leukotrienes (Groff et al. 1995). Prostaglandins are 20-carbon fatty acids that contain a five-carbon ring. Variations in structure exist among them leading to different designations, e.g., PGD, PGE, PGF, PGI, PGG, and PGH. The letter indicates the nature and position of substituents on the cyclopentane ring. There is generally a subscript number that follows the letters. It indicates the number of double bonds in the alkyl side chain. Molecules with the "2" subscript are the most abundant (Groff et al. 1995). These molecules are synthesized from arachidonic acid.

Eicosanoids formed from EPA have three double bond in the alkyl side chain, and are designated by a "3" subscript. They are generally much less active physiologically than those formed from arachidonic acid. They are considered to have antiinflammatory, antiaggregatory, and vasodilatory properties, whereas, the eicosanoids formed from arachidonic acid are considered to be proinflammatory, proaggregatory, and vasoconstrictive.

An appropriate amount of unesterified (free) arachidonate is necessary for the formation of eicosanoids. Arachidonic acid must be released from membrane phospholipids by the activity of a specific hydrolytic enzyme called phospholipase A_2. The most abundant phospholipids in most cells are phosphatidylcholine and phosphatidylethanolamine. The release of arachidonate from the cell membrane phospholipids for eicosanoid synthesis is influenced by various stimuli. There are two main types of stimuli, physiologic (specific) and pathological (nonspecific) (Groff et al. 1995). Physiologic stimulation (a natural occurrence) results from such things as

increased concentrations of epinephrine, angiotensin II, and antigen-antibody (loss of blood), and membrane-active venoms (Groff et al. 1995).

Vitamin E Nomenclature

Evans and Bishop first reported Vitamin E activity in 1922 and identified it as an essential nutrient (VERIS 1993). Their work was done in rats fed diets deficient in certain lipids. Reproductive failure was noted. The deficient lipid component was characterized and named vitamin E.

The term vitamin E refers to eight naturally occurring compounds with varying biologic activity. These include: α- β-, γ-, and δ-tocopherol and α- β-, γ-, and δ-tocotrienol. All these molecules contain a benzene ring and a phytol side chain. The tocopherols differ in the number and location of methyl groups on the benzene ring. For example, the compound α-tocopherol is also referred to as 5, 7, 8-trimethyl tocol because there are three methyl groups at the carbons numbered 5, 7 and 8 on the benzene ring. (See **Figure 6** for the number associated with each carbon atom.) The β-tocopherol has its methyl groups located at carbons 5 and 8 on the benzene ring; γ-tocopherol has methyl groups at carbons 7 and 8; and δ-tocopherol has one methyl group located at carbon number 8 (Farrell and Roberts 1994). These differences can be seen in **Figure 3.** Besides the four above-mentioned tocopherols, there are four compounds similar to the tocopherols called tocotrienols. The only difference between a tocopherol and a tocotrienol is that the side chain of the tocotrienols is unsaturated

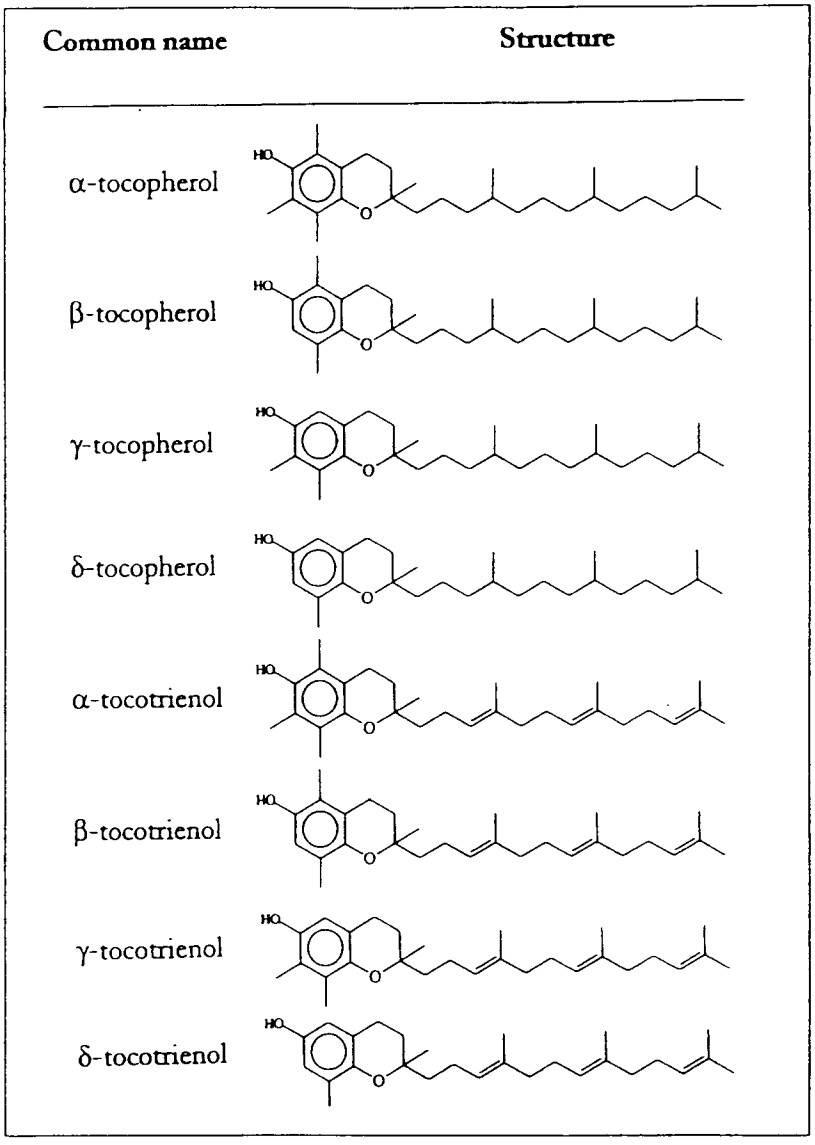


Figure 3. The eight naturally occurring compounds of vitamin E.

(hydrogen atoms have been removed, hence, the addition of three double bonds). The actual ring structure remains the same for the corresponding tocopherols and tocotrienols. Thus, there is an α- β-, γ-, and δ-tocotrienol that corresponds to each tocopherol (**Figure 3**). The number and position of the methyl groups affect the biologic activity.

What makes understanding vitamin E so difficult is that each of the tocopherols when synthesized commercially can exist in eight different stereoisomers (**Figure 4**). There are three asymmetrical carbon atoms, C-2 on the ring, and C-4' and C-8' on the side chain as seen in **Figure 5**. Each carbon in the long side chain is assigned a number (see **Figure 6**). If the letter following the number is "S" (i.e., 2S), when viewing a 3-D model of the compound the methyl group is facing towards oneself. On the other hand, if the letter following the number were "R" (i.e., 2R), the methyl group would face away from oneself. This "R" or "S" configuration may be present at any of the three asymmetrical carbons on the side chain, hence, leading to the eight stereoisomers. In an older system of nomenclature 2R 4'R 8'R was known as d-α-tocopherol whereas, 2S 4'R 8'R was known as l-α-tocopherol.

The natural and most abundant form of vitamin E (also known as 2R 4'R 8'R-α-tocopherol or d-α-tocopherol) exists as a single stereoisomer. Originally, synthetic vitamin E was synthesized from natural phytols. Natural phytol is a single stereoisomer, however, if used to make synthetic vitamin E, the product would be a 50/50 mixture of (2R 4'R 8'R) and (2S 4'R 8'R) (Pryor 1995). Natural phytol is no longer available and a synthetic phytol is used as the starting material. Synthetic phytol

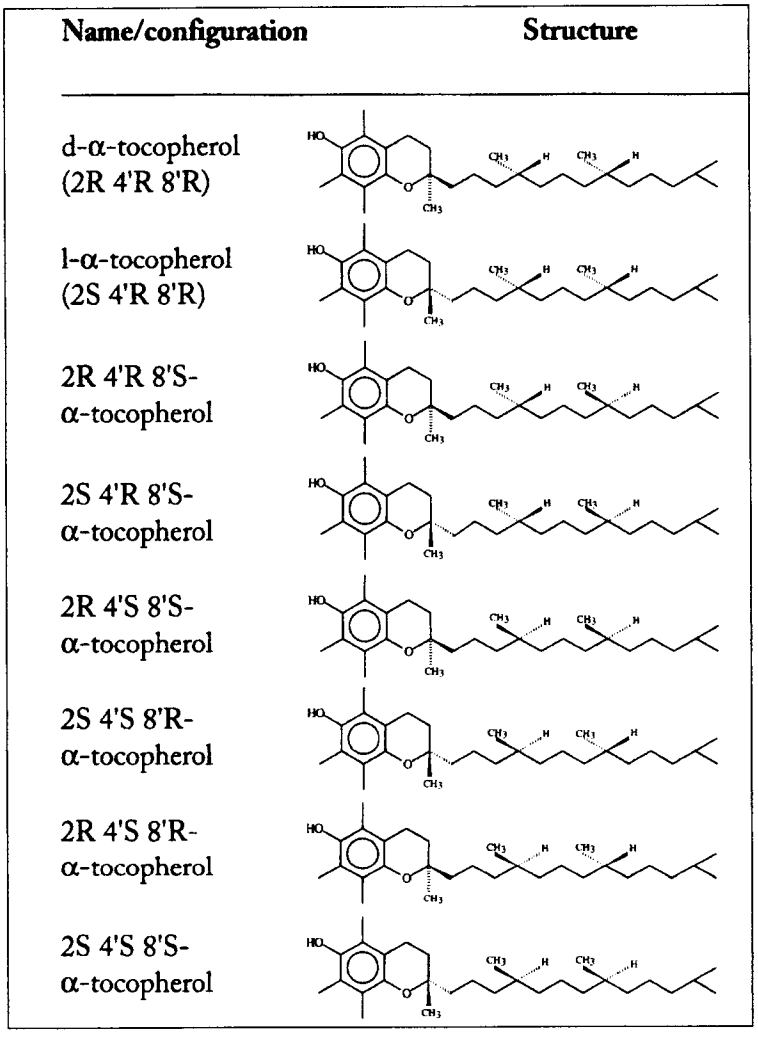


Figure 4. The eight stereoisomers of synthetic α-tocopherol.

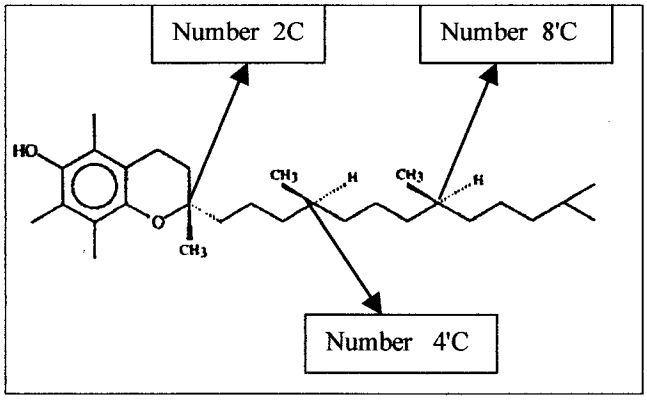


Figure 5. The three asymmetric carbons of α-tocopherol.

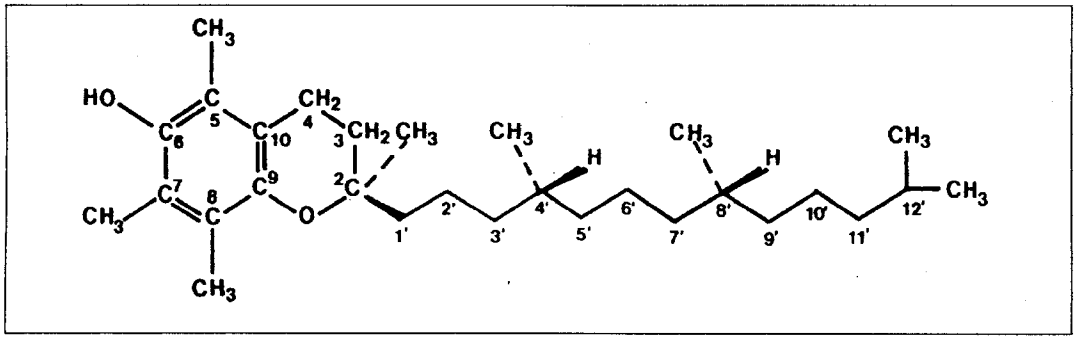


Figure 6. The numbering sequence for α-tocopherol.

is a mixture of stereoisomers. When it is used as the starting material, eight stereoisomers are formed and the mixture is called all-*rac*-tocopherol or dl-α-tocopherol (the term dl-α-tocopherol can be misleading though). These eight stereoisomers are illustrated in **Figure 4**.

Each of the different forms of vitamin E has different biopotencies. The methyl groups in the benzene ring are important determinants of the biologic activity of the tocopherol isomers. β-tocopherol has 40 to 50% of the biopotency of α-tocopherol, γ has 10 to 30%, and δ about 1%. The carbon group at the C-2 carbon is generally considered the most important in terms of the level of vitamin E activity (Pryor 1995). The naturally occurring d-α-tocopherol has the highest biological activity. Its activity, based on fetal resorption studies in rats, is 1.49 IU/mg. An IU (International Unit) is the amount of activity associated with 1 mg of dl-α-tocopheryl acetate.

Sources of vitamin E include vegetable oils (primarily soybean, sunflower, and corn oils), nuts and sunflower seeds. Whole grains as well as wheat germ are also important sources (Pryor 1995).

Interaction of Fatty Acids with the Immune System

An Overview of the Immune System

The word immunity comes from the latin word *immunis*, which means free or exempt (Clough and Roth 1997). Immunology is the study of the immune system. The

primary role of the immune system is to provide immunity. The immune system serves to protect or keep an individual free or exempt from various infectious diseases, certain toxins, and neoplastic diseases. In order for the immune system to provide protection it must first be able to differentiate cells and molecules that normally belong in the body from those that don't belong there. This is referred to as "self" recognition versus "non-self" recognition.

Being immune to a disease may or may not be absolute. Absolute would refer to a species of animals that are not susceptible to a particular disease, whereas another species might be. If a species is susceptible to a certain disease, there are various levels of immunity. Some individuals might be highly resistant to a disease and/or have mild symptoms, whereas other individuals might have a more severe form of the disease following exposure to an infectious agent.

The following is an overview of the major principles of immunology. Immunity to disease results from mechanisms including physical barriers, the action of white blood cells (collectively called lymphocytes), and a variety of protective molecules found in body fluids and/or on the surface of the body. Immunity can be classified by several different means. These include natural vs. acquired immunity, passive vs. active immunity, and humoral vs. cell-mediated immunity.

Immunity may be either natural or acquired. Natural immunity results from mechanisms that do not depend on previous exposure to infectious agents, whereas acquired immunity requires previous exposure to an infectious agent or vaccine.

Natural immunity is present on first exposure to an infectious agent. Mechanisms of natural immunity include such things as physical barriers to infection, as well as antimicrobial compounds. Physical barriers consist of intact skin and mucus membranes; a normal microbial flora; fatty acids in the skin; acid in the stomach; the mucociliary escalator in the trachea; enzymes found in the intestine, saliva, and tears; coughing; sneezing; vomiting; diarrhea; and fever. Antimicrobial compounds include the complement system, type 1 interferon (IFN-1), phagocytic cells, acute phase proteins, cationic peptides on epithelial surfaces, and natural killer (NK) lymphocytes (Clough and Roth 1997). Both people and animals have some degree of natural immunity to almost every infectious agent. However, natural defenses can be overwhelmed or impaired if exposed to high levels of infectious agents.

Complement is a form of natural immunity used by the immune system to respond to infectious agents. The complement system consists of a group of proteins found in the plasma that ultimately forms an enzyme cascade upon activation by invading pathogens. A small event can trigger a rapid and aggressive response. Complement activation initiates a series of events which helps to control many infectious agents (Clough and Roth 1997). This occurs by damaging the bacterial membrane, attracting neutrophils to the site of infection, increasing blood flow (vasodilation), increasing vascular permeability, and opsonizing bacteria for phagocytosis. Too much activation of the complement system can, however, end up damaging the host's tissue.

Acquired immunity requires previous exposure to a particular pathogen or a vaccine containing antigen from an infectious agent. Any infectious agent or molecule that stimulates an acquired immune response is called an antigen. An antigen is a molecule or cell that induces the production of antibodies and/or a T cell-mediated immune response (to be discussed later) by binding to specific receptors on lymphocyte surfaces (Clough and Roth 1997). An antibody is a protein molecule that is produced by B cells, which have differentiated into plasma cells in response to specific antigens that they bind. Antibodies are also called immunoglobulins.

An acquired immune response takes several days to develop after initial exposure to an antigen. This is because production of antibody from B cells and the T cell response to antigen take several days to develop. The immune response is much quicker when the individual is re-exposed to the same antigen. This is because of immunologic memory. It usually takes several days to a week to develop a response to the first exposure. This first exposure is also called the primary response. If and when an individual comes in contact with the same infectious agent, the B and T lymphocytes respond much more rapidly. This is called the secondary response.

Acquired immunity results from antibodies produced from B lymphocytes or from cell-mediated immunity by the actions of T lymphocytes. Antibodies produced by B lymphocytes include IgM, IgG, IgA, and IgE. Cell-mediated immunity consists of cytokines produced by T lymphocytes and cytotoxic T lymphocytes. Of the two types of immunity, the individual with acquired immunity is more resistant to an infectious agent than the individual with natural immunity (Clough and Roth 1997).

Acquired immunity may be acquired passively by transfer of antibodies or lymphocytes from one individual to another, or produced actively by ones own lymphoid system. Passive immunity is a type of acquired immunity whereby the individual receives either antibodies or lymphocytes from another individual. The donor individual must have previously come in contact with the infectious agent and have developed an immune response to the particular antigen. This is most commonly seen in mothers passing their own antibodies to their offspring through either the placenta or their colostrum. If the newborn didn't receive these antibodies from its mother, then it would be highly susceptible to disease because it would only have natural immune defense mechanisms to help it resist disease. However, passive immunity is short lived and disappears after a few weeks or months. At this time the individual's own active immune system must replace the passive response (Clough and Roth 1997).

When an infectious agent penetrates the body's natural defense systems, one of two things might occur. If the pathogen remains extracellular, e.g., a bacteria that does not invade the host's cells, an antibody or humoral-mediated immune (HMI) response occurs. If the pathogen is intracellular, e.g., a viral pathogen, a cell-mediated immune (CMI) response occurs.

In the HMI response, antibodies are made against antigen. The antigen reacts with a certain B cell, also known as a bursa cell, with a corresponding antibody on it surface. When the B cell binds to the antigen, the B cell undergoes a process known as clonal expansion and differentiation. During clonal expansion the B cell replicates

producing many copies of itself called daughter cells. Daughter cells become memory B cells or go on to become plasma cells.

A memory B cell is important upon subsequent reinfection with the same pathogen. These memory cells allow the body to produce a quicker response, i.e., production of antibody, the next time that particular pathogen is encountered. In other words, the B cells have become primed to that antigen.

Daughter B cells can also become plasma cells. Plasma cells are the cells that produce antibodies. When a B cell first encounters an antigen, plasma cells produce primarily IgM. (The abbreviation Ig stands for immunoglobin.) The primary response is usually slow and takes a couple of days to get started. This is in contrast to the secondary response. With a secondary response, the B cells have already been primed and "remember" that particular antigen such that they can mount an antibody attack much faster. This time, however, more IgG antibody is produced. The function of antibodies is to coat the foreign agent so that it may be phagocytosed, lysed, or neutralized, thus blocking its ability to attach and colonize.

Another function of the HMI response is to destroy host cells that have become infected with an intracellular pathogen and subsequently display antigen on their surface. The antibody will react to the antigen on the host cell (now called a target cell) and destroy it. This process is called antibody-dependent cellular cytotoxicity (ADCC).

In the case of the CMI response, an intracellular pathogen invades a host cell causing it to become a target cell. Certain cells in the body known as macrophages,

cytotoxic T cells (Tc), and NK cells are able to recognize and respond to these target cells without the assistance of antibodies. The antigen (also known as an epitope) reacts with an antibody-like receptor on a Tc cell called the Tc cell receptor (TCR). Once the target cell and Tc cell are bound together, the Tc cell undergoes clonal expansion similar to B cells. Again, this expansion produces memory Tc cells. This time, instead of plasma cells, effector cells are produced. These effector Tc cells bind to other target cells and inject cytotoxic granules into the target cells causing them to lyse. It is possible for both the HMI and CMI responses to occur simultaneously, and this is often the case.

Neither the HMI and/or CMI responses would be very effective by themselves without the aid of the helper response. An antigen is broken down and presented to T-helper cells by an antigen processing (or presenting) cell (APC). There are two types of T-helper cells, Th-1 and Th-2 cells. Th-2 cells produce cytokines (e.g., IL-4, IL-5, IL-9, IL-10 and IL-12) that aid in further expansion of B cells and allow for a more vigorous response to the antigen. Th-1 cells produce cytokines (e.g., IL-2, IFN-γ, and TNF-β) that help Tc cells. The Th cells also undergo clonal expansion like the B and Tc cells. Cytokines may also activate macrophages and NK cells. Macrophages and NK cells, along with the Tc cells, help to kill target cells (cell lysis and death).

Normally, the immune system is able to show tolerance, which means the body recognizes self from non-self. In certain individuals, the immune system can react to self, causing self-inflicted damage to tissues and organs. This results in autoimmune disease. When disease results from the immune system, the diseases are called

hypersensitivity disorders. On the other hand, if the immune response is slowed or deficient, the individual may be more susceptible to infectious agents.

Immunological Techniques

There are many techniques used to evaluate immune status or function. These techniques can help to determine if the immune system is working adequately. When performing immunological tests, three general categories are considered. These are *in vivo*, *ex vivo*, or *in vitro* tests. *In vivo* testing requires that the test be performed in the individual, e.g., the DTH test is an *in vivo* test wherein antigen is injected into the subject and the reaction occurs inside the body. *Ex vivo* testing is done outside of the body. The individual is stimulated (injected, fed, etc.) so that some kind of reaction takes place within the body and then cells are removed and various parameters are examined using these cells. *In vitro* testing would be if cells were manipulated in the lab, e.g., (incubated with a specific nutrient) and then stimulated *in vitro* to produce a measurable product, such as a cytokine.

Tests included in the *in vivo* category are summarized below. A DTH test, also known as type IV hypersensitivity reaction, is a general measure of cell-mediated immunity. The principle behind this test is that Langerhans cells will present cutaneously encountered antigens to T lymphocytes (Clough and Roth 1997). This results in antigenic-specific activation of T lymphocytes in local tissues. Inflammatory cytokines produced by these stimulated T lymphocytes cause other mononuclear cells

Table 1. Examples of tests used to evaluate immune function

❑ *In Vivo*	DTH test Titer response to vaccine Antibody response to a protein challenge Circulating lymphocyte concentration Immunophenotype analysis of lymphocytes
❑ *Ex Vivo*	Phagocytic and bactericidal capacity Cytokine production in stimulated cells Lymphocyte response to mitogenic compounds
❑ *In Vitro*	Similar tests as above (*ex vivo*), however, cells are "fed" in a test tube or petri dish rather than collected from an animal

(lymphocytes and macrophages) to migrate to the area and proliferate. To perform this test, foreign antigen is injected under the epidermis of the skin, e.g., in a dog. The CMI branch of the immune system responds to this antigen by producing a small raised wheal that can be measured 24 to 96 hours after injection. The larger the wheal, the greater the CMI response. It may take several weeks to months for DTH lesions to resolve completely.

A titer response to a vaccine is another type of *in vivo* test. An animal is vaccinated with a foreign substance. This substance acts like an antigen and will elicit an immune response. Most B lymphocytes require help from helper T (Th-2) lymphocytes to produce large amounts of antibody with a high degree of affinity. There are some simple antigens with highly repeated subunits in their structure, e.g., carbohydrates, that are T-independent antigens, meaning they do not require help from

helper T (Th-2) lymphocytes. Most antigens, however, including proteins and their derivatives, are T-dependent antigens. Following vaccination, antibody titers to the antigen can be measured years later to assess the B cell response (Clough and Roth 1997). An example of this would be to measure the tetanus titer in an individual who has been previously vaccinated with a tetanus vaccine.

Measuring the antibody response to a protein challenge is very similar to measuring the titer response. Again, the animal is injected with a protein substance. Since proteins make very good antigens, a good immune response (i.e., high antibody titer) is usually mounted against the foreign substance. The antibody titer can be assessed two weeks after the second booster challenge.

Circulating lymphocyte numbers can be determined from the total white blood cell count and differential. Various cell surface markers on the lymphocytes can be stained with fluorescent dye. These cells can then be sorted using flow cytometry. The flow cytometer utilizes optical and/or electronic devices to sort cells by size and/or to detect the presence of cell-bound fluorochrome-labelled antibody. Cells can be negatively or positively selected. Antibody-bound fluorescent cells pass through the flow cytometer and are deflected by electromagnetic fields. The cells are differentially deflected and the strength and direction varies according to the measured intensity of the fluorescence signal. This allows for cell separation by way of surface antibody binding (Field 1996).

In general, the Th cells express CD4 (cluster designation) molecules and recognize antigen bound to class II major histocompatibility complex (MHC), while,

cytotoxic and suppressor T lymphocytes express CD8 and recognize MHC class I bound antigens. These are, however, just useful guidelines as some CD8+ cells perform other functions as do some CD4+ cells (Clough and Roth 1997). Thus, T lymphocyte subsets that are defined based on cell surface molecules may have overlap in function between the subsets.

Ex vivo tests can be used to assess phagocytic and bactericidal capacity. This can be done using a fluorescent bead assay. In this assay, monocytes are incubated with fluorescent latex beads. The monocytes engulf the latex beads via phagocytosis. The number of monocytes ingesting beads and the number of beads ingested per monocyte can be determined using flow cytometry. This assay is a measure of monocyte function, i.e., their ability to ingest foreign particles.

Cytokine production in stimulated cells can be measured using an enzyme linked immunoadsorbent assay (ELISA) test. Monocytes in a blood sample are isolated and maintained in cell culture. Purified lipopolysaccharide (LPS) is added to the cell culture to stimulate the monocytes. The monocytes respond to this stimulus by producing increasing levels of cytokines, e.g., IL-1, IL-2, IL-6, and TNF. This assay provides another means of assessing monocyte function, i.e., it assesses the monocytes' ability to produce cytokines in response to antigen challenge.

The two most popular assays for measuring cytokines are bioassays or immunoassays. Bioassays measure biologically active cytokine; however, immumoassays detect both biologically active and inactive cytokines. In addition, immunoassays are simpler, less time consuming, and don't require special laboratory

facilities. Even though immunoassays are faster and easier to perform, certain factors can influence the quality and validity of the data. Soluble cytokine receptors in clinical samples can affect the recognition of cytokines by the immunoassay. Some cytokines are biologically active in dimeric or trimeric forms, whereas others can spontaneously form oligomers that are only active in the monomeric form. The presence of various binding proteins like human serum can affect the recognition of cytokines. Different means of preparing or storing the samples can alter the validity of the cytokine concentrations. Lastly, the type of immunoassay used can influence the validity of the data. Measuring exact levels of cytokines in biological samples and cell supernatants, especially on an absolute scale, is very difficult. There are, however, immunoassay kits that allow one to make valid comparisons among experimental treatment groups within a study.

Mitogen-stimulated lymphocyte proliferation is a widely used means of measuring *ex vivo* responses because it is believed to be a reliable measure of lymphocyte function *in vivo*. Using this type of study, T and B cells are activated by mitogens, which are molecules capable of activating lymphocytes in a non-antigen-specific manner. Most T cells can be stimulated by the mitogens phytohaemagglutinin (PHA) and Con A. However, direct comparisons are difficult to make because of variability among studies. In addition, it now appears that the results of *ex vivo* lymphocyte proliferation measurements are strongly influenced by the cell culture conditions, especially the nature of the serum used. For example, rats or mice fed diets rich in (n-6) PUFA, such as maize, soybean, safflower or sunflower oils, have

suppressed *ex vivo* mitogen-stimulated proliferation of spleen lymphocytes, compared to rats or mice fed diets rich in saturated fatty acids (Kollmorgen et al. 1979), (Erickson et al. 1980), (Levy et al. 1982), (Locniskar et al. 1983), (Morrow et al. 1985), (Marshall and Johnston 1985), (Kramer et al. 1986), (Olson et al. 1987), and (Calder 1995). There are other studies that oppose this data, indicating no effect of dietary (n-6) fatty acid supplementation on T-cell proliferation (Cathcart et al. 1987) or enhanced T-cell proliferation *ex vivo* (Ossman et al. 1980). The differences between these studies can be attributed to different levels of fat fed, duration of feeding, and cell culture conditions used when assessing T-cell proliferation *ex vivo*.

In vitro tests are similar to *ex vivo* tests. T-lymphocyte proliferation in response to mitogen, cytokine production, Tc cell activity, and NK cell activity can be assessed. T-lymphocytes have receptors for PGE_1 and PGE_2 and these compounds have been shown to suppress T-lymphocyte proliferation, T-cell mediated cytotoxicity, IL-2 production and NK cell activity *in vitro* (Calder 1996).

Viable cells are needed to perform these immunologic tests. Fortunately, obtaining viable cells to evaluate the immune system is relatively easy. Immune cells can come from solid organs as well as body fluids. The primary lymphoid organs include the thymus and bone marrow. Secondary lymphoid organs include the spleen, lymph nodes, Peyer's patches, tonsils, skin, and gut. Dissociating lymphoid tissue can be done with little difficulty since the cells are not bound together by tight junctions or large amounts of connective tissue. To obtain cells with reasonable viability, one

simply crushes or teases the organ to release large amounts of lymphocytes (Field 1996).

The most common population of immune cells sampled are those that come from peripheral blood. Mononuclear cells (lymphocytes and monocytes) and neutrophils can be obtained by cardiac puncture in rodents or by taking samples from large veins in bigger animals (and humans). The cells are then separated by density centrifugation through Ficoll-Hypaque gradients using commercially available solutions (Field 1996).

After obtaining immune cells, the easiest and most common way to count and examine their morphology, and to check for microbial contamination or presence of debris, is by use of a hemocytometer under a light microscope. If the counts are not needed right away, then the cells may be sent to a clinical laboratory for counting. Cells are often counted using a Coulter counter. This procedure uses an electronic particle counter, which can discriminate cells on the basis of volume (amount of resistance) as they pass through an electrical potential. Generally, this procedure is slower and less feasible than using the hemocytometer because it requires more cells and it means that the cells must be resuspended in different media (Field 1996).

Not only is yield important but so is the viability of the cells. Dead cells are hard to distinguish from viable ones. The quickest way to determine if a cell is viable is through dye-exclusion tests. For example, when cells are stained with trypan blue, counting the number of cells that have not taken up the dye assesses viable cells. Only dead cells will take up the dye (permeate the membrane). Other more descriptive dyes

include fluorescein diacaetate, which is used in combination with a fluorescent microscope. Another method for detecting live cells involves the use of flow cytometry. Cells are incubated with propidium iodide, which is a DNA stain (Field 1996). Only the viable cells take up the propidium iodide dye.

Mechanisms by which Fatty Acids May Affect Immune Cell Function

The following has been reviewed by (Calder 1996). The roles of fatty acids in cell function are diverse. Fatty acids may affect membrane phospholipids, eicosanoid production, and signal transduction pathways. These in turn affect receptors, enzymes, and cytokines, all of which affect cells of the immune system.

Changing the types of fatty acids alters membrane phospholipids, which in turn affects the fluidity of cell membranes. The fatty acid composition of membrane phospholipids and the fluidity of cell membranes affect cell membrane activities such as ion and substrate transport, receptor functioning and membrane-bound enzyme activity.

Some phospholipids are involved in intracellular signaling mechanisms, e.g., phosphatidylinositol-4,5-bisphosphate and phosphatidylcholine. They act as precursors for second messengers such as inositol-4,5-triphosphate and diacylglycerol. Changing the fatty acid composition of certain phospholipids may alter the signal transduction process because the phospholipid's ability to act as a substrate for phospholipases is no longer the same (Calder 1996). The activity of intracellular second messengers such as phospholipase A_2 and protein kinase C are also altered by direct action of unsaturated

fatty acids (Calder 1996). Because changing the types of fatty acids available to cells alters the composition of the membrane phospholipids, this may lead to changes in immune cell function, i.e., lymphocyte or macrophage function.

Eicosaniods are oxygenated derivatives of arachidonic acid and EPA. Eicosanoids include prostagladins (PG) and thromboxanes, which together are termed prostanoids, and leukotrienes. Usually the primary precursor for these compounds is arachidonic acid. The eicosanoids produced from arachidonic acid seem to be more biologically potent than those formed from EPA. The precursor PUFA, e.g., arachidonic acid or EPA, is derived from membrane phophatidylcholine by the action of phopholipase A_2 or from membrane phophatidylinositol-4,5-bisphosphate by the action of phospholipase C and a diacylglycerol lipase. The availability of arachidonic acid, the activities of phospholipase A_2 and phospholipase C, and the activities of both cyclooxygenase and lipoxygenase enzymes all determine which types of eicosanoids and how much of each type are synthesized.

The main products of the cylcooxygenase pathway are PGA_2, PGE_2, PGI_2 (prostacyclin), $PGF_{2\alpha}$, and thromboxane A_2, collectively called prostaglandins and thromboxanes. In general, these compounds have a short half life, e.g., as short as 30 seconds for PGI_2 and thromboxane A_2 released from platelets. They act upon the cell from which they were released. These products of the cyclooxygenase pathway are produced after cells are stimulated by such substances as cytokines, growth factors, endotoxin, zymosan, free radicals, antigen-antibody complexes, bradykinin, collagen, and thrombin. Once produced these biologically active products then turn around and

modify the response to the stimulus. It is possible that the actions of different prostanoids can cancel each other out by having opposite effects. For example, thromboxane A_2 increases platelet aggregation whereas PGI_2 inhibits platelet aggregation.

EPA and DHA, both (n-3) PUFA, competitively inhibit oxygenation of arachidonic acid via the cyclo-oxygenase pathway. In addition, EPA (but not DHA) is able to act as a substrate for both cyclo-oxygenase and 5-lipoxygenase. Ingestion of fish oils that contain (n-3) PUFA results in decreased levels of membrane arachidonic acid. In addition, there is a decrease in the capacity to synthesize eicosanoids from arachidonic acid.

The eicosanoids produced from EPA often have different biological properties from their analogues produced from arachidonic acid. EPA gives rise to the 3-series prostaglandins and thromboxanes and the 5-series leukotrienes. Thromboxane A_3 derived from EPA is less active than thromboxane A_2 derived from arachidonic acid in regards to aggregating platelets and constricting blood vessels. Leukotriene B_5 derived from EPA is less active than leukotriene B_4 derived from arachidonic acid in regards to chemotaxis and aggregation of human neutrophils. PGE_3 derived from EPA is less potent of an inhibitor of lymphocyte proliferation than PGE_2, which is derived from arachidonic acid. However, PGI_3 is as active as PGI_2 in inhibiting platelet aggregation and in promoting vasodilation.

Changes in the types and/or amounts of eicosanoids produced may lead to changes in the expression of cell surface molecules via cytokine production. Cytokines

are soluble protein mediators produced by cells after being activated by specific stimuli. Cytokines can act either on the cells that produced them (autocrine) or on cells away from those that produced them (paracrine). High concentrations of PGE_2 inhibit production of TNF by macrophages *in vitro*. This is likely caused by increased levels of cyclic AMP. On the other hand, low concentrations of PGE_2 appear to stimulate cyclic GMP production and lead to ehanced TNF-α levels. Inhibiting the production of prostaglandin *in vivo* leads to increased amounts of circulating TNF and IL-6 during acute endotoxemia in humans (Spinas et al. 1991).

Reports indicate that IL-1 production is also inhibited by PGE_2, yet some reports condradict this (Endres et al. 1991) and (Scales et al. 1989). Conversely, leukotriene B_4 and leukotriene C_4 enhance *in vitro* IL-1 production by macrophages and monocytes, yet again there are some contradictory reports. IL-6 production *in vitro* is enhanced by leukotriene B_4 (using human monocytes). T lymphocytes, have receptors for PGE_1 and PGE_2 and these compounds suppress IL-2 production *in vitro*. In summary, PGE_2 suppresses production of TNF, IL-1, IL-6 and IL-2 whereas the 4-series leukotrienes enhance the production of these cytokines (Calder 1997).

Sanderson et al. (1995) proposed that the immunomodulatory effects of dietary lipids may result from decreased expression of key lymphocyte surface molecules. The mechanisms by which diets containing PUFA alters the expression of cell surface markers is not known. Decreased PGE_2 may lead to changes in the expression of certain cell surface molecules via cytokine production as discussed above. Therefore, the precise effect of feeding fatty acids upon production of macrophage-derived

cytokines may relate to an altered balance in production of PGE_2 and leukotriene B_4 and/or the different capacities of the different types of cells to produce prostaglandins and leukotrienes (Calder 1997).

(n-3) Fatty Acids and their Effects on Cells of the Immune System

The effects of fatty acids on lymphocyte proliferation have been studied since the 1970's. These studies have used both saturated and unsaturated fatty acids in their protocols. Most studies agree that inhibition of lymphocyte proliferation is dependent, in part, on the degree of unsaturation of the fatty acid along with the chain length. Early studies suggested that arachidonic acid caused the greatest inhibition, while later studies rate EPA and arachidonic acid as being equal. While some studies have shown that saturated fatty acids can inhibit proliferation of lymphoctyes, saturated fatty acids cause less inhibition than unsaturated fatty acids (Calder 1996).

The proliferation of lymphocytes, both *in vivo* and *in vitro*, depends on the presence of various cytokines. The most important cytokine for proliferation of lymphocytes is the production of IL-2. It appears that fatty acids suppress proliferation of lymphocytes by inhibiting the production of IL-2. This has been demonstrated by Calder and Newsholme (1992b). They reported a 40 to 50 % decrease in the concentration of IL-2 in culture medium when rat lymph-node lymphocytes were stimulated with unsaturated fatty acids compared to saturated fatty acids. Furthermore, Richieri et al. (1990) showed that oleic, linoleic, α-linoloenic and arachidonic acid

inhibited the extracellular release of the contents of the granules which are responsible for target-cell killing by rat spleen cytotoxic T-lymphocytes (CTL). This suggests that these unsaturated fatty acids should inhibit CTL activity. (n-3) PUFA inhibit lymphocyte proliferation, however, this is not mediated by eicosanoids (Santoli et al. 1990), (Calder and Newsholme 1992b), (Kumar et al. 1992), and (Soyland et al. 1993). EPA, DHA, and α-linolenic acid (ALA), all (n-3) PUFA, suppress IL-2 production by rat or human lymphocytes.

It is also known that fatty acids directly affect the function of monocytes and phagocytic cells. Adding fish oil to monocytic cells in culture inhibits their proliferation (Watson et al. 1990). It has been shown that (n-3) PUFA influence the production of cytokines by cultured monocytic cells and human peripheral blood monocytes *in vitro* (Baldie et al. 1993). Other studies report that EPA and DHA both activate superoxide production by human neutrophils. DHA also enhances the response of two known neutrophil agonists (N-formyl-L-methionyl-L-leucyl-L-phenylalanine and phorbol ester) (Poulos et al. 1991). Opposing this data, Chen et al. (1994) reports that EPA and DHA significantly suppress phorbol ester-stimulated superoxide generation by human neutrophils. DHA inhibits the IFN-γ-stimulated tumoricidal action of murine peritoneal macrophages (Dustin et al. 1990). Of the two, DHA is a better inhibitor than EPA. The effects of DHA are not due to changes in the amount or type of eicosanoids produced (Khair-el-Din et al. 1995).

The effects of feeding (n-3) PUFA *ex vivo* have also been studied. There have been numerous studies that report that feeding rapeseed, linseed, or fish oil to

laboratory animals (such as rats, mice, chickens, and rabbits) suppresses the response of spleen lymphocytes to mitogenic stimuli, including Con A, PHA and pokeweed mitogen. Some studies (Yaqoob et al. 1994a), (Yaqoob and Calder 1995), and (Sanderson et al. 1995) have reported that feeding rats a diet containing 200 g/kg fish oil for 10 to 12 weeks resulted in a marked suppression of Con A- and PHA-stimulated spleen, thymus, lymph node and peripheral blood lymphocyte (PBL) proliferation *ex vivo*. However, the fish oil diet did not affect the proportions of T-, B-, CD4+, or CD8+ cells or macrophages in the spleen, thymus, lymph nodes or blood of the rats. Interestingly, the fish oil lowered the proportions of spleen lymphocytes bearing the IL-2 receptor (IL-2R), the proportion of thymic lymphocytes bearing the IL-2R and transferrin receptor, and the proportion of lymph-node lymphocytes bearing the transferrin receptor after Con A stimulation (Yaqoob et al. 1994a). Sanderson et al. (1995) noted spleen lymphocytes from animals fed this diet also showed a 60% lower level of expression of the IL-2R following Con A stimulation.

As far as lymphocyte-mediated cytolysis is concerned, CTL activity is significantly diminished after feeding an (n-3) PUFA-rich diet to mice or chickens (Fritsche and Johnstone 1990) and (Fritsche and Cassity 1992). Spleen NK cell activity is decreased when young mice are fed a diet containing 100 g/kg fish oil for 6 weeks compared to feeding chow or maize oil, which are (n-6) PUFA. However, there was no difference in NK cell activity when the fish oil was fed to older mice (Meydani et al. 1988). Other studies find similar results of decreased spleen NK activity after feeding (n-3) PUFA versus other oils or (n-6) PUFA. The same holds true for lymphokine-

activated killer cells. The activity of these cells was diminished if the animals were fed a fish oil-rich diet (Berger et al. 1993) and (Yaqoob et al. 1994b).

Cytokine production by macrophages is also influenced by dietary (n-3) PUFA. Because cytokine production by macrophages is regulated by eicosanoids and because dietary lipids affect eicosanoid production, one would expect that these lipids, especially (n-3) PUFA, would affect cytokine production. As with most studies, there are reports that (n-3) PUFA enhance (Chaet et al. 1994) TNF production *ex vivo*, while others claim decreased (Renier et al. 1993) or no effect (Hubbard et al. 1994) following fish oil feeding. Studies also show that dietary fish oil increases IL-6 production in rats (Tappia and Grimble 1994) and increases IL-1 production. Again, there are contradictory reports. The reason for the conflicting data may be attributable to variations in experimental protocols. Studies differ in regards to species of animals studied, anatomical site of origin of the cells, state of activation of the cells, stimulus used, nature of culture conditions, level of fish oil in the diet, duration of feeding, and methods used to quantify cytokine concentrations (Calder 1996).

Diets containing (n-3) PUFA also affect lymphocyte-derived cytokines. Relatively few studies have been conducted on this subject but three studies using humans as the model have shown that supplementation of the diet with (n-3) PUFA decreases *ex vivo* IL-2 production by peripheral blood lymphocytes (Endres et al. 1993), (Gallai et al. 1995), and (Meydani et al. 1993). Gallai et al. (1995) also reported decreased production of IFN-γ *ex vivo*. There has been one animal study that showed decreased production of IL-2 (*ex vivo*) following linseed oil or fish oil feeding.

In contrast, Fernandes et al. (1994) found that feeding 100 g/kg fish oil resulted in enhanced *ex vivo* IL-2 production by spleen lymphocytes stimulated by Con A. Another report indicated no significant effect of feeding 200 g/kg fish oil to weanling mice on *ex vivo* IL-2 or IFN-γ production after Con A stimulation (Yaqoob and Calder 1995).

Lastly, we will look at *in vivo* effects of feeding (n-3) PUFA. Because (n-3) PUFA decrease the amount of arachidonic acid derived eicosanoid, it would be expected that (n-3) PUFA would exert anti-inflammatory activities. However, Yoshino and Ellis (1987) showed that feeding rats EPA (500 mg/kg/day) and DHA (333 mg/kg/day) did not affect either antigen-induced inflammation of the air pouch or carrageenan-induced inflammation of the footpad. This was despite a significant reduction of the pro-inflammatory eicosanoids PGE_2 and LTB_4. Another study (Lefkowith et al. 1990) in rodents showed that dietary (n-3) PUFA inhibited neutrophils from penetrating the peritoneal cavity after intraperitoneal injection of zymosan. Other researchers, however, have reported different findings. Reddy and Lokesh (1994) found that feeding rats 100 g/kg fish oil for 10 weeks significantly lowered (40%) the inflammatory response to carrageenan injection in the footpad. Nakamura et al. (1994) showed that rats fed high-fat diets containing 20 g/kg EPA or DHA as ethyl ester resulted in a 50% reduction in swelling of the footpad in response to the carrageenan injection. The results were the same for both (n-3) PUFA.

The *in vivo* response to endotoxin was measured by Mascole et al. (1988) when guinea pigs were force fed an emulsion rich in fish oil. Survival after an intraperitoneal

injection of LPS was enhanced in those guinea pigs fed the fish oil diet compared to those fed safflower oil. These same workers found that feeding fish oil (145 g /kg body weight) to guinea pigs for 6 weeks significantly increased survival after intraperitoneal injection of LPS (Mascoli et al. 1989). In addition to enhancing survival against the lethal effects of endotoxin, Mulrooney and Grimble (1993) reported that feeding 100 g/kg fish oil for 8 weeks to weanling rats significantly decreased the responses to intraperitoneal TNF-α. These responses included a rise in liver and plasma C3 concentrations, a fall in plasma albumin concentration and an increase in liver, kidney, and lung protein synthesis. In addition, the fish oil diet also diminished the pyrogenic and anorexic effects of IL-1 and TNF-α (Mulrooney and Grimble 1993) (Hellerstein et al. 1989).

The DTH skin response is another test that can be measured *in vivo*. There are several reports that show the DTH response in rodents is significantly reduced after feeding diets rich in (n-3) PUFA (Yoshino and Ellis 1987) (Kelley et al. 1989). Taki et al. (1992) reported a suppressed DTH response to sheep erythrocytes in mice following tail-vein injection of emulsions of triacylglycerol rich in EPA or DHA. Kelley et al. (1991) and Meydani et al. (1993) showed that supplementing the diet of humans with (n-3) PUFA diminished the DTH response to seven recall antigens. Wander et al. (1997) also showed a significant suppression of the DTH skin test in dogs fed an enriched (n-3) fatty acid diet.

Ex vivo studies have shown that (n-6) fatty acids are not as good at suppressing immune cell activity as the (n-3) PUFAs. Olson et al. (1987) showed that feeding

weanling mice 50 or 200 g/kg soybean oil for nine months resulted in suppression of spleen CTL activity with the cells from the 200 g/kg group having the lowest activity. Erickson (1984) also found that feeding mice 80 or 200 g/kg safflower oil for four weeks decreased spleen CTL activity. Fritsche and Cassity (1992) found that feeding chickens 70 g/kg fish oil or linseed oil significantly suppressed spleen CLT activity compared to feeding chickens diets containing either lard (saturated fatty acid) or maize oil (n-6 PUFA).

Another report by Erickson and Schumacher (1989) found no effect on spleen NK-cell activity when mice were fed 50 or 200 g/kg palm oil (saturated fatty acid) or safflower oil (n-6 PUFA). Likewise, Leung and Ip (1986) reported little effect on NK activity of spleen cells in mice when fed 200 g/kg maize oil (n-6 PUFA). In addition, Fritsche and Johnston (1989) showed no difference in rat spleen NK-cell activity when fed diets rich in maize (n-6) or linseed oil. There are, however, contrasting studies. Morrow et al. (1985) reported that feeding weanling mice 90 g/kg lard or maize oil decreased spleen NK-cell activity compared to feeding 10 g/kg maize oil. In addition, the 90 g/kg lard diet was more suppressive than the 90 g/kg maize oil diet. In conjuction with this study, Meydani et al. (1988) found that feeding young mice a diet containing 100 g/kg fish oil for six weeks resulted in a larger decrease in spleen NK-cell activity compared to feeding chow or 100 g/kg maize oil. Yaqoob et al. (1994b) reported that NK-cell activity was lowest in weanling rats when fed 200 g/kg fish oil for10 weeks as compared to 200 g/kg hydrogenated coconut, olive, safflower, or evening primrose oil. Based on results from the above mentioned studies, little or no

effect was seen on NK-cell activity when either saturated fatty acids or (n-6) fatty acids were given. On the other hand, fish oil appeared to decrease NK-cell activity.

There have been a number of studies that show some saturated fatty acids inhibit lymphocyte proliferation. However, the majority of these studies concluded that saturated fatty acids cause less inhibition than unsaturated fatty acids (Mertin and Hughes 1975), (Calder et al. 1991), (Calder 1992), (Calder and Newsholme 1992a), (Calder et al. 1992), (Soyland et al. 1993), and (Rotondo et al. 1994). There have also been a number of reports indicating no inhibition of proliferation after feeding diets containing lauric and myristic acids (Weyman et al. 1975), (Weyman et al. 1977), (Weyman et al. 1975), (Tsang et al. 1977), (Calder et al. 1991), (Calder et al. 1992), (Calder 1992), (Calder and Newsholme 1992a), and (Soyland et al. 1993). In addition, there appears to be some general agreement that stearic acid is the most potent inhibitor of lymphocyte proliferation amongst the saturated fatty acids, causing a degree of inhibition similar to that of oleic acid (Calder et al. 1991), (Calder et al. 1992), (Calder 1992), (Calder and Newsholme 1992a), and (Soyland et al. 1993). Therefore, among the saturated fatty acids, the order of potencies appears to be:

lauric = myristic < palmitic < stearic

The Effects of α-tocopherol on Immune Function

α-Tocopherol is very important for regular body functions including protecting cell membranes from oxidative damage. It is a constituent of all cellular membranes

and is found in high concentration in the membranes of immune cells because they are at especially high risk for oxidative damage (Coquette et al. 1986), (Hatman and Kayden 1979), and (Machlin 1991). α-Tocopherol is essential for normal immune system function. In fact, vitamin E deficiency leads to a diminished ability of the immune system to respond to infectious microorganisms, to produce a DTH response, and to mount an antibody response to an antigen (Scott 1980), (Tengerdy 1990), and (Jeejeebhoy 1994).

Determining the proper amount of vitamin E needed for ideal immune function depends on how it interacts with other antioxidants and pro-oxidant nutrients, especially PUFA, and on other factors that modulate the immune response such as age and stress (Meydani and Tengerdy 1991) and (Eskew et al. 1986). The conventional methods for determining the recommended dietary allowance (RDA) are adequate for the amount of vitamin E needed to prevent clinical deficiency symptoms, but may not be adequate for the optimal level of vitamin E required to maintain immunological health.

The DTH reaction is a good way to evaluate immune function. Tests show that supplementation with vitamin E above the RDA can increase the DTH response in some situations. This can be readily seen in elderly persons and older laboratory animals (Meydani and Tengerdy 1991) and (Meydani et al. 1986). Old mice supplemented with vitamin E (500 ppm) for 30 days had a significantly higher DTH response to 2,4-dinitrofluorobenzene than mice fed the RDA (Meydani et al. 1986). The same types of results were seen in elderly persons who were supplemented with 88

mg vitamin E for 30 days. Also, long-term supplementation proved useful in increasing the DTH response (Meydani et al. 1997).

There have been many studies that looked at vitamin E and its effects on the immune response, especially antibody production. In general, there is little effect on specific antibody responses when domestic animals are deficient in vitamin E. However, domestic animals supplemented with vitamin E have potentiated antibody responses to many different organisms administered as either killed vaccine preparations or experimental infections. Some of the various studies include chickens showing an increase in agglutination antibodies (total IgG1, IgG2 and IgM) from *E. coli* (both live and vaccine) (Heinzerling et al. 1974) and (Tengerdy and Brown 1977), pigs showing an increase in both primary and secondary antibody responses to *E. coli* vaccine (Ellis and Vorhies 1976), and sheep showing increased primary antibody response to KLH (Rittacco et al. 1986).

The CMI responses involving lymphocyte functions are very sensitive to changes in vitamin E levels (Beharka et al. 1997). The level of vitamin E influences proliferation of lymphocytes in response to mitogens *in vitro*. Several different studies have shown that animals deficient in vitamin E show a depressed lymphocyte mitogenic response to T cell mitogens (Bendich 1990), (Eskew et al. 1985) and (Beharka et al. 1997). However, this effect can be reversed following vitamin E supplementation.

The addition of vitamin E above the RDA enhances mitogenesis in mixed populations of lymphocytes from both humans and animals compared to controls (Meydani and Tengerdy 1991). Moriguchi et al. (1990) reported that there is a

corresponding increase in rat splenic lymphocytes in response to Con A when the dietary intake of vitamin E is increased from 100 to 2500 mg/kg (a pharmacological level of vitamin E). In addition, Corwin and Shloss (1980) found that lymphocytes from mice supplemented with varying levels of vitamin E showed enhanced proliferation to Con A when the dietary levels were in excess of the vitamin content of normal chow.

Vitamin E also has an effect on cytokine production. IL-2 production can be influenced by the different levels of vitamin E in the diet. Meydani et al. (1986) showed that older mice fed diets containing 500 ppm vitamin E had enhanced IL-2 production compared to mice fed a diet containing 30 ppm. Similar results were found in elderly humans. Elderly humans supplemented with 800 IU/day of dl-α-tocopherol for 30 days showed increased Con A-induced IL-2 production versus nonsupplemented subjects (Meydani et al. 1990).

Other cytokines effected by vitamin E include TNF-α and IL-6 secreted by mononuclear phagocytes. Both humans and mice with acquired immunodeficiency syndrome (AIDS and MAIDS, respectively) have shown elevated levels of IL-6 and TNF-α as the disease progresses (Wang et al. 1995), (Boue et al. 1992) and (Wang et al. 1993). When mice were supplemented above the murine requirement for vitamin E, it appeared to lower the retrovirus-induced production of IL-6 and TNF seen in these mice (Wang et al. 1993) and (Wang et al. 1994). In other studies by Wang et al. (1993) and Wang et al. (1995) it was shown that mice infected with MAIDS and supplemented with vitamin E at 15 to 450 times the National Research Council (NRC)

recommendations had normalization of IL-6 and TNF-α production by spleenocytes during progression of the disease at all levels of supplementation.

The consumption of dietary alcohol (ethanol) decreases production of IL-6 and TNF-α (Beharka et al. 1997). Wahl et al. (1989) reported that in mice fed ethanol for 10 weeks and then supplemented with 15 times the NRC-recommended levels of vitamin E, the production of IL-6 by Con A-stimulated spleenocytes and IL-6 and TNF-α by LPS-stimulated spleenocytes was restored.

Vitamin E also affects phagocytic cell responses. In studies where rats were fed vitamin E deficient diets, they exhibited impaired macrophage chemotaxis, reduced ability to ingest complement-coated beads, and decreased protection from auto-oxidative damage (Rocha 1989). Supplementing with dietary vitamin E relieved endotoxin-induced inhibition of monocyte migration and phagocytosis in rats (Hill et al. 1983), reduced immunosuppressive cytokine production by macrophages in mice, and normalized monocyte chemotaxis in humans with diabetes (Yano et al. 1994). The ability of alveolar macrophages to phagocytize opsonized sheep red blood cells increased with increasing concentration of vitamin E. In conjunction with this, Moriguchi et al. (1990) showed a fivefold increase in phagocytosis in rats fed the diet with the highest vitamin E content compared to rats in the control group (Moriguchi et al. 1990). Yano et al. (1994) also demonstrated that alveolar macrophages from rats supplemented with vitamin E showed enhanced phagocytosis of opsonized particles.

The mechanism by which vitamin E produces immunostimulatory effects has not been clearly demonstrated (Meydani and Hayek 1992). Some of the effects of

vitamin E can be attributed to its antioxidative activity but clearly other mechanism are involved (Corwin and Shloss 1980) and (Sies 1991). It has been hypothesized that one alternative mechanism is the reduction of immunoinhibitory molecules secreted by immune cells themselves, especially macrophages. Activated macrophages secrete molecules like H_2O_2 (Fisher and Bostick-Bruton 1982) and (Metzger et al. 1980) and PGE_2; these molecules have been shown to depress lymphocyte proliferation (Fisher and Bostick-Bruton 1982), (Rola-Pleszczynski and Lemaire 1985) and (Webb et al. 1980), lymphokine production (Phipps et al. 1991) and the generation of cytotoxic T cells (Plaut 1979). Vitamin E is able to modulate the formation of these potentially immunosuppressive molecules, especially PGE_2. It has also been shown that peritoneal macrophages and splenocytes from mice supplemented with vitamin E produced less PGE_2 after being stimulated than unsupplemented control mice (Meydani et al. 1986) and (Romach et al. 1993). However, more research is needed to fully understand the mechanisms of how vitamin E affects immune cell function.

MATERIALS and METHODS

Animals- Thirty-two healthy female geriatric Beagles were chosen for this study. All of the dogs had been vaccinated for canine distemper, parvovirus, and rabies. The dogs were determined to be free of chronic systemic disease on the basis of physical examination, complete blood count (CBC), serum biochemical evaluations, urinalysis and fecal examination for parasites. For 90 days before the study, all dogs were consuming diets with a low concentration of (n-3) fatty acids compared to the concentration of (n-6) fatty acids (Science Diet Canine Maintenance, Hill's Pet Nutrition, Topeka, KS). The ratio of (n-6) fatty acids to (n-3) fatty acids was 19:8. The dogs were ranked according to body weight and assigned to six groups so that body weights were evenly distributed across all groups. During the course of the study the dogs were housed in indoor runs and fed once daily in the morning. The experimental protocol was reviewed and approved by the Oregon State University Animal Care and Use Committee according to the principles outlined by the National Institutes of Health (NCR 1985).

Diets- Six experimental diets were prepared, by Hill's Pet Nutrition, Topeka, KS, that varied in the amounts of (n-3) fatty acids and α-tocopherol they contained. Sources of (n-3) fatty acids and dl-α-tocopheryl acetate (TA) were added to a basal diet. Three of the diets contained a high ratio of (n-6) fatty acids to (n-3) fatty acids (the low n-3 diet); three contained a low (n-6) to (n-3) ratio (the high n-3 diet). At both of the (n-6) to (n-3) ratios, dl-α-tocopheryl acetate was added at three levels to

provide diets that were low, medium, and high in their vitamin E content. The dl-α-tocopheryl acetate composition and the physical characteristics of the dogs on each of the six diets are given in **Table 2**.

The basal diet ingredients by weight included 54.8% water, 20.3% turkey, 15.0% corn, 4.5% pork liver, 2.0% soy meal, 1.0% beet pulp, and 0.4% vitamin and mineral premixes. Rice hulls were used as the carrier for the vitamin premix, which contained 25,000 μg/kg cholecalciferol, 7500 mg/kg nicotinic acid, 5000 mg/kg calcium *D*-pantothenate, 21,770 mg/kg thiamine mononitrate, 1250 mg/kg riboflavin, 2,431 mg/kg pyridoxine hydrochloride, 250 mg/kg folic acid, 50 mg/kg biotin and 50 mg/kg vitamin B-12. Calcium carbonate was used a the carrier for the mineral mix, which contained 80 g/kg zinc oxide, 6.0 g/kg manganese as manganese oxide, 280 g/kg iodine as calcium iodate, 1.0 g/kg cobalt as cobalt carbonate, 180 mg/kg selenium as selenium selenite and 2.5 g/kg copper as copper chloride. The remaining 2% of the diet was provided by added oil. The source of oil for the (n-3) enriched diets was fish oil (Zapata Protein, Reedville, VA). The source of the oil for the (n-6) diets was corn oil (Mazola, Englewood Cliffs, NJ). The nutrient composition by weight was 77.4% moisture, 5.8 % protein, 4.5% fat, 1.3% ash, 0.6% crude fiber and the remainder carbohydrate. The fatty acid composition of the six diets is given in **Table 3**.

Study design- Dogs were fed their respective diets for 120 days. Body weight was measured once a week. Energy levels were adjusted so that dogs neither lost nor gained weight. Blood samples (~10 ml) were collected from the jugular vein into

Table 2. Physical characteristics of the Beagles [1] and dl-α-tocopheryl acetate composition of the diets

Dietary (n-3)	Low (n-3)			High (n-3)		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT
n	6	5	5	6	5	5
Age, y	8.6 ± 0.21	8.8 ± 0.20	9.2 ± 0.20	9.3 ± 0.17	8.9 ± 0.24	8.7 ± 0.44
Weight, kg	11.19 ± 0.97	9.93 ± 0.71	10.5 ± 0.95	12.10 ± 1.11	11.10 ± 0.83	11.16 ± 0.72
(n-6):(n-3)[3]	38.4:1	40.6:1	41.0:1	1.29:1	1.31:1	1.48:1
αT, μg/g[4]	17.2	106	446	16.6	95.0	448
αT, μg/g[5]	75.9	465	2010	76.2	436	1870

[1] Values are means ± SEM.
[2] dl-α-tocopheryl acetate.
[3] Woodson-Tenent Laboratories, Inc. (Des Moines, IA).
[4] Oregon State Univeristy Lipid Laboratory analysis; as fed.
[5] Dry matter basis

Table 3. Fatty acid composition of the six experimental diets[1]

Dietary (n-3)	Low (n-3)			High (n-3)		
Dietary αT	Low αT	Med αT	High αT	Low αT	Med αT	High αT
Fatty Acid	g/kg diet					
14:0	0.2	0.2	0.2	1.4	1.4	1.4
16:0	7.3	7.5	7.4	7.6	7.1	8.5
18:0	2.4	2.5	2.5	2.5	2.3	2.7
∑SFA[2]	9.9	10.2	10.1	11.6	10.9	12.7
16:1(n-7)	1.0	1.1	1.1	2.4	2.5	2.7
18:1(n-9)cis	13.9	14.5	14.5	10.2	9.0	11.4
∑MUFA[3]	15.1	15.8	15.8	13	11.8	14.5
18:2(n-6)cis	18.8	19.9	20.1	7.6	7.2	8.8
18:3(n-3)cis	0.5	0.5	0.5	0.6	0.5	0.6
20:4(n-6)	0.4	0.4	0.4	0.5	0.5	0.5
20:5(n-3)	<0.1	<0.1	<0.1	1.9	1.9	1.9
22:5(n-3)	<0.1	<0.1	<0.1	0.4	0.4	0.4
22:6(n-3)	<0.1	<0.1	<0.1	2.5	2.2	2.5
∑PUFA[4]	19.7	20.8	21.0	14.1	13.3	15.3
∑(n-6)[5]	19.2	20.3	20.5	8.1	7.7	9.3
∑(n-3)[6]	0.5	0.5	0.5	6.3	5.9	6.3
(n-6):(n-3)	38.4:1	40.6:1	41.0:1	1.29:1	1.31:1	1.48:1
DBI[7]	40.7	42.9	43.3	47.9	45	50.3

[1] Analysis performed by Woodson-Tenent Laboratories, Inc. (Des Moines, IA).

[2] Sum of the saturated fatty acids: 8:0 + 10:0 + 11:0 + 12:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0 + 22:0 + 24:0.

[3] Sum of the monounsaturated fatty acids: 14:1 + 15:1 + 16:1(n-7) + 17:1 + 18:1(n-9)cis + 18:1(n-7) + 18:1(n-9)trans + 20:1(n-9) + 22:1(n-9) + 24:1.

[4] Sum of the polyunsaturated fatty acids: 18:2(n-6)trans + 18:3(n-6)cis + 18:3(n-6) + 18:3(n-3)cis + 18:4(n-3) + 20:2(n-6) + 20:3(n-6) + 20:3(n-3) + 20:4(n-6) + 20:4(n-3) + 20:5(n-3) + 21:5(n-3) + 22:2(n-6) + 22:4(n-6) + 22:5(n-6) + 22:5(n-3) + 22:6(n-3).

[5] Sum of the (n-6) fatty acids.

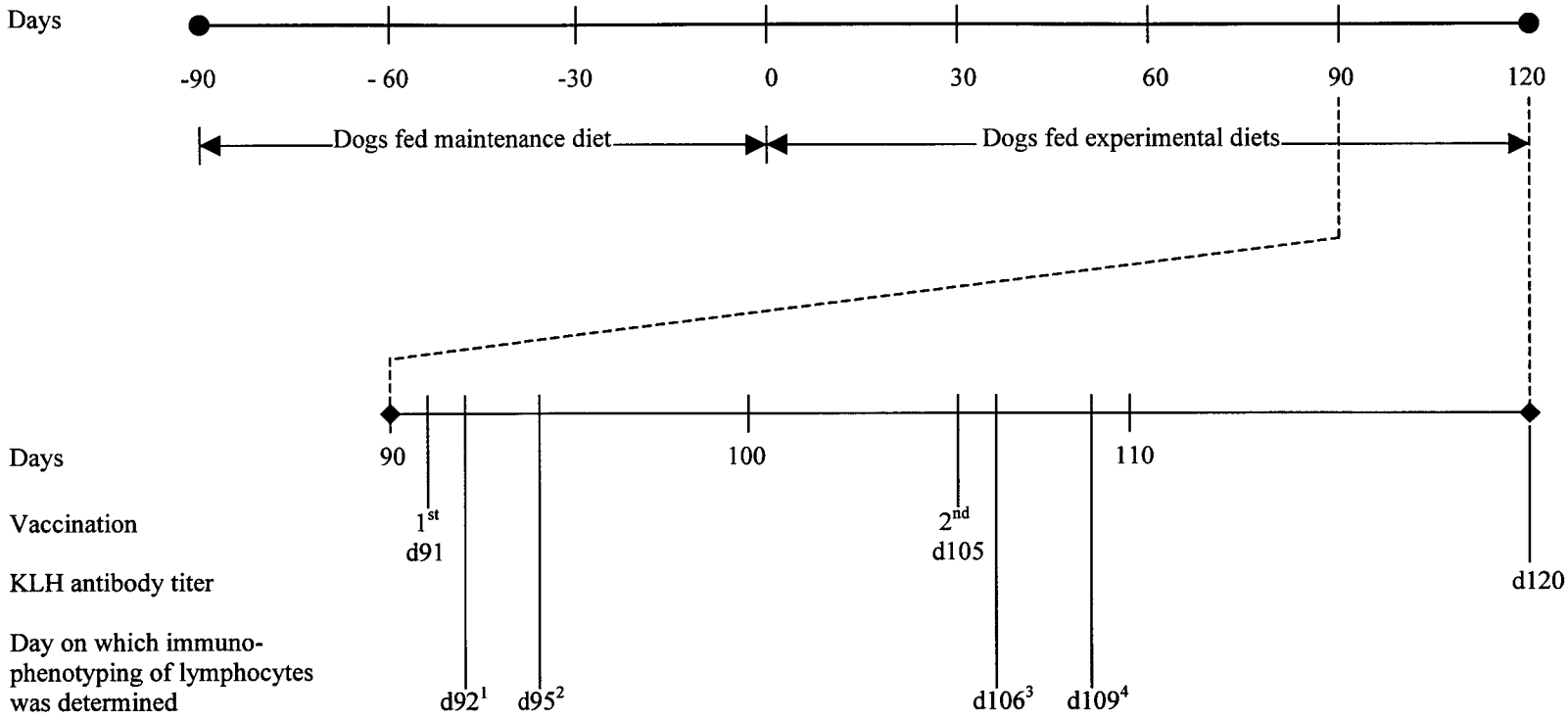
[6] Sum of the (n-3) fatty acids.

[7] DBI (double bond index) = 2[18:2(n-6) + 20:2(n-6)] + 3[18:3(n-3) + 20:3(n-6)] + 4[20:4(n-6)] + 5[20:5(n-3) + 22:5(n-3)] + 6[22:6(n-3)], where the concentration of each fatty acid is expressed as g/kg fatty acids.

evacuated tubes for serum, or tubes containing EDTA (0.1 ml of a 15% EDTA solution in a 10 ml tube) for plasma, after food was deprived for 24 h. This blood was used for used for biochemical and *in vitro* and *ex vivo* immunologic measurements, before beginning the study and after 82 days. All dogs were bled on the same day in the morning. The feeding trial was continued for an additional 38 days (for a total of 120 days) during which time *in vivo* immune response studies were performed. On the 91st day the dogs were vaccinated with KLH and blood was taken on days 92 and 95. A second "booster" vaccination was given two weeks after the first vaccination on day 105 and blood was collected 1 and 4 days (days 106 and 109) after the booster vaccination. On day 120 the KLH antibody titer was determined. This time line is given in **Figure 7**.

Biochemical Measurements

Plasma fatty acid profile- This was determined by gas chromatography as previously described (Wander et al. 1997) using heptadecanoic acid as the internal standard. In short, 15 µl of internal standard (17:0) was added to a 100 x 10 mm test tube with a teflon cap and blown dry using N_2. To this test tube, 0.2 ml of plasma was added and 0.8 ml saline to make a final volume of 1 ml. Then 3.75 ml of a 2:1 methanol/chloroform mixture was added to the test tube, which was then placed on a shaker (Labquake, Fisher Scientific, Pittsburgh, PA) for 1 hour. After shaking, the test tube was centrifuged at 2500 rpm for 10 min and the supernatant transferred to a 120 x 10 mm test tube to be used later. The residue in the original test tube was extracted



[1] One day after the first vaccination, the day on which CD69 expression on CD4 and CD8 positive lymphocytes was determined.
[2] Four days after the first vaccination, the day on which IL-2R expression on CD4 and CD8 positive lymphocytes was determined.
[3] One day after the second vaccination, the day on which CD69 expression on CD4 and CD8 positive lymphocytes was determined. The DTH skin test was also performed on this day.
[4] Four days after the second vaccination, the day on which IL-2R expression on CD4 and CD8 positive lymphocytes was determined.

Figure 7. Timeline for experimental protocol

using 1 ml distilled water and 3.75 ml of the methanol/chloroform mixture, then centrifuged again for 10 min. The supernatant from this spin was added to the supernatant from the first spin. To the supernatant test tube, 2.5 ml distilled water and 2.5 ml chloroform were added and the test tube was centrifuged for 10 min. At this point, two fractions were visible; the top fraction was removed using a water aspirator. The lower fraction (chloroform layer) was withdrawn using a pasteur pipette and placed into another 100 x 10 mm test tube and dried under nitrogen. After the solvent was completely evaporated, 0.2 ml benzene and 1 ml of boron trichloride was added to the tube, which was then filled with a nitrogen cap. The tube was then placed in a 95 °C heating block for 90 min. After heating, the test tube was removed and allowed to cool to room temperature. Once cooled, 5 ml of distilled water and 5 ml hexane were added and the tube was vortexed for 2 min. Following the mixing, the test tube was centrifuged at 1500 rpm for 10 min. Again, two fractions were visible. The top layer (hexane) was transfered to a 125 x 16 mm test tube. Three ml of hexane was added to the first tube, which was then mixed for 30 sec, and centrifuged at 1500 rpm for 10 min. The hexane layer (top) was combined with the first hexane layer that had been removed. Sodium sulfate (0.3 g) was added to these combined extracts and the tube was vortexed for 45 sec. After mixing, the extract was transferred to a 100 x 10 mm test tube and the hexane evaporated under nitrogen. The samples were reconstituted in 0.25 ml isooctane and 2 µl was injected into the gas chromatograph. The concentration of the fatty acids was expressed as g/100 g fatty acids.

Plasma thiobarbituric reactive substances (TBARS)- These were measured as previously discussed (Wander et al. 1996). Briefly, 0.5 ml of plasma was placed in a 16 ml centrifuge tube and 2.5 ml of a 20% trichloroacetic acid (TCA) solution was added. A precipitate appeared immediately. The test tube was left to stand for 10 min at room temperature. The test tube was then centrifuged for 10 min at room temperature. The supernatant was discarded and 2.5 ml 0.05 M H_2SO_4 was added, to wash the precipitate, and the test tube was then vortexed and centrifuged for 10 min. Again, the supernatant was discarded and 2.5 ml 0.05 M H_2SO_4 plus 3.0 ml 0.2% thiobarbituric acid (in 2 M Na_2SO_4) was added to the precipitate and the test tube was placed in a boiling water bath for 30 min. The test tube was then placed in a cold water bath. n-Butyl alcohol (4.0 ml) was added to the extract. The resulting chromogen formed and the test tube was vortexed. The organic phase was separated by centrifugation for 10 min and its absorbance was determined at a wavelength of 530 nm.

Plasma α-tocopherol- This was determined by HPLC using a fluorometric detector as discussed previously (Wander et al. 1997). In short, α-tocopherol was extracted into hexane, dried, resuspended in methanol, then injected into a C18, reversed-phase column (250 X 4.6 mm, 5 μm CLS-ODS, Shim-pack, Shimadzu). The column was fitted with a precolumn filter (0.5 μm frit, A-318; Upchurch Scientific , Oak Harbor, WA), the temperature was maintained at 35 °C, and the flow rate for 100% methanol was 1.5 ml/min. The excitation wavelength was 292 nm; the emission wavelength was 330 nm. δ-Tocopherol was used as the internal standard.

Plasma total lipids, cholesterol and triglycerides- These were measured separately from the serum biochemistry profile, using techniques previously discussed (Wander et al. 1996). The sum of cholesterol and triglycerides was taken as an estimate of total lipid (Wander et al. 1996).

Serum biochemistries- Serum was frozen and later analyzed using a Roche FARA II system (Roche, Inc., Somerville, NJ).

Urinalyses- These were performed on the same day the urine was collected by a certified medical technologist. The urine was collected by placing each dog into a metabolism cage with a clean, sterile beaker underneath the cage for urine collection. Urine was obtained as a "free catch" from the beaker, or if the dogs did not urinate on their own, cystocentesis was performed.

Complete blood count (CBC) and white cell differential- The CBC was determined using a hematology analyzer (Baker 9010, Serono-Baker Instrument, Inc., Allentown, PA). The white cell differential was determined by a certified medical technologist upon microscopic examination of blood smears after Wright-Giemsa staining.

Immunologic Measurements

Delayed-type hypersensitivity (DTH) skin test- A DTH skin test was performed on day 106 as described below. The DTH skin test is an *in vivo* indicator of specific cell-mediated immune responsiveness by T cells and is measured as swelling and induration following an intradermal challenge. Dogs were initially sensitized with a

KLH (Calbiochem-Behring Diagnostics, La Jolla, CA) suspension administered intramuscularly (500 μg of KLH emulsified in 1.0 mg of T1501 adjuvant for a total volume of 0.5 ml) 91 days after the initiation of the feeding (first vaccination). The KLH and adjuvant were combined in an oil-water emulsion as described by Woodard (1989), except that the ingredients were sonicated instead of ground. In short, 1.0 g/L KLH, 50 ml/L hexadecane, 35 ml/L Tween 80, 15 ml/L Span 80 and 2 ml/L T1501 adjuvant were emulsified and added to normal saline solution. Fourteen days later (day 105) a second 0.5-ml intramuscular injection was given (the second or "booster" vaccination). One day later (day 106) the intradermal skin test was performed. To accomplish this, a large rectangular patch was gently clipped on the lateral side of the chest of each dog. Individual disposable tuberculin syringes were filled with heat-aggregated KLH. Heat aggregated KLH is needed for the DTH skin test unlike that which was used for vaccination. The vaccination does not require heat aggregation. Saline (0.9%) was used as the negative control. Histamine base (0.1 g/L) was used as a positive control (Histatrol, Center Laboratories Port Washington, NY). A 25-gauge needle was used to inject 0.05 ml of each of these (KLH, positive and negative controls) intradermally. The 0.05-ml dose of heat-aggregated KLH consisted of ~3 mg of KLH. The KLH was heat aggregated according to the method of Exon et al. (1990). Briefly, 120-mg of soluble KLH was added to 6 ml of sterile, normal saline solution and then heat aggregated in an 80 °C water bath for 1 h. The resultant gel was centrifuged twice at 400 x g for 10 min, removing the saline layer each time. The gel

was then dispersed by passing it through a 23-gauge needle once, and through a 25-gauge needle twice, carefully avoiding formation of air bubbles.

The sites of injection were marked with a felt marker. No chemical restraint was used or needed for the dogs. Examinations were made at 15 and 30 min, and at 24, 48, 72, and 96 h after intradermal injections. Reactions were recorded according to the diameter of induration and degree of erythema. A reaction larger than the negative control was considered to be a positive reaction. If a positive reaction to the saline control was observed (it was for all the dogs at 15 and 30 min), the diameter of its induration was subtracted from the other positive reactions. However, by 24 h no reaction to the saline control was noted. Histamine produced an induration typically 20 mm larger than the saline control at 15 min, after which the reaction subsided. These controls ruled out trauma or the volume of substance injected as the cause of the DTH response. The same person administered the test to all dogs.

Quantitation of PGE_2 from stimulated mononuclear cells- Peripheral blood mononuclear cells were isolated according to the methods of Coligan et al. (1992) and Krakowka et al. (1987). In short, cells were separated from a 1:1 dilution of blood and Dulbecco's phophate-buffered saline (DPBS; Sigma Chemical, St. Louis, MO) by layering the blood-DPBS mixture over Histopaque 1077 (Sigma) and centrifuging 30 min at 900 x g. Cells were washed twice in Hank's balanced salt solution (HBSS, Sigma) and resuspended in RPMI 1640 supplemented with 100,000 U/L penicillin, 100 mg/L streptomycin, 2 mmol/L L-glutamine and 10% fetal calf serum (Sigma). An aliquot of the cell suspension was used to count cells with a Coulter counter (Coulter

Electronics Inc., Hialeah, FL), and cell viability was assessed by trypan blue exclusion. Trypan blue was added to the aliquot of cells and allowed to stand for 2 min. Following this, 20 µl of this mixture was added to a counting chamber for viewing under a light microscope. If cells stained blue, they were considered non-viable. The remaining mononuclear cells were centrifuged for 10 min at 400 x g and resuspended in RPMI 1640 containing 5% fetal calf serum for a final concentration of 2×10^9 cells/L.

Mononuclear cells (5 ml of 2×10^9 cells/L suspension) were transferred to 25-ml tissue culture flasks (Corning, Corning, NY) and incubated for 4 h at 37 °C in an atmosphere of 95% air and 5% CO_2. Nonadherent cells were decanted and the adherent cells were washed twice with 5 ml RPMI 1640 (no fetal calf serum) to remove any residual nonadherent cells. Five milliliters of RPMI 1640 (5% fetal calf serum) containing 30 mg/L LPS (*E. Coli* 055:B5; Sigma) was added to the adherent cells (mainly macrophages), which were then incubated for 40 h at 37 °C in 95% air and 5% CO_2. Previous timed-incubation studies showed maximal production of PGE_2 at 40 h. The supernatant was clarified by centrifugation for 10 min at 1000 x g and filtered (0.45 m filter) (UNIFLO- Plus; Scheicher and Schuell, Keene, NH). Cell-free supernatant was stored at -70 °C for subsequent PGE_2 analysis. A flask containing medium only was processed identically and supernatant was harvested for use as a control. PGE_2 concentration was determined by Dr. Ken Allen, Department of Food Science and Human Nutrition, Colorado State University, using a radioimmuno assay (RIA) (Bottje et al. 1993).

Keyhole limpet hemocyanin antibody titer- Dogs were injected intramuscularly with 0.5 ml KLH vaccine, as described above (the same injections as for the DTH skin test), on days 91 and 105. Serum was collected for KLH antibody titer on day 120, 15 days after the booster vaccination was given. The humoral immune response to KLH was measured by a modification of an indirect ELISA procedure previously described by Woodard (1989). In short, microtiter plates (ICN Biomedicals, Horsham, PA) were coated with 0.1 ml of Dulbecco's phosphate-buffered saline (DPBS; Sigma Chemical, St. Louis, MO) (pH 7.4; 0.01 mol/L) containing 5 mg/L of KLH (which adheres to the microtiter plate), covered with parafilm to prevent evaporation, and refrigerated overnight or until needed. Before use, plates were inverted to remove excess coating buffer and washed three times with DPBS containing 0.05% Tween 20. Serum samples (in quadruplicate) were then placed into wells and serial 1:4 dilutions made. To start the dilutions, 10 µl of sera was added to 990 µl of PBS with Tween 20; this makes a 1:100 dilution. To each of the dilution wells on the microtiter plate, 150 µl of Tween 20 was added. Then 50 µl of the 1:100 dilution was added to the first row of dilution wells making a 1:400 dilution. Fifty µl of the 1:400 dilution was added to the second row making a 1:1600 dilution. This was continued four more times making a final dilution of 1:409,600. The final volume in each well was 0.05 ml.

Two positive and two negative control wells were included on each plate. The positive control was serum from a dog vaccinated in a previous experiment. The negative control was serum from a non-vaccinated animal. Coated plates containing serum samples were incubated for 1 h at 37 °C while rotating on a platform at 120 rpm.

Plates were washed three times with DPBS-Tween 20 to remove unbound antibody. Antibody against dog IgG was conjugated with alkaline phosphatase (Jackson ImmunoResearch Laboratories, West Grove, PA) and diluted 1:5000 with DPBS-Tween 20. Subsequently, 0.1 ml was added to each well for a 1-h incubation at 37 °C. Plates were washed three times with DPBS-Tween 20. Alkaline phosphatase (p-nitrophenyl phosphate disodium $\cdot 6H_2O$; 1 g/l; Sigma) was then added to each well (0.15 ml/well). After addition of substrate, plates were allowed to incubate at room temperature until the mean absorbency of the two positive controls equaled 1.0. Because not all plates developed color at the same rate, a positive control serum was used rather than a specifically timed incubation. Otherwise, data obtained from different plates and days could not be compared statistically. After allowing the positive controls to reach a mean absorbance of 1.0, the entire plate was read at two wavelengths, 405 and 492 nm, on a MR 700 spectrophotometer (Dynatech Laboratories, Alexandria, VA). Two wavelengths were used because one was a reference wavelength (492 nm) and the other was a test wavelength (405 nm). Both a minimum and a maximum absorbance were recorded and the minimum was subtracted from the maximum. The results were expressed as the log of the titer.

Isolation of peripheral blood mononuclear cells for analysis of surface-marker expression by flow cytometric analysis- Peripheral blood mononuclear cells were isolated according to the methods of Coligan et al. (1992), which were modified as follows. Cells were separated from a 1:1 dilution of blood and Dulbecco's phosphate-buffered saline (DPBS; Sigma) by layering the blood-DPBS mixture over Histopaque

1077 (Sigma) and centrifuging 30 min at 900 x g at 4°C. Cells harvested from the mononuclear cell layer were washed in three times the volume of DPBS and centifuged 10 min at 400 x g. The resultant pellet was resuspended in approximately 0.5 ml DPBS with 0.1% sodium azide (Sigma) and 1.0 % bovine serum albumin (Sigma). (This combined solution is referred to as PAB.) The cell concentration was determined using a Coulter ZBI Counter (Coulter Electronics Inc.). A representative aliquot of the 0.5 ml PAB suspension was used to assess purity of the peripheral blood mononuclear cells (conducted by a clinical pathology technician, who stained and viewed the cells under light microscopy for cellular uniformity). Another aliquot of the suspension was used to assess cell viability by trypan blue exclusion. Cells (1×10^6) were added to each well of a 96-well, round bottom microtiter plate (Corning). PAB was added to each well in sufficient volume to allow the antibody and antigen to mix, such that the final volume was 50 to 150 µl.

The appropriate reagents were then added to the wells. The reagents used were fluorescein-labeled monoclonal antibodies for canine CD4 (anti-canine CD4-FITC [CM 12.125]) and CD8 (anti-canine CD8-FITC [CM 1.140]); biotin-labeled monoclonal antibody for CD69 (anti cat/dog B/T activation marker [CM 2.58], Custom Monoclonals, West Sacramento, CA); phycoerythrin-conjugated human recombinant IL-2 (R&D Systems, Minneapolis, MN); and the following immunoglobulins: fluoroscein-labled mouse IgG_1 (kappa), biotin-labeled mouse IgG_1 (kappa), and phycoerythrin-labeled rat IgG_{2a} (kappa) (all from PharMingen, San Diego, CA). The above mentioned immunoglobins were negative controls for the above mentioned

positive monoclonal antibodies and were isotypically-matched controls for non-specific fluorescence. Streptavidin-Spectral Red (Southern Biotechnology Associates, Inc., Birmingham, AL) was added as the second-step reagent for biotin-labeled monoclonal antibodies.

The canine CD4 and CD8 monoclonal antibodies were isolated by the methods of Gebhard and Carter (1992) and supplied to Custom Monoclonals. We then purchased these monoclonal antibodies from Custom Monoclonals. Canine IL-2 was detected using human IL-2 as reported by Somberg et al. (1992).

The amount of immunologic reagents used in each test was determined from recommendations of manufacturers and preliminary titration experiments. After the reagents were added, the microtiter plate was vortexed and the cells incubated in the dark for 30 min on ice. After incubation, 125 µl of PAB was added to each well. The plate was gently vortexed, and then centrifuged at 210 x g for 3 min. Then cells were washed once more with 125 µl of PAB and then resuspended in 175 µl of PAB. Cells were then filtered into a flow vial and rinsed with an additional 300 µl of PAB. At this time (just prior to sorting by flow cytometry), cell viability was confirmed by propidium iodide staining. Using this method, any cells that stained positive for propidium iodide were considered dead or damaged. The cell suspensions were evaluated immediately by flow cytometric analysis as discussed below.

Fluorescent bead assay- Mononuclear cells were isolated as described above for the PGE_2 assay in order to measure the amount of fluorescent bead engulfment. The mononuclear cells that remained from the PGE_2 assay were suspended in 20 ml of

RPMI-10, placed in 75 cm^2 tissue culture flasks (Corning, Corning, NY), and incubated for 4 hours at 37 °C, 5% CO_2. After incubation the supernatant was decanted and the adherent cells (mainly macrophages) were rinsed with 10 ml DPBS. These cells were then harvested with 15 ml Eagle's minimum essential medium (Gibco) plus 10% fetal calf serum (EMEM-10) and centrifuged for 10 min at 400 x g. The supernatant was discarded and the cells resuspended in 1 ml EMEM-10. Cells were then counted and 1 x 10^6 cells placed in a 1.5 ml microcentrifuge tube. An appropriate volume of beads (63 µl) was added to the suspension which resulted in a final bead to macrophage ratio of 25:1. The final volume in the tubes was adjusted to 1 ml with EMEM-10. The cells were incubated on a Labquake (Fisher Scientific) for 2 h at 37 °C. Fluorescent bead engulfment was assayed using an EPICS V flow cytometer (Coulter Electronics Inc.,).

Analysis of surface-marker expression by flow cytometry- Multicolor flow cytometric analysis was performed using an EPICS V flow cytometer (Coulter Electronics Inc.). All data were collected by listmode acquisition and analyzed using WinList software (Verity Software House, Inc., Topsham, ME).

The percentages of T cells stained with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies for CD4 (or CD8) in the mononuclear cell population were determined by subtracting the FITC-only isotypic control values from these values. The percentages of the FITC-positive CD4 (or CD8) T cells that were phycoerythrin-IL-2R positive or biotin-CD69 positive were determined by setting the region on the isotypic controls for nonspecific staining at an average of approximately 5% on each analysis date.

Four histogram sets (designated CD4+, CD4-, CD8+, and CD8-) were produced for each dog on each sample date for data analysis. Included here are two representative histogram sets (CD4+ and CD4-) for dog number 1 on the April 10, 1997 examination date (see **Figure 8**). The "CD4+ histogram set" was stained for CD4, IL-2R, and CD69; the "CD4- histogram set" was stained for CD4 only (as well as the negative isotypic controls for IL-2R and CD69). Therefore in reality, both the CD4+ and CD4- histogram sets are positive for CD4, but only the CD4+ set is positive for IL-2R and CD69. (The isotypic control for CD4 also was examined, but not included in these histograms.)

Values for the different surface markers (for example, using the data for dog number 1 on the April 10, 1997 examination date), were determined as follows: 1) The CD4 positive cells were determined from region 2 (R2) of both the CD4+ and CD4- histogram sets by adding the value from each set, then dividing by 2, such that an average number of CD4 positive cells was obtained (24.8 + 29.2 = 54 divided by 2 = 27% CD4 positive cells) (see **Figure 8**). 2) The IL-2R value was calculated from region 3 (R3) by subtracting the CD4- value from the CD4+ value (5.7 - 3.4 = 2.3% IL-2R positive cells) (see **Figure 8**). 3) The CD69 value was calculated from region 5 (R5) in the same manner as the IL-2R value (23.1 - 3.3 = 19.8) (see **Figure 8**). 4) The above process was repeated with the CD8+ and CD8- histogram sets for the percentage of CD8 positive cells, and the percentages of the CD8 positive cells that were also expressing IL-2R and CD69.

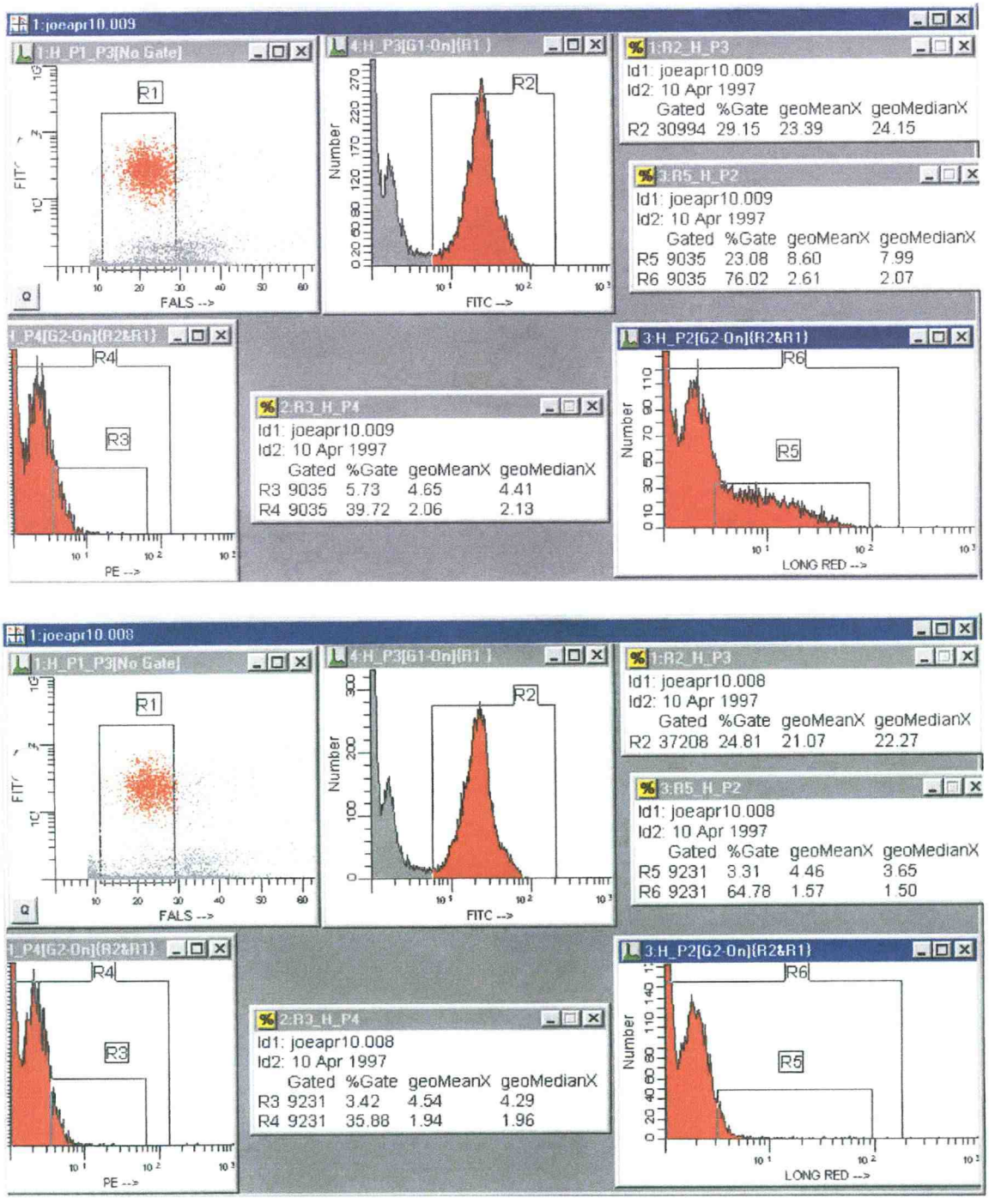


Figure 8. Representative flow cytometry histograms of CD4 cell surface markers. Histogram set "joeapr10.009" is from dog number 1 on April 10, 1997 and has positive stains for CD4, IL-2 and CD69 (designated "CD4+"). Histogram set "joeapr10.008" is also from dog number 1 on April 10, 1997 and has a positive stain for CD4, but negative isotypic controls for IL-2 and CD69 (designated "CD4-").

Kinetics of canine Il-2R and CD69 expression- The expression of canine IL-2R and CD69 surface markers on CD4 cells following KLH vaccination were determined in separate studies. CD69 is considered an early activation marker, thus, it shows up quickly after cells have been stimulated. IL-2R is slower to appear and does not show up until several days after stimulation.

Two mini experiments were performed in our laboratory (not associated with this study) to determine which day(s) each of the markers appeared. In the first mini study, IL-2R expression was assessed by flow cytometry on days 0 through 5 after the booster injection of KLH (Hall et al. 1999). Since IL-2R expression peaked on day 4 following KLH sensitization, IL-2R expression was assessed on day 4 post KLH vaccination in this study. In the second mini study, CD69 expression was assessed by flow cytometry on days 0 through 4 after the booster injection of KLH in two dogs not used in the present study. The kinetics for CD69 expression were less definitive. CD69 reportedly is a very early activation marker, which diminishes or disappears after 1 or 2 days of activation (Sandor et al. 1995) and (Hartnell et al. 1993). The percentage of CD4 positive cells expressing CD69 was marginally higher on day 1 after KLH sensitization, maintained an elevated level of expression through day 3, and returned to baseline on day 4. Therefore, CD69 and IL-2R expression on CD4 positive and CD8 positive cells were assessed on days 1 and 4 post KLH vaccination (days 92 and 106 for CD69 and days 95 and 109 for IL-2R) in all dogs in this study.

Statistical Analysis

A two-factor ANOVA, with the two levels of dietary (n-6):(n-3) fatty acid ratio and the three levels of α-tocopheryl acetate as the two factors, was used to determine significant differences among the six diets for the values at the start of the study, the end of the feeding trial (day 82) and the first and fourth days after the first (days 92 and 95) and second (days 106 and 109) vaccinations. To determine the effect of diet on the dependent variables the difference between day 82 and day 0 was calculated. To determine if the response to vaccination differed among the dogs fed the different diets, the differences between day 82 and the first and fourth days after the first vaccination (days 92 and 95) and after the second vaccination (days 106 and 109) were calculated. Although data was collected for all time points for all these variables, the fourth day after vaccination was used for CD4+ with IL-2R, and CD8+ with IL-2R determinations, and the first day after vaccination was used for CD4+ with CD69 and CD8+ with CD69. If there was a significant interaction, cell means, not main effect means, were compared. The cell means are each of the individual numbers for the six different diets and main effect means are a composite of either the two levels of (n-3) fatty acids or the three levels of α-tocopheryl acetate. If the interaction was a result of the intake of α-tocopheryl acetate, the three cell means at each level of (n-3) fatty acid intake were compared using a 1-way ANOVA. If the interaction was a result of the ratio of (n-6):(n-3) fatty acids then the two cell means in the low, medium, and high α-tocopheryl acetate columns were compared as if they were separate experiments. If there was not a significant interaction, main effect means were compared. If there was

a main effect of the level of α-tocopheryl acetate in the diet, a protected least-square difference (LSD) post-hoc test was used to compare means. If the main effect was the result of the ratio of (n-6):(n-3) fatty acids then a protected least-square difference (LSD) post-hoc test was used to compare means. To look at the effect of vaccination at each of the time points after vaccination, the same procedure was followed using differences between day 82 and the vaccination date. This allowed us to determine at which time point(s) the significant difference(s) occurred.

All other statistical tests (not involving flow cytometric analysis) were done using a 2-way ANOVA comparing day 0 to day 82, taking the initial data and comparing it to the final data (day 82). Values were considered significant at $P \leq 0.05$. A general linear model procedure (SAS 6.12 Cary, NC) was used for the statistical analyses.

RESULTS

The fatty acid composition of the plasma at the beginning of the study was similar for all the dogs regardless of the projected dietary intervention. After feeding the six diets that contained different (n-6):(n-3) fatty acid ratios and different levels of α-tocopheryl acetate for 82 days, the fatty acid composition changed significantly. There was not a significant interaction between the fatty acid ratio and level of α-tocopheryl acetate in the diet for any of the individual fatty acids, nor was there a main effect of α-tocopheryl acetate. However, the sum of the (n-3) fatty acids was higher in the dogs given the diets with the higher amount of (n-3) fatty acids ($p = 0.0001$) as seen in **Figure 9**. This resulted primarily from the 32-fold increase in EPA and the 10-fold increase in DHA. In addition to increases in the level of (n-3) fatty acids in the plasma, the sum of the (n-6) fatty acids decreased ($p = 0.0001$). This occurred mostly because of lower concentrations of linoleic acid and arachidonic acid in the dogs consuming the high (n-3) fatty acid diet. The average linoleic acid concentration for all dogs fed the high (n-3) diet was 21.38 ± 1.02 g/100 g fatty acids, which was lower than that in all dogs fed the low (n-3) fatty acid diet (29.07 ± 1.05 g/100 g fatty acids,). The concentration of aracahidonic acid was significantly higher for the dogs fed the low (n-3) fatty acid diet (23.97 ± 0.82 g/100 g fatty acids) compared to the dogs fed the high (n-3) fatty acid diet (14.25 ± 0.77 g/100 g fatty acids).

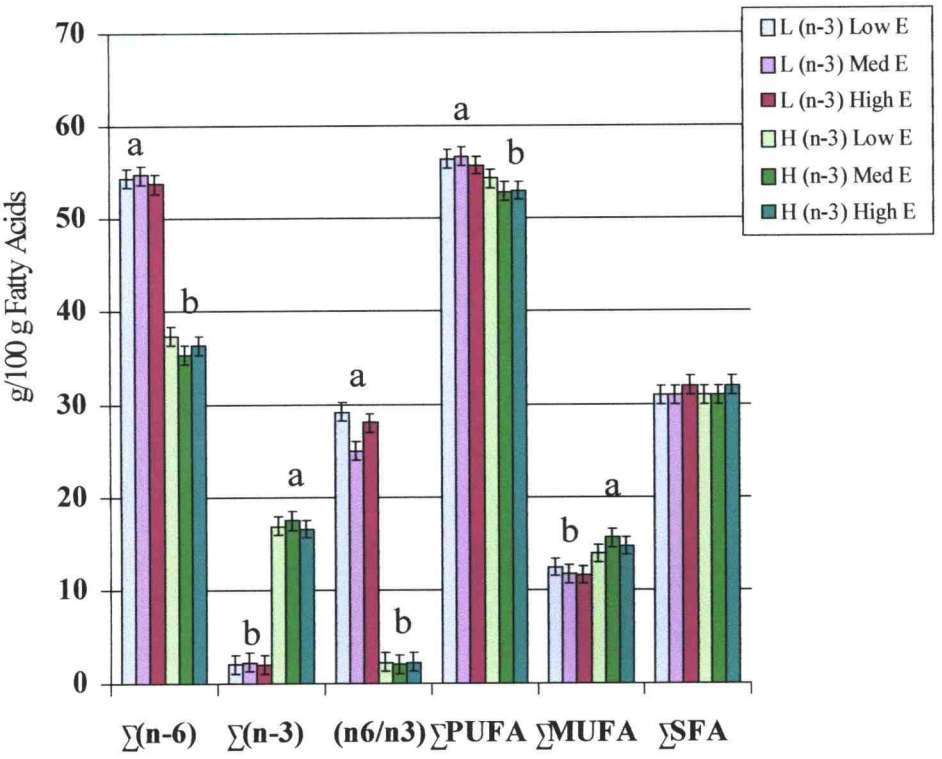


Figure 9. Effects of diets differeing in the amount of (n-3) fatty acids and α-tocopheryl acetate on the concentration of fatty acids in plasma of geriatric Beagle dogs. Each bar represents the g/100 g fatty acids concentration (mean ± SEM) after dogs had consumed the respective diets for 82 days. Within a group of fatty acids, bars with different letters above them are significantly different ($p \leq 0.05$).

The concentration of saturated fatty acids was not influenced by either the dietary (n-6):(n-3) fatty acid ratio or the level of α-tocopheryl acetate. The concentration of MUFA and PUFA was not influenced by the dietary α-tocopheryl acetate level but was by the (n-6):(n-3) fatty acid ratio. The sum of the MUFA

increased ($p = 0.007$) and the sum of the PUFA decreased ($p = 0.003$) in the dogs fed the high (n-3) fatty acid diets.

The plasma fatty acid profile data were also expressed as the difference in the final and initial values. A very similar analysis emerged as occurred for the data obtained at the end of the 82 day feeding interval with the exception that several fatty acids (16:0, 18:1(n-9)*c*, 18:3 (n-3), 20:2(n-6), and 23:0) no longer exhibited significant changes.

There were no significant differences among the plasma α-tocopherol concentrations in the dogs before the study began. There was a significant interaction between the dietary ratio of (n-6):(n-3) fatty acids and dietary α-tocopheryl acetate content on the plasma α-tocopherol values measured at the end of the 82 day feeding trial ($p = 0.02$) (**Figure 10**). This was also true when the data were expressed as the difference between the values measured before and after the feeding trial ($p = 0.01$). This interaction occurred because when the dogs ate the diet with the high amount of (n-3) fatty acids, the plasma α-tocopherol concentration did not increase as much as it did when they consumed the low (n-3) fatty acid diet.

There were no significant differences among the plasma α-tocopherol concentrations when expressed relative to the lipid content of the diets (cholesterol and triglycerides) at the beginning of the study. After consumption of the diets for 82 days, there was an effect of the amount of α-tocopheryl acetate in the diet on the level of plasma α-tocopherol (**Figure 11**). The p value was 0.0001 and the three means were 0.08, 0.11, and 0.19 (all $\pm$ 0.007) for the low, medium and high levels of

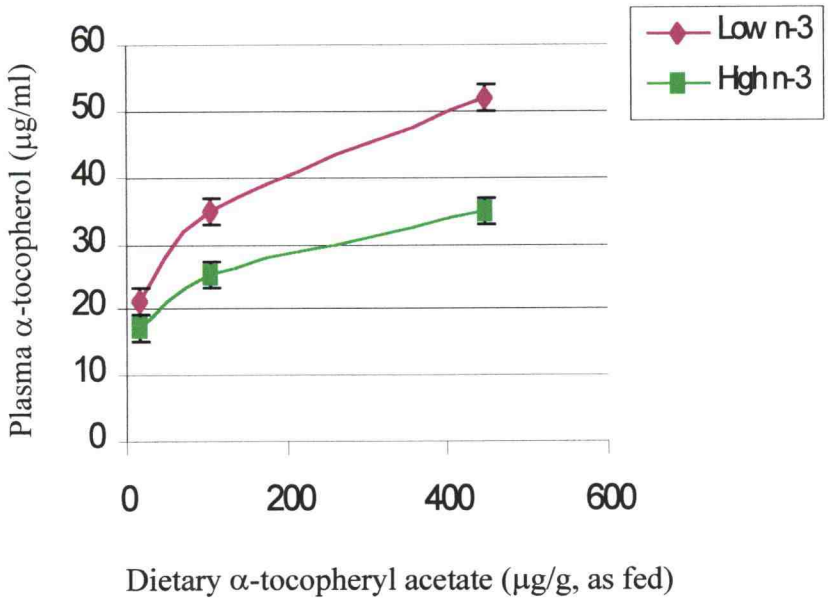


Figure 10. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on plasma α-tocopherol concentrations in Beagle dogs for day 82.

α-tocopheryl acetate, respectively. All three dietary levels were significantly different from one another (p= 0.0051, 0.0001 and 0.0001). Similar results occurred when the data are expressed as the difference between day 82 and day 0. After consumption of the diets for 82 days there was no interaction between the amount of α-tocopheryl acetate in the diet and the dietary ratio of (n-6):(n-3) fatty acids on plasma α-tocopherol. Also, there was no effect of the dietary ratio of (n-6):(n-3) fatty acids on plasma α-tocopherol.

When the data for plasma α-tocopherol concentrations are expressed relative to the double bond index (DBI) of the respective plasma data there is a significant

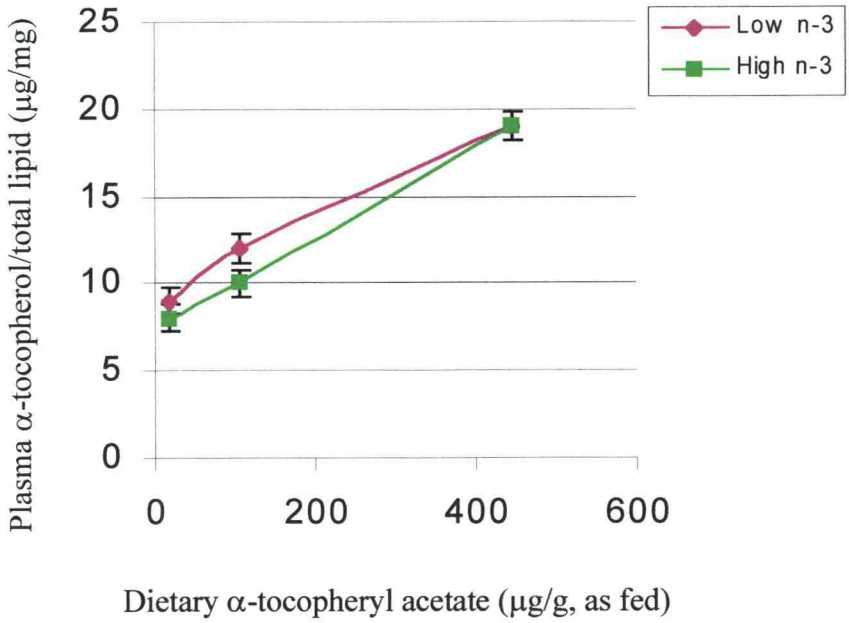


Figure 11. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on plasma α-tocopherol concentrations expressed relative to plasma lipids (cholesterol and triglyceride) in Beagle dogs for day 82.

interaction at the beginning of the study ($p = 0.03$). This interaction is the result of variability among the dogs and did not alter the interpretation of the data. At the end of the feeding trial there was also a significant interaction ($p = 0.0041$) (**Figure 12**). This interaction is caused by the fact that the α-tocopherol concentration of the plasma relative to plasma DBI rose more in the low (n-3) fatty acid diet than it did in the high (n-3) fatty acid diet. Similar results occurred when the data are expressed as the difference between day 82 and day 0.

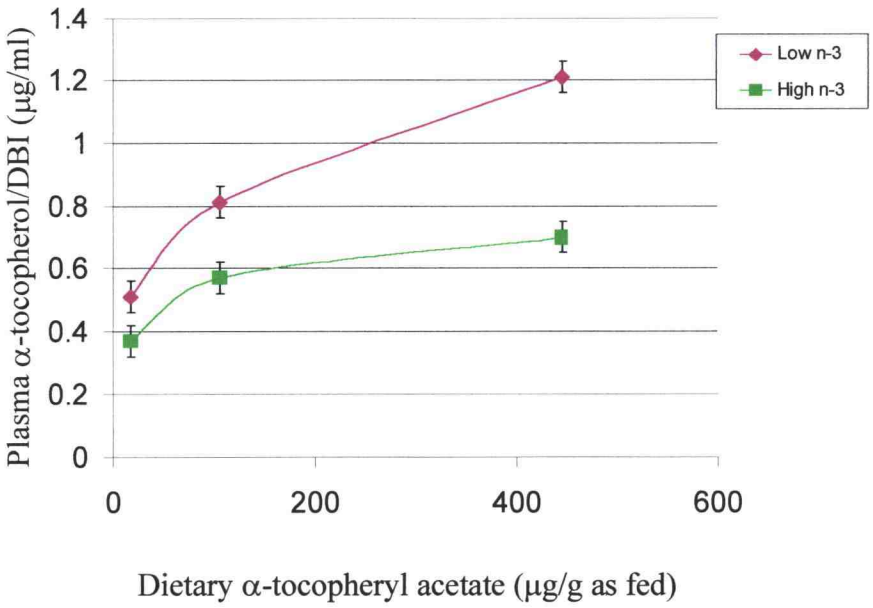


Figure 12. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on plasma α-tocopherol concentrations expressed relative to the double bond index (DBI) in Beagle dogs for day 82.

TBARS concentration was measured to assess lipid peroxidation. There were no significant differences among the dogs consuming the six diets at the beginning of the study for plasma TBARS (**Figure 13**). TBARS concentration was not significantly altered after 82 days of feeding. There was a significant interaction, however, between the amounts of (n-3) fatty acids and α-tocopheryl acetate in the diet when the difference between day 82 and day 0 was calculated ($p = 0.02$). When the low (n-3) fatty acid diets were fed, there was no effect on the amount of TBARS measured regardless of the level of α-tocopheryl acetate in the diets (0.29 ± 0.21, 0.15 ± 0.23, and 0.14 ± 0.23 nmol/ml for the low, medium and high α-tocopheryl acetate,

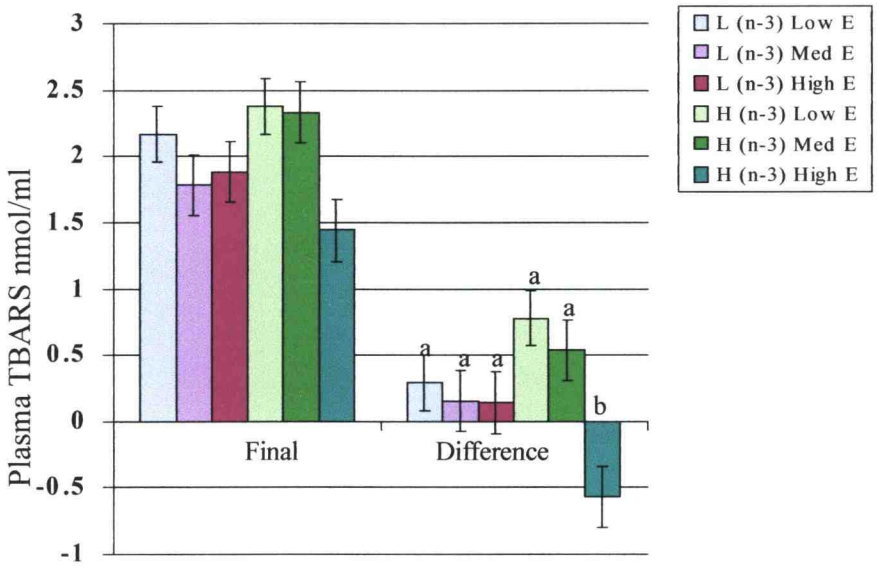


Figure 13. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on plasma TBARS in Beagle dogs for day 82.

respectively). When the high (n-3) fatty acid diets were fed, the amount of TBARS measured decreased as the level of α-tocpheryl acetate increased in the diets (0.78 ± 0.21, 0.54 ± 0.23, and –0.57 ± 0.23 nmol/ml for the low, medium and high α-tocopheryl acetate, respectively).

Prior to starting the dietary intervention, neither the plasma concentrations of cholesterol nor triglyceride differed significantly among the dogs consuming the six different diets. After the 82 day feeding period, there was no interaction between the dietary ratio of (n-6):(n-3) fatty acids and the concentration of α-tocopheryl acetate nor was there a main effect of α-tocopheryl acetate on these two variables. There

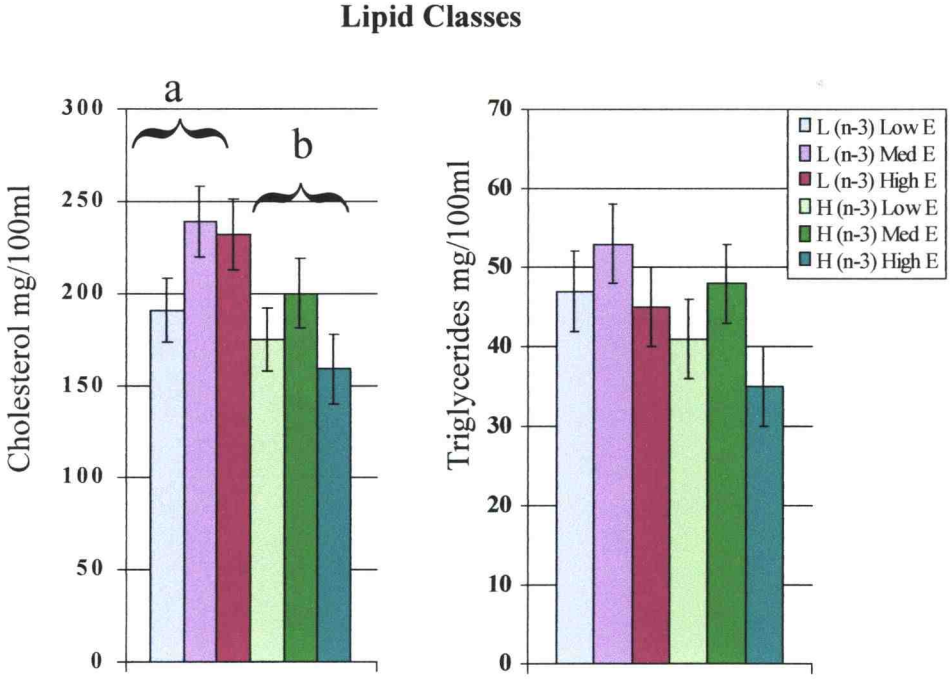


Figure 14. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on plasma lipid concentrations (cholesterol and triglyceride) in Beagle dogs for day 82.

was, however, an effect of the dietary fatty acid content on the plasma concentration of cholesterol (**Figure 14**). After the dietary intervention, the plasma cholesterol concentration was 20% lower in the high (n-3) fatty acid group than in the low (n-3) fatty acid group (220.7 ± 18.5 vs 177.6 ± 18.5 mg/100 ml, $p = 0.008$). The concentration of triglyceride in the plasma was not significantly different after the dietary intervention.

Consumption of the diets had minimal effects on the serum biochemistries. At the beginning of the study there were no significant differences among the dogs consuming the six different diets for any of the serum biochemistries tested. There was

a significant interaction for blood urea nitrogen (BUN) when the difference between day 82 and day 0 was calculated ($p = 0.04$). However, the only dog groups that were significantly different from each other were the dogs consuming the low (n-3) fatty acid/high α-tocopheryl acetate diet and the dogs consuming the high (n-3) fatty acid/high α-tocopheryl acetate diet ($p = 0.02$).

There were no other significant interactions between the ratio of (n-6):(n-3) fatty acids and the level of α-tocopheryl acetate for the dogs fed the six different diets. A significant difference was noted for albumin in both the final (day 82) and the difference between day 82 and day 0 ($p = 0.04$ and 0.05, respectively). Serum albumin concentration in dogs consuming the low (n-3) fatty acid diets were lower than in dogs consuming the high (n-3) fatty acid diets (3.1 ± 0.14 and 3.5 ± 0.14, respectively for day 82). The pattern was the same for the difference in albumin. The same scenario was seen with alkaline phosphatase (ALP). A significant difference was noted for day 82 and the difference ($p = 0.03$ and 0.02 respectively). The effects were opposite this time, however, with the values being lower in the dogs fed the high (n-3) fatty acid diets compared to the dogs fed the low (n-3) fatty acid diets (83.0 ± 25.3 and 163.4 ± 25.3, respectively for day 82 and -1.2 ± 19.2 and 62.3 ± 19.2, respectively for the difference).

CBC and white cell differential counts showed some significant interactions between the ratio of (n-6):(n-3) fatty acids and the level of α-tocopheryl acetate for the dogs fed the six different diets at the beginning of the study. Those that were significant included hemoglobin (HGB) $p = 0.03$, hematocrit (HCT) $p = 0.04$, and red

blood cell count (RBC) $p = 0.05$. These differences were the result of variability among the dogs and not the result of dietary intervention. There were no significant differences between dogs consuming the six diets at the end of the feeding trial for the leukogram and hemogram parameters measured.

When concentrating on just the white blood cell (WBC) and more specifically, the lymphocyte numbers from the CBC panel, there were no significant interactions between the ratio of (n-6):(n-3) fatty acids and the level of α-tocopheryl acetate in the dogs fed the six different diets for any of the time points assessed. There were also no significant differences noted as a result of either the ratio of (n-6):(n-3) fatty acids or the level of α-tocopheryl acetate in the diets on the WBC numbers. There were significant differences seen in the lymphocyte numbers, however. When the difference between day 92 and day 82 was calculated, a significant difference was seen as a result of the level of α-tocopheryl acetate in the diets ($p = 0.05$). The dogs receiving the high level of α-tocopheryl acetate were significantly different from the dogs receiving the low level of α-tocopheryl acetate (1960 ± 300 and 1169 ± 270, respectively). There was also a significant difference noted in lymphocyte numbers (as a result of the level of α-tocopheryl acetate in the diets) when the difference between day 109 and day 82 was calculated ($p = 0.01$). Again, the dogs receiving the high level of α-tocopheryl acetate were significantly different from both the low and medium levels of α-tocopheryl acetate diets (2320 ± 280, 1454 ± 250, and 1937 ± 280, lymphocytes respectively). There was a significant difference seen as a result of the ratio of (n-6):(n-3) fatty acids when the difference between day 95 and day 82 was calculated ($p =$

0.05). In the dogs receiving the high (n-3) fatty acid diets, the lymphocyte numbers were significantly higher compared to the dogs receiving the low (n-3) fatty acid diets (2240 ± 220 and 1690 ± 220, lymphocytes respectively).

The DTH skin test showed no significant interaction between the ratio of (n-6):(n-3) fatty acids and the level of α-tocopheryl acetate in the dogs fed the six different diets at 15 min, 30 min, 24 h, and 48 h (**Figure 15**) ($p = 0.77$, 0.27, 0.87, and 0.45, respectively). At 72 hr and 96 h, however, there were significant differences among the diet groups. There was a significant interaction between the dietary ratio of (n-6):(n-3) fatty acids and the α-tocopheryl acetate levels in the diets for the values measured at these two times ($p = 0.0006$ and 0.003, respectively). The interaction occurred because when the dogs ate diets with the high amount of (n-3) fatty acids, the DTH skin test did not differ to any great extent at the three different levels of α-tocopheryl acetate. However, when they consumed the diets that contained the low amount of (n-3) fatty acids, the DTH skin response was markedly larger in the dogs fed the medium level of α-tocopheryl acetate.

There was no significant difference among any of the dogs consuming the six diets at the beginning of the study for the production of PGE_2 from peripheral blood mononuclear cells stimulated with LPS. After feeding the experimental diets for 82 or 104 days, there were still no significant differences among any of the dogs consuming the six diets.

The humoral immune response was assesed after an initial vaccination and a subsequent booster vaccination by measuring the production of antibody to KLH.

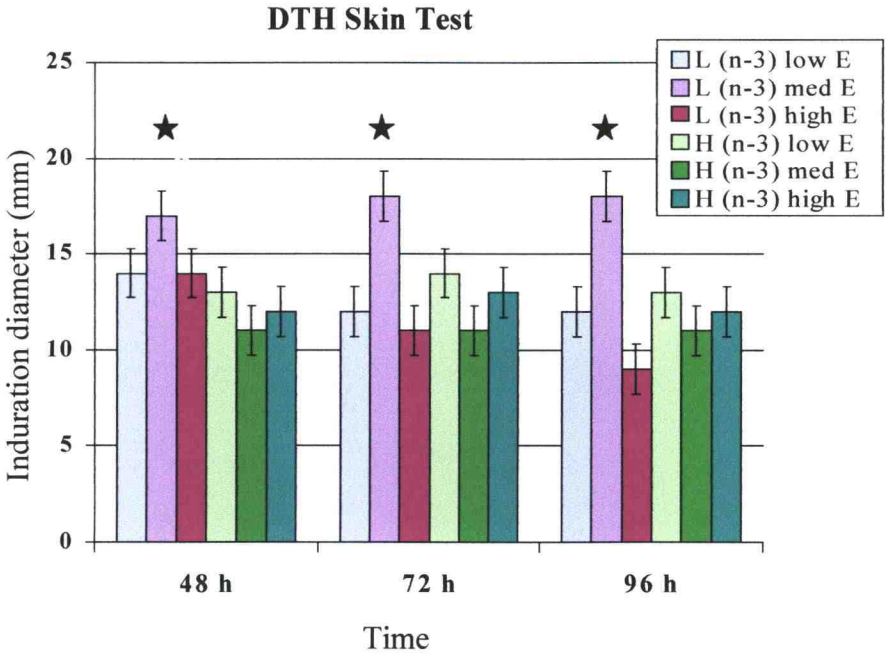


Figure 15. Effect of feeding dogs diets that differed in the (n-6):(n-3) fatty acid ratio and α-tocopheryl acetate (αT) on the delayed-type hypersensitivity (DTH) skin test in Beagle dogs. The dogs were challenged with an intradermal injection of KLH to which they had been previously sensitized. Each bar represents the induration diameter in mm (mean ± SEM) after the dogs had consumed their respective diets for 120 days. Within a time period, bars with a star above them are significantly different ($p \leq 0.05$).

Results are expressed as log titers. There was no significant difference among any of the dogs consuming the six diets (**Figure 16**).

The percent of fluorescent beads engulfed did not differ significantly among the dogs consuming the six diets at the start of the feeding study. After 82 days of feeding fish oil or corn oil and various levels of α-tocopheryl acetate, no significant differences were seen among the dogs consuming the six diets ($p = 0.37$).

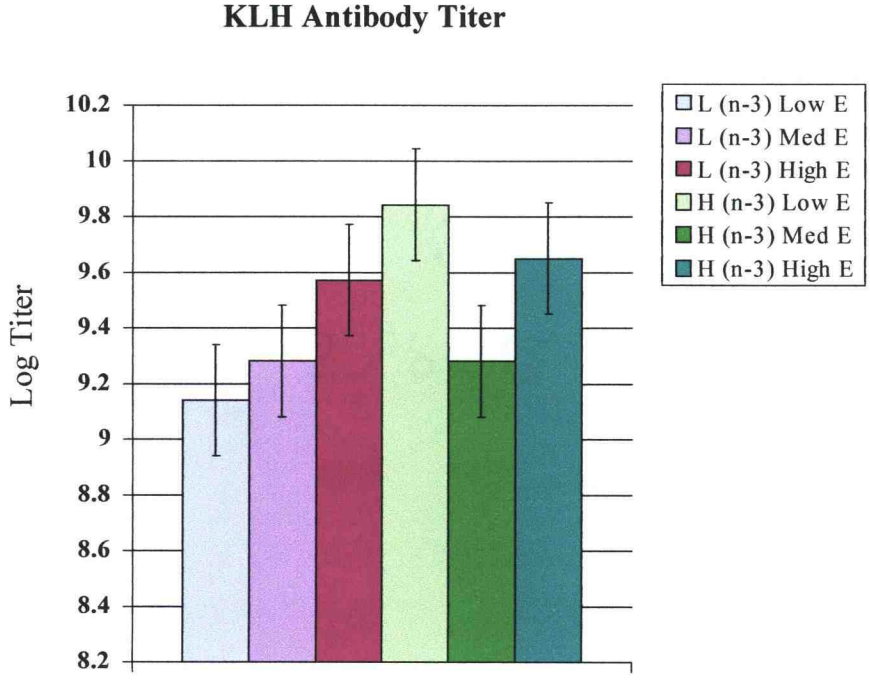


Figure 16. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and α-tocopheryl acetate (αT) on KLH antibody response in Beagle dogs. There were no significant differences among the dogs consuming the six diets.

There were no significant interactions between the two dietary factors in the dogs consuming the six diets for any of the cell surface markers on the two T-cell subpopulations (CD4+ and CD8+ lymphocytes) except for the ratio between the percent of T-cells expressing CD4 to the percent expressing CD8 (four days after the booster vaccination) ($p = 0.05$). This difference was significant between the low (n-3) fatty acid/low α-tocopheryl acetate diet and the high (n-3) fatty acid/low α-tocopheryl acetate diet (-0.3 ± 0.23 and 0.5 ± 0.23, respectively $p = 0.01$). In addition, dogs consuming the high (n-3) fatty acid/low α-tocopheryl acetate diet and the high (n-3)

fatty acid/medium α-tocopheryl acetate diet were also significantly different (0.5 ± 0.23 and –0.3 ± 0.25, respectively p = 0.02).

At the beginning of the study there were no significant differences in the dogs consuming the six diets for the percent of T-cells that expressed CD4. In these dogs, the ratio between the percentage of T-cells that expressed CD4 compared to those expressing CD8 was also not significantly different. The percent of cells positive for IL-2R on the T-cells that expressed CD4 was not significantly different among the dogs consuming the six diets, nor was the percent of cells positive for IL-2R on the T-cells that expressed CD8. The same holds true for the percentage of CD4+ and CD8+ T-cells that expressed CD69. There was a significant difference in lymphocytes expressing CD8 on day zero, which was caused by the level of α-tocopheryl acetate in the diets (p = 0.002). Those dogs receiving diets with the medium level of α-tocopheryl acetate had a higher percentage of CD8+ cells compared to dogs receiving diets with low and high levels of α-tocopheryl acetate (27.6 ± 1.8, 18.1 ± 1.7, and 19.4 ± 1.8, respectively).

After 82 days of feeding, there was a significant difference in dogs consuming the six diets based on percentage of cells expressing CD8 (p = 0.001). This difference was attributed to the amount of α-tocopheryl acetate in the diets. The diets with low, medium, and high levels of α-tocopheryl acetate were 13.7 ± 1.6, 23.4 ± 1.7, and 18.5 ± 1.7, respectively for the percentage of cells expressing CD8. Dogs consuming the low and medium α-tocopheryl acetate diets were significantly different from one another (p = 0.0003) as were dogs consuming the low and high α-tocopheryl acetate

diets ($p = 0.05$). The ratio of cells expressing CD4 to CD8 was also significantly different in the dogs consuming the six diets on day 82 ($p = 0.02$). This difference was also attributed to the amount of α-tocopheryl acetate in the diets. Dogs consuming the low α-tocopheryl acetate diets (2.3 ± 0.3) were significantly different from dogs consuming the medium α-tocopheryl acetate diets (1.5 ± 0.3) ($p = 0.01$), as well as the high α-tocopheryl acetate diets (1.5 ± 0.3) ($p = 0.02$). Dogs consuming the medium and high α-tocopheryl acetate diets were not significantly different from one another.

When the difference between day 82 and day 0 was calculated, the ratio of those cells expressing CD4 to CD8 was significantly different among the dogs consuming the six diets based on the levels of α-tocopheryl acetate in the diets ($p = 0.05$). The dogs that were significantly different from each other were those consuming the low α-tocopherol diets and the high α-tocopherol diets (0.5 ± 0.2 and -0.06 ± 0.2, respectively; $p = 0.02$).

After the first vaccination there were no differences among the dogs consuming the six diet for any of the variables tested, regardless of whether the impact was measured on day 92 (one day later) as was done for the CD4+ and CD8+ T-cells expressing CD69 or on day 95 (four days after the vaccination) as was done for the CD4+ and CD8+ T-cells expressing IL-2R. After the second "booster" vaccination there was no interaction between the ratio of (n-6):(n-3) fatty acids and the level of α-tocopheryl acetate in the diet for the dogs consuming the six diets nor was there an effect on any of the variables, except for the ratio of CD4+/CD8+ T-cells ($p = 0.05$). This was attributed to the level of α-tocopheryl acetate in the diet. The ratio of

CD4+/CD8+ T-cells was higher in the dogs fed the low level of α-tocopheryl acetate (2.5 ± 0.2) compared to those fed the medium and high levels of α-tocopheryl acetate diets (1.5 ± 0.2 and 1.5 ± 0.2, respectively). There was no effect of the dietary (n-6):(n-3) fatty acid ratio on these variables. The level of dietary α-tocopheryl acetate significantly influenced the percentage of CD8+ T-cells after 109 days of feeding the six diets ($p = 0.03$). The percentage of CD8+ T-cells was highest in dogs fed the medium level of α-tocopheryl acetate diet (18.5 ± 1.6) compared to the dogs fed the low and high levels of α-tocopheryl acetate (12.2 ± 1.6 and 15.8 ± 1.7, respectively).

DISSCUSSION

There have been many studies, both human and animal, that have reported beneficial effects of feeding fish oil (a source of (n-3) fatty acids) or fish on the immune response. In addition, studies have also pointed out some negative aspects of feeding fish oil, i.e., increased lipid peroxidation (Meydani et al. 1991a) and (Wander et al. 1996), although recently this issue has come under question (Wander et al. 1998). α-Tocopherol also plays a significant role in the immune response. One of the most important functions of α-tocopherol is to protect cell membranes from oxidative damage, for which cells of the immune response are at an especially high risk (Beharka et al. 1997). α-Tocopherol is essential for the immune system to function correctly. A deficiency in vitamin E actually diminishes the immune system's ability to respond to infectious microorganisms, to produce a DTH reaction, or to mount an antibody response to an antigen (Beharka et al. 1997).

To help prevent or lessen some of the presumed adverse effects of high levels of (n-3) fatty acids, i.e., increased lipid peroxidation, α-tocopherol is usually also supplemented. α-Tocopherol is given to enhance the normal antioxidant mechanisms, which are generally used up by the lipid peroxidation from the (n-3) fatty acids. However, (n-3) fatty acids are influenced by the level of α-tocopherol present in the diet, hence a balance between α-tocopherol and (n-3) fatty acids is critical in determining the overall functional outcome (Calder 1998b).

The purpose of this study was to determine the effect of varying the amount of α-tocopheryl acetate in diets supplemented with (n-3) fatty acids on the immune response in healthy geriatric Beagle dogs. Older dogs were chosen because immune system responsiveness has been shown to decline with age. In addition, lipid peroxidation may also be more pronounced in this population (Harman 1982). Furthermore, a study by Meydani et al. (1991b), and a study by Suzuki et al. (1985), indicated that more EPA and DHA are incorporated into plasma lipids of older women, and older rats, respectively, when fed fish oil.

The changes in the fatty acid profiles after feeding the six experimental diets were as expected. The levels of arachidonic acid were significantly lower in the dogs fed the high (n-3) fatty acid diets. In addition to this, the levels of linoleic acid in the dogs fed the high (n-3) fatty acid diets were also significantly decreased. The reason for this is that the precursor of the (n-6) fatty acid family is linoleic acid (18:2 (n-6)) and in animal tissues, linoleic acid is converted into arachidonic acid. So, with less linoleic acid to work with, less arachidonic acid was produced. Furthermore, the levels of EPA and DHA were significantly increased in the dogs fed the high (n-3) fatty acid diet. Again, these results are expected. These results can also be seen in the sums of the (n-6) and (n-3) fatty acids, as well as in the ratio of the (n-6):(n-3) fatty acids.

Plasma α-tocopherol levels increased significantly in dogs fed the high level of α-tocopheryl acetate compared to dogs fed the low level of α-tocopheryl acetate at both levels of (n-3) fatty acid diets. This increase was significant even when plasma α-tocopherol levels were expressed relative to total plasma lipids (cholesterol and

triglycerides) or when expressed relative to the DBI. The latter represents the more accurate way of looking at the plasma α-tocopherol because it relates α-tocopherol concentration to the actual number of double bonds present in the plasma (Wander et al. 1997). An important finding was that the increase in α-tocopherol was less pronounced in the dogs receiving the high (n-3) fatty acid diets.

Plasma cholesterol was lower in the dogs fed the high (n-3) fatty acid diets compared to the dogs receiving the low (n-3) fatty acid diets regardless of the level of α-tocopheryl acetate in the diet. Plasma triglycerides tended to be lower in the dogs fed the high (n-3) fatty acid diets compared to the dogs receiving the low (n-3) fatty acid diets regardless of the level of α-tocopheryl acetate in the diet. Although total cholesterol is not routinely influenced by dietary (n-3) fatty acids, triglycerides are (Harris 1989).

Lymphocyte numbers were influenced by the level of α-tocoopheryl acetate in the diet on both days 92 and 109. Lymphocyte numbers increased as a result of vaccination in dogs receiving the high levels of α-tocopheryl acetate in their diet. A similar increase was also noted in a previous study by Hall et al. (1999).

The results of this study showed a significant interaction between the ratio of (n-6):(n-3) fatty acids and the level of α-tocopheryl acetate in the diet when examining the differences between the TBARS values measured on the 82nd day after eating the treatment diets and the values measured before treatment was initiated (day 0). This interaction was caused by the level of α-tocopheryl acetate in the diets. The value of the TBARS was lowest in the dogs fed the highest level of α-tocopheryl acetate on the

high (n-3) fatty acid diets. The value of the TBARS was equivalent for all three levels of α-tocopheryl acetate when the low (n-3) fatty acid diets were fed. Generally, an increase in the value of plasma TBARS is seen with higher levels of (n-3) fatty acids in the diet. This was seen in studies by Wander et al. (1996) and Meydani et al. (1991b) where they both showed increases in TBARS when high (n-3) fatty acids were fed to their respective subjects (young and elderly women). However, it appears that when high levels of α-tocopheryl acetate are given in conjunction with the high levels of (n-3) fatty acids, there is a decrease in the level of TBARS measured. TBARS data can sometimes be misleading, and there are now better ways to assess lipid peroxidation that give more accurate results. Some of these newer methods include measuring conjugated dienes, use of high performance liquid chromatography (HPLC) to measure the amount of lipid peroxidation in cholesterol esters and the use of HPLC to measure the amount of phospholipid peroxidation.

The T-cell mediated immune response, assessed by the DTH skin test, showed a significant interaction between the ratio of (n-6):(n-3) fatty acids and the level of α-tocopheryl acetate at both 72 and 96 h. At both 72 and 96 h, when high levels of (n-3) fatty acids were fed to the dogs, the DTH skin test results were not significantly different from one another for dogs consuming each of the three levels of α-tocopheryl acetate. However, when the low (n-3) fatty acids were fed, dogs consuming either the low or high level of α-tocopheryl acetate diets had a similar DTH skin test response. These two groups of dogs were both significantly different from the dogs fed the medium level of α-tocopheryl acetate. The DTH reaction is an *in vivo* assay used to

assess cell-mediated immune function. DTH is antigen-specific, T-cell dependent, and is a recall response that manifests itself as an inflammatory reaction that does not become apparent until roughly 24 h after exposure to the antigen and may not reach peak intensity for several days (Clough and Roth 1998).

Studies have indicated that α-tocopherol supplementation alone can significantly enhance the DTH response in elderly human subjects. Meydani et al. (1990) reported that supplementation with 800 IU/d for one month resulted in a significantly increased DTH response in healthy elderly persons. Furthermore, this study reported a threefold increase in lymphocyte vitamin E levels in the supplemented group that correlated with enhanced IL-2 production. Other findings in this study were enhanced lymphocyte proliferation, decreased production of PGE_2, and decreased levels of immunosuppressive serum lipid peroxides.

Meydani et al. (1997) reported increases in the DTH response of elderly human subjects when supplemented with 60, 200 or 800 IU (mg/d dl-α-tocopherol). The greatest responses were seen in the group receiving 200 mg/d. Therefore, these researchers concluded that 200 mg/d represented the optimal level of dl-α-tocopherol supplement needed to maximize the DTH response in elderly human subjects. This observation of optimal immune response at 200 mg/d suggests that there may be a threshold level for the immunostimulatory effect of α-tocopherol. In our present study, the dogs receiving low levels of (n-3) fatty acids with medium α-tocopheryl acetate in the diet showed the maximum DTH response, which seems to correlate with the results of Meydani et al. (1997).

The recommended daily requirement of vitamin E for an adult dog is 20 IU/kg of diet based on a diet supplying 3,300 kcal metabalizable energy/kg (NRC 1985). (All diets in this study supplied approximately 950 kcal/kg of diet. Thus, the adjusted daily requirement of vitamin E for these diets would be 6 IU/kg of diet based on a diet supplying 950 kcal/kg). Another way of expressing the requirement of vitmain E is that approximately 1.1 IU/kg body weight should be given to dogs daily (NRC 1985). The level recommended for humans is 8 IU of tocopherol equivalents for women and 10 IU of tocopherol equivalents for men (Rimm 1999). Using the assumption that an average adult Beagle weighs 10 kg, this comes out to 11 IU/dog a day. Our dogs were fed 16.6 and 17.2 mg/kg α-tocopheryl acetate, which is equivalent to ~17 IU/dog on the low α-tocopheryl acetate diets, 95.0 and 106 mg/kg α-tocopheryl acetate, which equates to ~100 IU/dog for the medium α-tocopheryl acetate diets, and 446 and 448 mg/kg α-tocopheryl acetate, which comes out to roughly 447 IU/dog for the high α-tocopheryl acetate diets. Thus, none of the diets in this study were deficient in vitamin E and the diets containing the most vitamin E had approxiamately 45 times the NRC requirement.

The mechanisms by which vitamin E enhances the immune system have yet to be fully explained, however, the evidence suggests that vitamin E works by either reducing prostaglandin synthesis (Meydani et al. 1986, 1990) and/or decreasing the formation of free radicals (Corwin and Shloss 1980). One area of research suggests that free radical damage to cells can lead to the pathological changes associated with aging. Free radicals can also be formed as a result of environmental pollutants, the

diet one consumes, by damage to the components of the immune system and many other causes (Meydani and Beharka 1998). Reactive oxygen species, especially H_2O_2, which is produced by activated macrophages, depresses lymphocyte proliferation (Metzger et al. 1980). Vitamin E has been shown to decrease H_2O_2 formation by polymorphonuclear cells (Baehner et al. 1977). Furthermore, the immunosuppressive effect of vitamin E deficiency appears to be linked to increased free radical reactions, which lead to greater production of PGE_2. Beharka et al. (1997) reported that supplementation with vitamin E improved T-cell responsiveness by reducing macrophage PGE_2 production.

In our study, PGE_2 levels were not significantly different for any of the six experimental diets. In fact, the levels of PGE_2 were extremely high and no differences were seen among any of the groups of dogs fed the experimental diets. In Wander et al. (1997) the concentration of PGE_2 decreased as the concentration of fish oil in the diet increased. We expected to see a lower concentration of PGE_2 for the dogs fed high levels of α-tocopheryl acetate in their diets and high levels of (n-3) fatty acids in this study.

It is possible that during centrifugation some of the cells (for example 5%) might rupture because of high shear conditions (the actual force of the spinning). This would allow release of cellular constituents, including microsomal cylooxygenase (COX), into the supernatant. If this were to occur, PGE_2 production would continue even after centrifugation. Adding an aspirinate (aspirin) solution (0.5 volume of 43

mM aspirin added to media buffer) to the supernatant immediately after centrifugation would inactivate the COX, thus preventing further PG production (Allen 1999).

The humoral immune response can be assessed by measuring the production of antibodies to a foreign protein, such as KLH. A foreign protein is anything that the body's immune system has never come into contact with before. Because the body does not recognize this particle, it is considered "foreign" by the body. The log of the antibody titer to KLH was unaffected in dogs by either the low or high (n-3) fatty acid diets regardless of the level of vitamin E fed. These results are consistent with our previous study (Wander et al. 1997) where antibody production to KLH was not effected by the ratio of (n-6):(n-3) fatty acid in the diet. Work published by Meydani et al. (1997) showed that antibody titers to tetanus were not significantly altered with vitamin E supplementation in normal healthy elderly subjects. In a recent study reported by Kelley et al. (1998a), supplementation with arachidonic acid (either 200 mg/d or 1.5 g/d for the first 15 days of the study) to young healthy men showed no effect on serum antibody titers against influenza vaccine (A/Texas and B/Panama). Thus, as noted above, other studies have shown that supplementation with fatty acids can have no effect on antibody titers.

An *ex vivo* test of the immune system was the measurement of fluorescent bead engulfment by macrophages. This data was not significantly different at any time point during the study (baseline, after 82 days, or the difference between the two). The results that we expected to see were a decrease in the amount of beads engulfed by macrophages from the dogs fed the high (n-3) fatty acid diets. The reason for this is

that fish oil (along with olive and evening primrose oil) has been shown to suppress the expression of IL-2R following mitogenic stimulation of spleen lymphocytes (Sanderson et al. 1995). Because fish oil suppresses the expression of IL-2R and since IL-2 activates macrophages (Calder 1998a), we expected to see less engulfment of beads by macrophages in the dogs fed high (n-3) fatty acid diets.

Changes in cell surface markers, or phenotypes, of T-cells were also assessed. The percent of cells expressing CD4 was not significantly altered by any of the six experimental diets. The percent of cells expressing CD8 at the start of the study was significantly different in those dogs fed the medium level of α-tocopheryl acetate regardless of the level of (n-3) fatty acids ($p = 0.002$). These same dogs were also significantly different at the conclusion of the feeding trial ($p = 0.001$). When the difference between the end of the feeding trial and the beginning of the feeding trial was calculated, the significance diminished ($p = 0.09$), demonstrating that the six experimental diets had a minimal effect on cells expressing either CD8 or CD4 in the T-cell subpopulations. The ratio of CD4+/CD8+ cells was significantly altered by the level of α-tocopheryl acetate in the diet ($p = 0.02$).

For the most part, vaccination had little effect on cell surface markers. After the second vaccination (day 109), there was a tendency for CD4+ cells to decrease ($p= 0.09$) and CD8+ cells to decrease ($p=0.03$). The significance in both cases was a direct result of the level of α-tocopheryl acetate in the diet. The CD4+/CD8+ ratio also was significantly decreased after the second vaccination, only this time a significant interaction between the ratio of (n-6):(n-3) fatty acids and the level of α-tocopheryl

acetate in the diets was seen ($p= 0.05$). This interaction was caused mostly by the α-tocopheryl acetate factor. Even though changes in the percentages of CD4+ and CD8+ cells were not large, when the two terms were combined into a ratio term, the changes became a more powerful representation of the two individual markers. Vaccination had no effect on the IL-2R or CD69 cell surface makers on either CD4+ or CD8+ T-cells.

Although the results in this study do not correlate with a previous study (Hall et al. 1999) (a decrease in the percent of CD4+ cells after vaccination), there is a similar trend in this study. The results were just not large enough to be considered significant. The results from this study disagree with those of Meydani et al. (1993) and Wu et al. (1996), however, there are other studies that agree with our current findings. Kelley et al. (1998b) found that when healthy young men were supplemented with DHA, there was no effect on the number of B, total T, helper T, suppressor T, helper/suppressor ratio, Tc lymphocytes, or NK cells in circulation as assessed by flow cytometry. In addition, the number of T cells producing IL-2R was not changed by DHA supplementation. Another study by Kelley et al. (1998a) showed similar results when young men were supplemented with arachidonic acid. Supplementing with arachidonic acid did not alter the absolute number or the percentage (for B, T, helper, suppressor, and NK cells) of these lymphocytes in the circulation, nor did it alter the ratio between helper and suppressor T lymphocytes. A vaccine was given in the second study by Kelly et al. (1998a). These two studies by Kelley et al. showed that feeding

either high (n-3) or high (n-6) fatty acid diets had no significant effect on composition of lymphocyte subsets, nor did vaccination.

Based on these results, we conclude that there is an interaction between dietary levels of α-tocopheryl acetate and the ratio of (n-6):(n-3) fatty acids, which effects the immune system as measured by the DTH skin test in geriatric Beagle dogs. Our results agree with the work done by Meydani et al. (1997) in that there appears to be an upper limit to the beneficial effects of vitamin E. A dose response study (for α-tocopheryl acetate with more doses in the range between 100 and 400 μg/g diet) of α-tocopheryl acetate should be performed to verify that there is an upper limit to the immunostimulatory effects of dietary vitamin E.

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APPENDIX

Table 1. Plasma fatty acid profile of Beagles before the dietary intervention [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Fatty Acid	g/100g fatty acids								
14:0	0.37 ± 0.05	0.32 ± 0.06	0.29 ± 0.05	0.39 ± 0.05	0.30 ± 0.07	0.26 ± 0.05	0.89	0.22	0.93
16:0	12.50 ± 0.34	12.26 ± 0.37	12.89 ± 0.37	12.73 ± 0.34	12.67 ± 0.37	12.86 ± 0.37	0.50	0.53	0.84
18:0	19.44 ± 0.35	19.08 ± 0.38	19.27 ± 0.38	19.34 ± 0.35	19.21 ± 0.38	19.95 ± 0.38	0.43	0.49	0.57
18:1(n-9) *t*	0.29 ± 0.01	0.30 ± 0.01	0.24 ± 0.01	0.26 ± 0.01	0.29 ± 0.02	0.27 ± 0.01	0.90	0.10	0.26
18:1(n-9) *c*	10.87 ± 0.38	10.61 ± 0.42	10.48 ± 0.42	10.94 ± 0.38	10.74 ± 0.42	9.77 ± 0.42	0.61	0.16	0.54
18:1(n-7)	3.05 ± 0.10	3.27 ± 0.11	3.00 ± 0.11	3.02 ± 0.10	2.94 ± 0.11	2.94 ± 0.11	0.12	0.47	0.33
18:2(n-6)	23.63 ± 0.46	22.94 ± 0.51	23.33 ± 0.51	22.73 ± 0.46	22.81 ± 0.51	23.43 ± 0.51	0.45	0.61	0.57
20:0	0.19 ± 0.03	0.22 ± 0.03	0.21 ± 0.03	0.19 ± 0.04	0.27 ± 0.04	0.26 ± 0.03	0.25	0.30	0.76
18:3(n-3)	0.28 ± 0.03	0.23 ± 0.03	0.24 ± 0.02	0.26 ± 0.02	0.41 ± 0.05	0.22 ± 0.03	0.15	0.10	0.04
20:2(n-6)	0.36 ± 0.07	0.23 ± 0.07	0.25 ± 0.06	0.25 ± 0.06	0.34 ± 0.06	0.20 ± 0.12	0.65	0.25	0.86
20:3(n-6)	1.19 ± 0.33	1.15 ± 0.16	0.97 ± 0.23	0.91 ± 0.23	1.42 ± 0.23	1.20 ± 0.23	0.73	0.55	0.54
22:0	0.16 ± 0.01	0.18 ± 0.00	0.18 ± 0.01	0.17 ± 0.01	16.00 ± 0.01	0.16 ± 0.01	0.72	0.81	0.48
20:4(n-6)	24.01 ± 0.46	24.34 ± 0.51	24.61 ± 0.51	24.10 ± 0.46	23.31 ± 0.57	24.57 ± 0.51	0.44	0.33	0.51
23:0	0.22 ± 0.04	0.31 ± 0.08	0.25 ± 0.05	0.25 ± 0.08	0.24 ± 0.05	0.22 ± 0.08	0.69	0.80	0.78
20:5(n-3)	0.23 ± 0.08	0.32 ± 0.07	0.30 ± 0.08	0.29 ± 0.12	0.38 ± 0.08	0.21 ± 0.08	0.90	0.46	0.65
24:0	0.22 ± 0.02	0.23 ± 0.02	0.27 ± 0.03	0.26 ± 0.04	0.29 ± 0.06	0.26 ± 0.04	0.47	0.77	0.69

Table 1. Plasma fatty acid profile of Beagles before the dietary intervention [1] (Continued)

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Fatty Acid	g/100g fatty acids								
24:1	1.88 ± 0.24	1.62 ± 0.28	2.56 ± 0.34	2.18 ± 0.24	1.55 ± 0.24	1.82 ± 0.24	0.46	0.10	0.20
22:5(n-3)	1.80 ± 0.08	1.85 ± 0.08	1.91 ± 0.09	1.85 ± 0.08	1.76 ± 0.08	1.86 ± 0.08	0.67	0.66	0.69
22:6(n-3)	0.62 ± 0.05	0.67 ± 0.05	0.57 ± 0.05	0.57 ± 0.05	0.60 ± 0.05	0.62 ± 0.05	0.67	0.67	0.55
Others[6]	0.41 ± 0.10	0.53 ± 0.11	0.56 ± 0.11	0.54 ± 0.10	0.46 ± 0.13	0.43 ± 0.11	0.79	0.97	0.47
∑ SFA[7]	32.87 ± 0.29	32.47 ± 0.32	32.98 ± 0.32	32.72 ± 0.29	32.95 ± 0.36	33.61 ± 0.32	0.24	0.17	0.42
∑ MUFA[8]	16.04 ± 0.55	15.76 ± 0.60	15.36 ± 0.60	16.70 ± 0.55	15.80 ± 0.60	14.75 ± 0.60	0.94	0.09	0.55
∑ PUFA[9]	50.67 ± 0.60	51.22 ± 0.66	51.08 ± 0.66	50.03 ± 0.60	50.28 ± 0.66	51.19 ± 0.66	0.35	0.47	0.71
∑ N6[10]	48.03 ± 0.52	48.35 ± 0.57	48.54 ± 0.57	47.38 ± 0.52	47.68 ± 0.57	48.53 ± 0.57	0.34	0.33	0.80
∑ N3[11]	2.64 ± 0.14	2.86 ± 0.15	2.81 ± 0.17	2.65 ± 0.14	2.60 ± 0.15	2.66 ± 0.15	0.27	0.78	0.65
N6/N3	18.44 ± 0.94	17.08 ± 1.04	17.35 ± 1.16	17.91 ± 0.94	18.86 ± 1.04	18.38 ±1.04	0.37	0.95	0.50
DBI[12]	157.85± 2.47	161.05±2.70	159.57±2.70	156.77±2.47	157.98±2.70	160.44±2.70	0.61	0.53	0.76

[1] Least square means ± SEM
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate
[6] Unidentified fatty acids
[7] Sum of the saturated fatty acids: 8:0 + 10:0 + 11:0 + 12:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0 + 22:0 + 24:0.

Table 1. Plasma fatty acid profile of Beagles before the dietary intervention [1] (Continued)

[8] Sum of the monounsaturated fatty acids: 14:1 + 15:1 + 16:1(n-7) + 17:1 + 18:1(n-9)*cis* + 18:1(n-7) + 18:1(n-9)*trans*+ 20:1(n-9) + 22:1(n-9) + 24:1.

[9] Sum of the polyunsaturated fatty acids: 18:2(n-6) + 18:3(n-6) + 18:3(n-3) + 18:4(n-3) + 20:2(n-6) + 20:3(n-6) + 20:3(n-3) + 20:4(n-6) + 20:4(n-3) + 20:5(n-3) + 21:5(n-3) + 22:2(n-6) + 22:4(n-6) + 22:5(n-6) + 22:5(n-3) + 22:6(n-3).

[10] Sum of the (n-6) fatty acids.

[11] Sum of the (n-3) fatty acids.

[12] DBI (double bond index) = 2[18:2(n-6) + 20:2(n-6)] + 3[18:3(n-3) + 20:3(n-6)] + 4[20:4(n-6)] + 5[20:5(n-3) + 22:5(n-3)] + 6[22:6(n-3)], where the concentration of each fatty acid is expressed as g/kg fatty acids.

Table 2. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) to Beagles for 82 days on plasma fatty acid profile [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Fatty acid	g/100g fatty acids								
14:0	0.23 ± 0.13	0.18 ± 0.13	0.24 ± 0.13	0.34 ± 0.08	0.52 ± 0.08	0.42 ± 0.08	0.03	0.83	0.60
16:0	9.70 ± 0.40	9.40 ± 0.44	10.43 ± 0.40	10.07 ± 0.36	11.03 ± 0.40	10.92 ± 0.40	0.01	0.15	0.26
18:0	20.28 ± 0.78	19.54 ± 0.78	20.57 ± 0.78	19.93 ± 0.71	18.72 ± 0.78	19.75 ± 0.78	0.30	0.34	0.93
18:1(n-9) *t*	0.63 ± 0.05	0.62 ± 0.05	0.60 ± 0.50	0.52 ± 0.04	0.61 ± 0.05	0.66 ± 0.05	0.61	0.60	0.24
18:1(n-9) *c*	7.15 ± 0.63	6.68 ± 0.71	6.94 ± 0.63	7.69 ± 0.58	7.98 ± 0.63	8.22 ± 0.63	0.05	0.92	0.79
18:1(n-7)	2.36 ± 0.33	2.48 ± 0.37	2.37 ± 0.33	3.02 ± 0.30	3.83 ± 0.33	3.10 ± 0.33	0.002	0.33	0.54
18:2(n-6)	30.13 ± 1.05	27.57 ± 1.05	29.51 ± 1.05	20.81 ± 0.96	21.00 ± 1.05	22.35 ± 1.05	0.0001	0.29	0.38
20:0	0.21 ± 0.03	0.20 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.16 ± 0.02	0.17 ± 0.03	0.30	0.73	0.83
18:3(n-3)	0.13 ± 0.01	0.13 ± 0.01	0.15 ± 0.01	0.23 ± 0.01	0.23 ± 0.01	0.21 ± 0.01	0.0007	0.91	0.27
20:2(n-6)	0.37 ± 0.04	0.34 ± 0.04	0.32 ± 0.04	0.26 ± 0.04	0.18 ± 0.06	0.20 ± 0.05	0.006	0.90	0.48
20:3(n-6)	0.75 ± 0.07	0.99 ± 0.07	0.73 ± 0.07	0.73 ± 0.07	0.62 ± 0.08	0.60 ± 0.07	0.014	0.26	0.09
22:0	0.15 ± 0.02	0.21 ± 0.02	0.19 ± 0.02	0.16 ± 0.03	0.14 ± 0.04	0.16 ± 0.04	0.22	0.81	0.44
20:4(n-6)	23.25 ± 0.79	24.26 ± 0.88	23.34 ± 0.79	15.65 ± 0.72	13.82 ± 0.79	13.28 ± 0.79	0.0001	0.34	0.16
23:0	0.16 ± 0.03	0.21 ± 0.2	0.18 ± 0.02	0.23 ± 0.02	0.24 ± 0.03	0.25 ± 0.02	0.034	0.56	0.81
20:5(n-3)	0.19 ± 0.69	0.38 ± 0.56	0.18 ± 0.98	8.07 ± 0.40	8.53 ± 0.43	7.37 ± 0.43	0.0001	0.56	0.76

Table 2. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate to Beagles for 82 days on plasma fatty acid profile [1] (Continued)

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Fatty acid	g/100g fatty acids								
24:0	0.25 ± 0.03	0.30 ± 0.03	0.32 ± 0.03	0.28 ± 0.03	0.29 ± 0.03	0.26 ± 0.03	0.69	0.62	0.49
24:1	1.18 ± 0.24	1.02 ± 0.24	1.02 ± 0.27	0.93 ± 0.31	1.04 ± 0.24	0.86 ± 0.27	0.56	0.90	0.87
22:5(n-3)	1.15 ± 0.22	1.19 ± 0.22	1.23 ± 0.22	2.44 ± 0.20	2.35 ± 0.22	2.59 ± 0.22	0.0001	0.80	0.89
22:6(n-3)	0.72 ± 0.25	0.67 ± 0.25	0.60 ± 0.25	6.22 ± 0.23	6.48 ± 0.25	6.51 ± 0.25	0.0001	0.90	0.71
Others[6]	0.62 ± 0.10	0.67 ± 0.10	0.76 ± 0.10	0.60 ± 0.09	0.64 ± 0.10	0.59 ± 0.10	0.39	0.83	0.73
∑ SFA[7]	30.61 ± 0.47	30.94 ± 0.47	31.92 ± 0.47	31.15 ± 0.43	30.93 ± 0.47	31.72 ± 0.47	0.77	0.10	0.71
∑ MUF[8]	12.40 ± 1.15	11.69 ± 1.29	11.59 ± 1.15	13.93 ± 1.05	15.55 ± 1.15	14.71 ± 1.15	0.0066	0.90	0.59
∑ PUFA[9]	56.35 ± 1.06	56.74 ± 1.19	55.72 ± 1.06	54.31 ± 0.97	52.87 ± 1.06	52.97 ± 1.06	0.0031	0.64	0.69
∑ N6[10]	54.40 ± 0.95	54.74 ± 1.07	53.78 ± 0.95	37.37 ± 0.87	35.40 ± 0.95	36.36 ± 0.95	0.0001	0.61	0.46
∑ N3[11]	1.95 ± 0.57	2.18 ± 0.57	1.93 ± 0.57	16.94 ± 0.52	17.46 ± 0.57	16.60 ± 0.57	0.0001	0.61	0.86
N6/N3	29.17 ± 1.85	24.97 ± 1.85	28.13 ± 1.85	2.23 ± 1.69	2.03 ± 1.85	2.20 ± 1.85	0.0001	0.46	0.53
DBI[12]	166.54 ± 4.33	169.61 ± 4.85	165.01 ± 4.85	197.34 ± 3.96	192.58 ± 4.33	189.16 ± 4.33	0.0001	0.49	0.62

[1] Least square means ± SEM
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate

Table 2. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate to Beagles for 82 days on plasma fatty acid profile [1] (Continued)

[6] Unidentified fatty acids
[7] Sum of the saturated fatty acids: 8:0 + 10:0 + 11:0 + 12:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0 + 22:0 + 24:0.
[8] Sum of the monounsaturated fatty acids: 14:1 + 15:1 + 16:1(n-7) + 17:1 + 18:1(n-9)*cis* + 18:1(n-7) + 18:1(n-9)*trans* + 20:1(n-9) + 22:1(n-9) + 24:1.
[9] Sum of the polyunsaturated fatty acids: 18:2(n-6) + 18:3(n-6) + 18:3(n-3) + 18:4(n-3) + 20:2(n-6) + 20:3(n-6) + 20:3(n-3) + 20:4(n-6) + 20:4(n-3) + 20:5(n-3) + 21:5(n-3) + 22:2(n-6) + 22:4(n-6) + 22:5(n-6) + 22:5(n-3) + 22:6(n-3).
[10] Sum of the (n-6) fatty acids.
[11] Sum of the (n-3) fatty acids.
[12] DBI (double bond index) = 2[18:2(n-6) + 20:2(n-6)] + 3[18:3(n-3) + 20:3(n-6)] + 4[20:4(n-6)] + 5[20:5(n-3) + 22:5(n-3)] + 6[22:6(n-3)], where the concentration of each fatty acid is expressed as g/kg fatty acids.

Table 3. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on the difference between day 82 and day 0 values of the plasma fatty acid profile in Beagle dogs [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Fatty Acid	g/100g fatty acids								
14:0	-0.05 ± 0.18	-0.20 ± 0.18	-0.03 ± 0.13	-0.03 ± 0.08	0.30 ± 0.10	0.16 ± 0.08	0.05	0.70	0.28
16:0	-2.43 ± 0.40	-2.50 ± 0.45	-2.46 ± 0.40	-2.65 ± 0.37	-1.64 ± 0.40	-1.94 ± 0.40	0.26	0.49	0.40
18:0	0.77 ± 0.66	0.46 ± 0.66	1.30 ± 0.66	0.58 ± 0.60	-0.49 ± 0.66	-0.20 ± 0.66	0.10	0.53	0.59
18:1(n-9) *t*	0.33 ± 0.06	0.34 ± 0.07	0.36 ± 0.06	0.24 ± 0.05	0.32 ± 0.07	0.38 ± 0.05	0.61	0.35	0.63
18:1(n-9) *c*	-3.50 ± 0.70	-3.63 ± 0.79	-3.53 ± 0.70	-3.25 ± 0.64	-2.76 ± 0.70	-1.55 ± 0.70	0.08	0.46	0.46
18:1(n-7)	-0.70 ± 0.29	-0.84 ± 0.33	-0.62 ± 0.29	-0.00 ± 0.27	0.89 ± 0.29	0.16 ± 0.29	0.0002	0.45	0.19
18:2(n-6)	6.45 ± 0.87	4.62 ± 0.87	6.18 ± 0.87	-1.92 ± 0.80	-1.80 ± 0.87	-1.07 ± 0.87	0.0001	0.41	0.53
20:0	0.01 ± 0.07	-0.04 ± 0.05	-0.08 ± 0.07	-0.00 ± 0.07	-0.11 ± 0.06	-0.11 ± 0.07	0.52	0.33	0.91
18:3(n-3)	ND[6]	-0.11 ± 0.01	-0.09 ± 0.01	-0.03 ± 0.01	-0.21 ± 0.01	-0.00 ± 0.01	0.66	0.02	0.02
20:2(n-6)	-0.04 ± 0.10	0.07 ± 0.13	0.11 ± 0.09	-0.19 ± 0.13	ND	ND	0.40	0.57	ND
20:3(n-6)	-0.30 ± 0.12	-0.22 ± 0.06	-0.29 ± 0.08	-0.21 ± 0.08	-0.73 ± 0.08	-0.54 ± 0.08	0.02	0.13	0.04
22:0	-0.00 ± 0.02	0.05 ± 0.01	0.00 ± 0.03	-0.01 ± .03	ND	ND	0.93	0.14	ND
20:4(n-6)	-1.13 ± 0.81	-0.43 ± 0.91	-1.27 ± 0.81	-8.45 ± 0.74	-8.91 ± 0.91	-11.29 ± 0.81	0.0001	0.11	0.25
23:0	-0.05 ± 0.02	-0.13 ± 0.04	-0.07 ± 0.03	-0.05 ± 0.04	0.08 ± 0.04	-0.02 ± 0.04	0.06	0.78	0.17
20:5(n-3)	ND	-0.26 ± 0.19	-0.14 ± 0.27	10.07 ± 0.27	7.80 ± 0.19	6.70 ± 0.19	0.0001	0.005	0.06

Table 3. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate on the difference between day 82 and day 0 values of the plasma fatty acid profile in Beagle dogs [1] (Continued)

Dietary (n-3)	Low			High			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Fatty Acid	g/100g fatty acids								
24:0	0.03 ± 0.02	0.07 ± 0.02	-0.00 ± 0.03	0.06 ± 0.04	-0.11 ± 0.06	-0.01 ± 0.04	0.15	0.30	0.10
24:1	-0.51 ± 0.50	-0.99 ± 0.50	-2.33 ± 0.86	-1.26 ± 0.86	-0.49 ± 0.43	-0.85 ± 0.50	0.45	0.39	0.33
22:5(n-3)	-0.69 ± 0.22	-0.66 ± 0.22	-0.78 ± 0.25	0.59 ± 0.20	0.59 ± 0.22	0.72 ± 0.22	0.0001	0.99	0.84
22:6(n-3)	0.07 ± 0.26	0.00 ± 0.26	0.03 ± 0.26	5.65 ± 0.24	5.87 ± 0.26	5.89 ± 0.26	0.0001	0.91	0.81
Others[7]	0.18 ± 0.09	0.14 ± 0.09	0.19 ± 0.09	0.06 ± 0.08	0.12 ± 0.10	0.15 ± 0.09	0.46	0.84	0.87
∑ SFA[8]	-2.03 ± 0.46	-1.53 ± 0.46	-1.06 ± 0.46	-1.57 ± 0.42	-1.99 ± 0.52	-1.89 ± 0.46	0.48	0.74	0.35
∑ MUFA[9]	-3.22 ± 1.10	-3.59 ± 1.23	-3.77 ± 1.10	-2.77 ± 1.01	-0.24 ± 1.10	-0.04 ± 1.10	0.011	0.52	0.27
∑ PUFA[10]	5.07 ± 1.13	4.96 ± 1.27	4.64 ± 1.13	4.28 ± 1.03	2.58 ± 1.13	1.77 ± 1.13	0.04	0.42	0.62
∑ N6[11]	5.87 ± 0.98	5.95 ± 1.09	5.24 ± 0.98	-10.01 ± 0.89	-12.27 ± 0.98	-12.16 ± 0.98	0.0001	0.32	0.48
∑ N3[12]	-0.80 ± 0.62	-0.68 ± 0.62	-0.96 ± 0.70	14.29 ± 0.57	14.86 ± 0.62	13.94 ± 0.62	0.0001	0.64	0.87
N6/N3	11.39 ± 2.29	7.88 ± 2.29	12.03 ± 2.57	-15.67 ± 2.09	-16.82 ± 2.29	-16.18 ± 2.29	0.0001	0.50	0.75
DBI[13]	6.38 ± 5.15	6.42 ± 5..75	5.44 ± 5.15	40.56 ± 4.70	34.60 ± 5.15	28.72 ± 5.15	0.0001	0.45	0.56

[1] Least square means ± SEM
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate

Table 3. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate on the difference between day 82 and day 0 values of the plasma fatty acid profile in Beagle dogs [1] (Continued)

[6] ND = not large enough to be defined
[7] Unidentified fatty acids
[8] Sum of the saturated fatty acids: 8:0 + 10:0 + 11:0 + 12:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0 + 22:0 + 24:0.
[9] Sum of the monounsaturated fatty acids: 14:1 + 15:1 + 16:1(n-7)+ 17:1 + 18:1(n-9)*cis* + 18:1(n-7) + 18:1(n-9)*trans* + 20:1(n-9) + 22:1(n-9) + 24:1.
[10] Sum of the polyunsaturated fatty acids: 18:2(n-6) + 18:3(n-6) + 18:3(n-3) + 18:4(n-3) + 20:2(n-6) + 20:3(n-6) + 20:3(n-3) + 20:4(n-6) + 20:4(n-3) + 20:5(n-3) + 21:5(n-3) + 22:2(n-6) + 22:4(n-6) + 22:5(n-6) + 22:5(n-3) + 22:6(n-3)
[11] Sum of the (n-6) fatty acids.
[12] Sum of the (n-3) fatty acids.
[13] DBI (double bond index) = 2[18:2(n-6) + 20:2(n-6)] + 3[18:3(n-3) + 20:3(n-6)] + 4[20:4(n-6)] + 5[20:5(n-3) + 22:5(n-3)] + 6[22:6(n-3)], where the concentration of each fatty acid is expressed as g/kg fatty acids.

Table 4. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on plasma α-tocopherol concentrations in Beagle dogs for day 0 (initial), day 82 (final) and the difference between initial and final[1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
	μg/ml								
Day 0	29.64 ± 1.96	30.31 ± 2.15	36.36 ± 2.15	26.39 ± 1.96	32.28 ± 2.15	29.10 ± 2.15	0.11	0.08	0.12
Day 82	20.84 ± 2.11	34.90 ± 2.31	52.36 ± 2.31	17.33 ± 2.11	25.44 ± 2.31	35.38 ± 2.31	0.0001	0.0001	0.0189
Difference	-8.79 ± 1.75	4.58 ± 1.92	16.00 ± 1.92	-9.06 ± 1.75	-6.84 ± 1.92	6.28 ± 1.92	0.0001	0.0001	0.0101

[1] Values are means ± SEM
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate

Table 5. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on plasma α-tocopherol concentrations expressed relative to plasma lipid (cholesterol and triglycerides) in Beagle dogs for day 0 (initial), day 82 (final) and the difference between initial and final [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
	μg/mg								
Day 0	0.11 ± 0.006	0.11 ± 0.006	0.11 ± 0.006	0.11 ± 0.006	0.10 ± 0.006	0.11 ± 0.006	0.73	0.43	1.00
Day 82	0.09 ± 0.008	0.12 ± 0.009	0.19 ± 0.009	0.08 ± 0.008	0.10 ± 0.009	0.19 ± 0.009	0.22	0.0001	0.82
Difference	-0.02 ± 0.01	0.01 ± 0.01	0.08 ± 0.01	-0.03 ± 0.01	0.00 ± 0.01	0.07 ± 0.01	0.38	0.0001	0.85

[1] Values are means ± SEM
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate

Table 6. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on plasma α-tocopherol concentrations expressed relative to the double bond index (DBI) in Beagle dogs for day 0 (initial), day 82 (final) and the difference between initial and final [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
	μg/ml								
Day 0	0.73 ± 0.04	0.71 ± 0.05	0.84 ± 0.05	0.55 ± 0.04	0.72 ± 0.05	0.58 ± 0.05	0.001	0.22	0.03
Day 82	0.51 ± 0.05	0.81 ± 0.05	1.21 ± 0.05	0.37 ± 0.05	0.57 ± 0.05	0.70 ± 0.05	0.0001	0.0001	0.0041
Difference	-0.22 ± 0.04	0.11 ± 0.04	0.37 ± 0.04	-0.19 ± 0.04	-0.15 ± 0.04	0.13 ± 0.04	0.0001	0.0001	0.0022

[1] Values are means ± SEM
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate

Table 7. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on plasma TBARS in Beagle dogs for day 0 (initial), day 82 (final) and the difference between initial and final [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
	nmol/ml								
Day 0	1.88 ± 0.15	1.63 ± 0.17	1.70 ± 0.17	1.60 ± 0.15	1.78 ± 0.17	2.01 ± 0.17	0.6533	0.6640	0.1688
Day 82	2.17 ± 0.27	1.78 ± 0.30	1.84 ± 0.30	2.38 ± 0.27	2.33 ± 0.30	1.44 ± 0.30	0.6187	0.0938	0.2836
Difference	0.29 ± 0.21	0.15 ± 0.23	0.14 ± 0.23	0.78 ± 0.21	0.54 ± 0.23	-0.57 ± 0.23	0.7514	0.0062	0.0215

[1] Values are means ± SEM.
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate

Table 8. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on plasma lipid concentrations (cholesterol and triglycerides) in Beagle dogs for day 0 (initial), day 82 (final) and the difference between initial and final[1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
	mg/100 ml								
Chol Day 0	246.0 ± 20.2	256.0 ± 22.2	275.4 ± 22.2	221.0 ± 20.2	272.0 ± 22.2	229.2 ± 22.2	0.30	0.35	0.37
Chol Day 82	191.3 ± 17.4	239.2 ± 19.1	231.6 ± 19.14	174.5 ± 17.4	199.8 ± 19.1	158.6 ± 19.1	0.008	0.15	0.32
Chol difference	-54.6 ± 20.1	-16.8 ± 22.0	-43.8 ± 22.0	-46.5 ± 20.1	-72.2 ± 22.0	-70.6 ± 22.0	0.17	0 .84	0.33
Trig Day 0	36.16 ± 4.8	38.8 ± 5.3	47.8 ± 5.3	36.33 ± 4.8	39.2 ± 5.3	34.0 ± 5.3	0.30	0.65	0.30
Trig Day 82	46.83 ± 4.6	53.2 ± 5.0	45.2 ± 5.0	41.16 ± 4.6	48.4 ± 5.0	35.2 ± 5.0	0.09	0.12	0.85
Trig difference	10.6 ± 7.0	14.4 ± 7.6	-2.6 ± 7.6	4.83 ± 7.0	9.2 ± 7.6	1.2 ± 7.6	0.69	0.26	0.77

[1] Values are means ± SEM
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate

Table 9. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on serum biochemistries in Beagle dogs on day 0 (initial), day 82 (final), and the difference between initial and final [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Variable									
BUN[6] (mg/dl) Day 0	11.51 ± 1.3	11.58 ± 0.42	13.02 ± 1.42	11.45 ± 1.29	10.98 ± 1.42	11.00 ± 1.42	0.43	0.86	0.76
BUN (mg/dl) Day 82	12.68 ± 1.5	12.28 ± 1.66	10.46 ± 1.66	10.68 ± 1.51	11.92 ± 1.66	13.42 ± 1.66	0.88	0.96	0.30
BUN (mg/dl) D[7]	1.16 ± 1.3	0.70 ± 1.37	-2.56 ± 1.37	-0.76 ± 1.25	0.94 ± 1.37	2.42 ± 1.37	0.32	0.80	0.04
Creatinine (mg/dl) Day 0	0.61 ± 0.04	0.54 ± 0.04	0.64 ± 0.04	0.63 ± 0.04	0.56 ± 0.04	0.58 ± 0.04	0.83	0.21	0.60
Creatinine (mg/dl) Day 82	0.66 ± 0.05	0.66 ± 0.05	0.64 ± 0.05	0.61 ± 0.05	0.66 ± 0.05	0.70 ± 0.05	0.94	0.86	0.60
Creatinine (mg/dl) D[7]	0.05 ± 0.04	0.12 ± 0.05	-0.00 ± 0.05	-0.01 ± 0.04	0.10 ± 0.05	0.12 ± 0.05	0.79	0.19	0.18
Glucose (mg/dl) Day 0	105.83 ± 3.4	97.80 ± 3.70	100.20 ± 3.70	106.16 ± 3.38	98.80 ± 3.70	102.20 ± 3.70	0.70	0.10	0.97
Glucose (mg/dl) day 82	82.50 ± 6.5	63.80 ± 7.13	70.00 ± 7.13	76.50 ± 6.50	69.80 ± 7.13	76.00 ± 7.13	0.72	0.19	0.59
Glucose (mg/dl) D[7]	-23.33 ± 6.1	-34.00 ± 6.71	-30.20 ± 6.71	-29.66 ± 6.13	-29.00 ± 6.71	-26.20 ± 6.71	0.86	0.73	0.61
Protein (g/dl) Day 0	5.95 ± 0.2	5.60 ± 0.18	6.22 ± 0.18	6.15 ± 0.16	6.32 ± 0.18	6.06 ± 0.18	0.09	0.62	0.07
Protein (g/dl) Day 82	6.55 ± 0.4	5.84 ± 0.46	6.90 ± 0.46	6.98 ± 0.42	7.02 ± 0.46	6.68 ± 0.46	0.21	0.67	0.32
Protein (g/dl) D[7]	0.60 ± 0.4	0.24 ± 0.38	0.68 ± 0.38	0.83 ± 0.35	0.70 ± 0.38	0.62 ± 0.38	0.49	0.79	0.79
Albumin (g/dl) Day 0	2.81 ± 0.1	2.58 ± 0.11	2.96 ± 0.11	2.83 ± 0.10	2.98 ± 0.11	2.82 ± 0.11	0.31	0.62	0.06
Albumin (g/dl) Day 82	3.25 ± 0.2	2.84 ± 0.24	3.10 ± 0.24	3.36 ± 0.22	3.60 ± 0.24	3.44 ± 0.24	0.04	0.93	0.39
Albumin (g/dl) D[7]	0.43 ± 0.2	0.26 ± 0.19	0.14 ± 0.19	0.53 ± 0.17	0.62 ± 0.19	0.62 ± 0.19	0.05	0.86	0.58
Bilirubin (mg/dl) Day 0	0.08 ± 0.006	0.10 ± 0.01	0.10 ± 0.007	0.10 ± 0.006	0.10 ± 0.007	0.10 ± 0.007	0.38	0.45	0.45
Bilirubin (mg/dl) Day82	0.13 ± 0.02	0.14 ± 0.02	0.16 ± 0.02	0.16 ± 0.02	0.14 ± 0.02	0.16 ± 0.02	0.64	0.79	0.79

Table 9. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate on serum biochemistries in Beagle dogs on day 0 (initial), day 82 (final), and the difference between initial and final [1] (Continued)

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Variable									
Bilirubin (mg/dl) D[7]	0.04 ± 0.02	0.04 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.04 ± 0.02	0.06 ± 0.02	0.79	0.76	0.93
ALP[8] (IU/L) Day 0	91.16 ± 16.51	95.00 ± 18.1	117.00 ± 18.1	75.33 ± 16.51	75.80 ± 18.09	101.60 ± 18.09	0.25	0.28	0.99
ALP (IU/L) Day 82	143.50 ± 41.08	124.60 ± 45.0	222.00 ± 45.01	64.66 ± 41.08	74.40 ± 45.01	110.00 ± 45.01	0.03	0.26	0.79
ALP (IU/L) D[7]	52.33 ± 31.19	29.60 ± 34.2	105.00 ± 34.2	-10.66 ± 31.19	-1.40 ± 34.17	8.40 ± 34.17	0.02	0.41	0.63
ALT[9] (IU/L) Day 0	60.00 ± 9.38	39.40 ± 10.3	39.00 ± 110.3	47.75 ± 9.38	45.20 ± 10.28	42.80 ± 10.28	0.91	0.35	0.60
ALT (IU/L) Day 82	56.66 ± 9.78	41.20 ± 10.7	57.60 ± 10.7	38.83 ± 9.78	56.80 ± 10.72	56.20 ± 10.72	0.88	0.64	0.28
ALT (IU/L) D[7]	-3.33 ± 12.75	1.80 ± 14.0	18.60 ± 14.0	-8.91 ± 12.75	11.60 ± 13.97	13.40 ± 13.97	0.97	0.26	0.81
Na[10] (mEq/l) Day 0	148.66 ± 1.09	148.80 ± 1.2	150.00 ± 1.2	149.16 ± 1.09	147.80 ± 1.19	146.60 ± 1.19	0.18	0.82	0.25
Na (mEq/l) Day 82	101.16 ± 33.39	59.40 ± 36.6	92.20 ± 36.6	75.83 ± 33.39	59.80 ± 36.58	90.00 ± 36.58	0.75	0.63	0.91
Na (mEq/l) D[7]	-47.50 ± 33.3	-89.40 ± 36.4	-57.80 ± 36.4	-73.33 ± 33.26	-88.00± 36.43	-56.60 ± 36.43	0.79	0.63	0.90
K[11] (mEq/l) Day 0	4.55 ± 0.10	4.46 ± 0.1	4.62 ± 0.1	4.50 ± 0.10	4.44 ± 0.11	4.42 ± 0.11	0.34	0.76	0.71
K (mEq/l) Day 82	3.18 ± 1.03	1.88 ± 1.1	2.96 ± 1.1	2.35 ± 1.03	1.82 ± 1.13	2.56 ± 1.13	0.63	0.64	0.93
K (mEq/l) D[7]	-1.36 ± 1.03	-2.58 ± 1.1	-1.66 ± 1.1	-2.15 ± 1.03	-2.62 ± 1.13	-1.86 ± 1.13	0.70	0.68	0.93
Cl[12] (mEq/l) Day 0	115.00 ± 1.02	115.60 ± 1.1	115.00 ± 1.12	115.33 ± 1.02	114.40 ± 1.12	114.60 ± 1.12	0.64	0.94	0.77
Cl (mEq/l) Day 82	77.16 ± 25.4	45.00 ± 27.9	70.60 ± 27.85	57.00 ± 25.42	45.60 ± 27.85	69.40 ± 27.85	0.75	0.62	0.90
Cl (mEq/l) D[7]	-37.83 ± 25.5	-70.60 ± 28.0	-44.40 ± 27.98	-58.33 ± 25.54	-68.80± 27.98	-45.20 ± 27.98	0.77	0.62	0.89
Ca[13] (mg/dl) Day 0	9.56 ± 0.16	9.50 ± 0.2	9.92 ± 0.17	9.56 ± 0.16	9.64 ± 0.17	9.72 ± 0.17	0.88	0.27	0.63

Table 9. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate on serum biochemistries in Beagle dogs on day 0 (initial), day 82 (final), and the difference between initial and final [1] (Continued)

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Variable									
Ca (mg/dl) Day 82	9.83 ± 0.46	9.26 ± 0.5	10.38 ± 0.51	10.28 ± 0.46	10.58 ± 0.51	10.38 ± 0.51	0.15	0.65	0.43
Ca (mg/dl) D[7]	0.26 ± 0.44	-0.24 ± 0.48	0.46 ± 0.48	0.71 ± 0.44	0.94 ± 0.48	0.66 ± 0.48	0.12	0.90	0.58
P[14] (mg/dl) Day 0	4.71 ± 0.32	4.36 ± 0.36	4.74 ± 0.36	4.63 ± 0.32	4.38 ± 0.36	4.02 ± 0.36	0.36	0.60	0.54
P (mg/dl) Day 82	5.20 ± 0.52	4.86 ± 0.57	4.22 ± 0.57	4.80 ± 0.52	4.88 ± 0.57	4.68 ± 0.57	0.95	0.59	0.73
P (mg/dl) D[7]	0.48 ± 0.52	0.50 ± 0.57	-0.52 ± 0.57	0.16 ± 0.52	0.50 ± 0.57	0.66 ± 0.57	0.53	0.75	0.38

[1] Values are means ± SEM
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate
[6] Blood Urea Nitrogen
[7] Difference between initial and final
[8] Alkaline Phosphatase
[9] Alanine aminotransferase
[10] Sodium
[11] Potassium
[12] Chloride
[13] Calcium
[14] Phosphorus

Table 10. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on complete blood count (CBC) in Beagle dogs for day 0 (initial), day 82 (final), and the difference between initial and final [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Variable									
HGB[6] (g/dl) Day 0	16.30 ± 0.63	17.22 ± 0.69	14.80 ± 0.69	18.08 ± 0.69	15.96 ± 0.69	17.22 ± 0.7	0.0898	0.2363	0.0290
HGB (g/dl) Day 82	16.30 ± 1.29	16.88 ± 1.29	12.56 ± 1.29	15.70 ± 1.17	15.70 ± 1.29	16.28 ± 1.4	0.5475	0.3353	0.1552
HGB (g/dl) D[7]	-0.06 ± 1.43	-0.34 ± 1.43	-2.24 ± 1.43	-1.60 ± 1.43	-0.26 ± 1.43	-0.85 ± 1.6	0.9846	0.7032	0.6140
HCT[8] (%) Day 0	47.87 ± 2.19	50.44 ± 2.40	43.76 ± 2.40	55.34 ± 2.40	46.36 ± 2.40	50.20 ± 2.4	0.1028	0.1505	0.0422
HCT (%) Day 82	46.38 ± 3.70	48.20 ± 3.70	35.18 ± 3.70	44.45 ± 3.38	43.64 ± 3.70	45.95 ± 4.2	0.6440	0.3228	0.1245
HCT (%) D	-1.82 ± 4.29	-2.24 ± 4.29	-8.58 ± 4.29	-8.46 ± 4.29	-2.72 ± 4.29	-4.13 ± 4.8	0.8059	0.6693	0.4636
RBC[9] (x10^6/ul) Day 0	7.14 ± 0.34	7.41 ± 0.37	6.32 ± 0.37	8.24 ± 0.37	6.84 ± 0.37	7.47 ± 0.4	0.0721	0.1045	0.0508
RBC (x10^6/ul) Day 82	6.83 ± 0.55	7.03 ± 0.55	5.11 ± 0.55	6.66 ± 0.50	6.33 ± 0.55	6.79 ± 0.6	0.5594	0.3120	0.1080
RBC (x10^6/ul) D	-0.28 ± 0.61	-0.38 ± 0.61	-1.21 ± 0.61	-1.28 ± 0.61	-0.54 ± 0.61	-0.66 ± 0.7	0.6961	0.7443	0.4763
WBC[10] (n^{11}/ul) Day 0	9.78 ± 0.89	10.38 ± 0.98	9.38 ± 0.98	10.22 ± 0.89	8.04 ± 0.98	10.20 ± 1.0	0.6437	0.6917	0.2226
WBC (n/ul) Day 82	8.72 ± 1.51	10.10 ± 1.66	8.34 ± 1.66	11.40 ± 1.51	8.76 ± 1.66	8.22 ± 1.7	0.7590	0.5374	0.4336
WBC (n/ul) D	-1.07 ± 1.22	-0.28 ± 1.33	-1.04 ± 1.33	1.18 ± 1.22	0.72 ± 1.33	-1.98 ± 1.3	0.4736	0.3647	0.4674
SEG[12] (n/ul) Day 0	6581 ± 678	7322 ± 678	6922 ± 678	7915 ± 619	5511 ± 678	7205 ± 678	0.9069	0.4394	0.0749
SEG (n/ul) Day 82	6166 ± 1364	6672 ± 1364	5906 ± 1364	8574 ± 1245	5980 ± 1364	5593 ± 1364	0.6736	0.4768	0.4548
SEG (n/ul) D	-414.8 ± 1036	-650.6 ± 1036	-1016 ± 1036	659.0 ± 946	469.0 ± 1036	-1613 ± 1036	0.5291	0.3345	0.6420

Table 10. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate on complete blood count (CBC) in Beagle dogs for day 0 (initial), day 82 (final), and the difference between initial and final [1] (Continued)

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Variable									
Band[13] (n/ul) Day 82	4.7 ± 37.47	51.6 ± 41.05	120.0 ± 41.05	16.00 ± 41.05	13.80 ± 41.05	62.8 ± 41.1	0.4068	0.1384	0.6809
Lymph[14] (n/ul) Day 0	1883.67 ± 298	2337.20 ± 327	2074.40 ± 327	1722.17 ± 298	2139.60 ± 327	2687.60 ± 330	0.7466	0.1701	0.3787
Lymph (n/ul) Day 82	1731.33 ± 367	2630.60 ± 402	1949.40 ± 402	2049.00 ± 367	2247.40 ± 402	1558.80 ± 400	0.6377	0.2119	0.5693
Lymph (n/ul) D	-152.33 ± 391	293.40 ± 429	-125.00 ± 429	326.83 ± 391	107.8 ± 429	-1129 ± 430	0.4925	0.1274	0.2147
Mono[15] (n/ul) Day 0	193.00 ± 73.9	374.40 ± 80.9	235.67 ± 104	222.8 ± 80.9	177.0 ± 80.91	273.33 ± 100	0.5550	0.6880	0.3015
Mono (n/ul) Day 82	322.83 ± 126	379.20 ± 138	146.20 ± 138	326.80 ± 138	301.00 ± 138	635.00 ± 140	0.2237	0.8799	0.1023
Mono (n/ul) D	129.83 ± 152	4.80 ± 167	-31.33 ± 216	104.00 ± 167	124.00 ± 167	114.67 ± 220	0.5979	0.9110	0.8681
Eos[16] (n/ul) Day 0	254.80 ± 67.9	346.20 ± 67.9	188.60 ± 67.9	156.00 ± 87.6	265.00 ± 75.9	238.67 ± 87.6	0.4954	0.3457	0.5866
Eos (n/ul) Day 82	123.33 ± 78.9	367.00 ± 86.4	218.60 ± 86.4	182.60 ± 86.4	217.40 ± 86.4	490.80 ± 86.4	0.3917	0.0673	0.0690
Eos (n/ul) D	-112.40 ± 124	20.80 ± 124	30.00 ± 124	121.67 ± 160	-45.25 ± 138	256.33 ± 160	0.2618	0.4907	0.4643
MCV[17] (fl[18]) Day 0	67.15 ± 0.77	68.20 ± 0.84	69.14 ± 0.84	67.00 ± 0.77	67.48 ± 0.84	67.30 ± 0.8	0.1832	0.3484	0.5902
MCV (fl) Day 82	67.43 ± 0.72	68.68 ± 0.79	69.22 ± 0.79	66.70 ± 0.72	68.96 ± 0.79	67.70 ± 0.8	0.3035	0.0630	0.5286
MCV (fl) D	0.28 ± 0.59	0.48 ± 0.64	0.08 ± 0.64	-0.27 ± 0.59	1.48 ± 0.64	0.40 ± 0.6	0.6190	0.2843	0.4580
MCHC[19] (g/dl) Day 0	34.08 ± 0.48	34.28 ± 0.53	33.96 ± 0.53	33.17 ± 0.48	34.50 ± 0.53	34.32 ± 0.5	0.7907	0.3126	0.3851
MCHC (g/dl) Day 82	35.65 ± 0.55	35.04 ± 0.60	35.74 ± 0.60	35.43 ± 0.55	35.98 ± 0.60	36.44 ± 0.6	0.3289	0.5540	0.5681

Table 10. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate on complete blood count (CBC) in Beagle dogs for day 0 (initial), day 82 (final), and the difference between initial and final [1] (Continued)

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Variable									
MCHC (g/dl) D	1.57 ± 0.59	0.76 ± 0.64	1.78 ± 0.64	2.27 ± 0.59	1.48 ± 0.64	2.12 ± 0.6	0.2607	0.3487	0.9449
MCH[20] (pg) Day 0	22.88 ± 0.45	23.36 ± 0.49	23.46 ± 0.49	22.22 ± 0.45	23.32 ± 0.49	23.08 ± 0.6	0.3725	0.1985	0.8047
MCH (pg) Day 82	24.00 ± 0.41	24.08 ± 0.45	24.76 ± 0.45	23.65 ± 0.41	24.82 ± 0.45	23.98 ± 0.5	0.7193	0.2958	0.2429
MCH (pg) D	1.12 ± 0.43	0.72 ± 0.48	1.30 ± 0.48	1.43 ± 0.43	1.50 ± 0.48	0.90 ± 0.5	0.5521	0.9108	0.4909

[1] Values are means ± SEM.
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate
[6] Hemoglobin
[7] Difference between initial and final
[8] Hematocrit
[9] Red Blood Cells
[10] White Blood Cells
[11] Number of cells
[12] Segmented neutrophils
[13] Band neutrophils
[14] Lymphocytes
[15] Monocytes
[16] Eosinophils

Table 10. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate on complete blood count (CBC) in Beagle dogs for day 0 (initial), day 82 (final), and the difference between initial and final [1] (Continued)

[17] Mean Corpuscular Volume
[18] Fentoliters
[19] Mean Corpuscular Hemoglobin Concentration
[20] Mean Corpuscular Hemoglobin

Table 11. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on white blood cell and lymphocyte numbers in Beagle dogs on day 0 (initial), day 82 (final) and the difference between initial and final. The differences between day 82 and designated dates post vaccination are also shown. [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
WBC Day 0	9.8 ± 0.89	10.4 ± 0.98	9.4 ± 0.98	10.2 ± 0.89	8.0 ± 0.98	10.2 ± 0.98	0.6437	0.6917	0.2226
WBC Day 82	8.7 ± 1.5	10.1 ± 1.7	8.3 ± 1.7	11.4 ± 1.5	8.8 ± 1.7	8.2 ± 1.7	0.7590	0.5374	0.4336
WBC D[6]	-1.1 ± 1.2	-0.3 ± 1.3	-1.0 ± 1.3	1.2 ± 1.2	0.7 ± 1.3	-2.0 ± 1.3	0.4736	0.3647	0.4674
WBC Day 92[7]	18.8 ± 2.2	10.9 ± 2.4	17.2 ± 2.4	13.8 ± 2.2	14.2 ± 2.4	18.8 ± 2.4	0.9812	0.0898	0.1777
WBC Day 95[8]	1.3 ± 1.4	0.4 ± 1.5	3.0 ± 1.5	1.2 ± 1.4	-0.1 ± 1.5	2.6 ± 1.5	0.7754	0.2336	0.9940
WBC Day 106[9]	20.2 ± 3.5	13.2 ± 3.8	17.4 ± 3.8	10.4 ± 3.5	10.3 ± 3.8	13.3 ± 3.8	0.0779	0.5628	0.6077
WBC Day 109[10]	2.4 ± 1.7	1.7 ± 1.8	2.0 ± 1.8	2.0 ± 1.7	-0.6 ± 1.8	2.6 ± 1.8	0.6292	0.5505	0.7265
Lymph Day 0	1884 ± 300	2337 ± 330	2074 ± 330	1722 ± 300	2140 ± 330	2688 ± 330	0.7466	0.1701	0.3787
Lymph Day 82	1731 ± 370	2631 ± 400	1949 ± 400	2049 ± 370	2247 ± 400	1559 ± 400	0.6377	0.2119	0.5693
Lymph D[6]	-152 ± 400	293 ± 400	-125 ± 400	327 ± 400	108 ± 400	-1129 ± 400	0.4925	0.1274	0.2147
Lymph Day 92[7]	-394 ± 34	-414 ± 380	-290 ± 380	-1047 ± 340	-391 ± 380	701 ± 380	0.6910	0.0508	0.0934
Lymph Day 95[8]	-408 ± 400	-872 ± 430	29 ± 430	10 ± 400	36 ± 430	817 ± 430	0.0511	0.1509	0.8255
Lymph Day 106[9]	-415 ± 420	-668 ± 460	234 ± 460	-492 ± 420	-79 ± 460	355 ± 460	0.5670	0.2080	0.7462
Lymph Day 109[10]	-520 ± 340	-637 ± 370	631 ± 370	-353 ± 340	-367 ± 370	502 ± 370	0.7291	0.0110	0.8545

[1] Values are means ± SEM

Table 11. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on white blood cell and lymphocyte numbers in Beagle dogs on day 0 (initial), day 82 (final) and the difference between initial and final. The differences between day 82 and designated dates post vaccination are also shown. [1] (Continued)

[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate
[6] Difference between day 82 and day 0
[7] Difference between day 92 and day 82
[8] Difference between day 95 and day 82
[9] Difference between day 106 and day 82
[10] Difference between day 109 and day 82

Table 12. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on the delayed type hypersensitivity skin test in Beagles at the various time points when measurements were taken [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
	mm of induration								
Time: 15 min	8.67 ± 0.93	8.00 ± 1.02	6.40 ± 1.02	7.50 ± 0.93	7.20 ± 1.02	6.60 ± 1.02	0.4714	0.2734	0.7747
Time: 30 min	7.33 ± 1.14	8.80 ± 1.24	7.60 ± 1.24	8.83 ± 1.14	6.40 ± 1.24	8.00 ± 1.24	0.8673	0.9196	0.2672
Time: 24 h	11.00 ± 1.74	10.80 ± 1.91	10.60 ± 1.91	11.33 ± 1.74	9.20 ± 1.91	10.40 ± 1.91	0.7494	0.8140	0.8652
Time: 48 h	14.00 ± 1.69	16.80 ± 1.85	13.60 ± 1.85	12.92 ± 1.69	11.40 ± 1.84	11.80 ± 1.85	0.0710	0.7525	0.4508
Time: 72 h	12.20 ± 1.10	18.20 ± 1.10	11.40 ± 1.10	14.00 ± 1.10	11.20 ± 1.10	12.80 ± 1.10	0.1730	0.0795	0.0006
Time: 96 h	11.60 ± 1.00	16.00 ± 1.00	9.40 ± 1.00	13.20 ± 1.00	11.20 ± 1.00	11.80 ± 1.00	0.7476	0.0214	0.0026

[1] Values are means ± SEM
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate

Table 13. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on PGE_2 production in Beagle dogs on day 0 (initial), day 82 (final) and the difference between initial and final [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
PGE_2	pg/ml								
Day 0	3.4 ± 1.6	2.6 ± 1.7	4.5 ± 1.7	4.5 ± 1.6	2.8 ± 1.7	7.8 ± 1.7	0.2734	0.1548	0.6619
Day 82	7307 ± 1981	6059 ± 2170	4416 ± 2170	5632 ± 1981	2986 ± 2170	6693 ± 2170	0.6362	0.6492	0.4494
Difference[6]	7304 ± 1981	6057 ± 2170	4412 ± 2170	5627 ± 1981	29983 ± 2170	6685 ± 2170	0.6356	0.6496	0.4499
Day 104	5227 ± 2670	7255 ± 2957	10006 ± 2957	12244 ± 2670	6055 ± 2957	8983 ± 2957	0.5019	0.6132	0.2607
Difference[7]	-2080 ± 3417	1195 ± 3743	5589 ± 3743	6612 ± 3417	3069 ± 3743	2290 ± 3743	0.4222	0.8628	0.2593

[1] Values are means ± SEM
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate
[6] The difference between day 82 and day 0
[7] The difference between day 104 and day 82

Table 14. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on KLH antibody log titer in Beagle dogs after vaccination with KLH [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Log Titer	9.14 ± 0.19	9.28 ± 0.21	9.57 ± 0.21	9.84 ± 0.19	9.28 ± 0.21	9.65 ± 0.23	0.1296	0.2959	0.1752

[1] Values are means ± SEM
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate

Table 15. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on fluorescent bead engulment by macrophages isolated from Beagle dogs for day 0 (initial), day 82 (final) and the difference between initial and final [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
	% of beads engulfed								
Day 0	24.69 ± 1.9	23.08 ± 2.1	24.27 ± 2.1	26.84 ± 1.9	25.71 ± 2.3	26.46 ± 2.1	0.1825	0.8009	0.9922
Day 82	29.31 ± 2.6	27.81 ± 2.9	26.99 ± 2.9	30.88 ± 2.6	21.95 ± 2.9	29.17 ± 2.9	0.7712	0.2353	0.3745
Difference	4.62 ± 3.0	4.73 ± 3.3	2.72 ± 3.3	4.03 ± 3.0	-1.25 ± 4.3	2.71 ± 3.29	0.4344	0.7376	0.6635

[1] Values are means ± SEM
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate

Table 16. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on characteristics of T-cell subpopulations in Beagle dogs [1]

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Variable	% of + cells								
CD4+, Day 0	32.4 ± 3	31.9 ± 4	27.3 ± 4	30.7 ± 3	31.2 ± 4	32.8 ± 4	0.28	0.40	0.48
Day 82	26.8 ± 3	28.0 ± 3	26.5 ± 3	31.7 ± 3	32.6 ± 3	28.3 ± 3	0.16	0.67	0.87
Difference	-5.6 ± 3	-3.9 ± 3	-0.8 ± 3	1.1 ± 3	-5.6 ± 3	-4.5 ± 3	0.85	0.63	0.15
Day 95[6]	2.8 ± 2	1.3 ± 2	2.7 ± 2	6.0 ± 2	1.1 ± 2	1.4 ± 2	0.77	0.40	0.61
Day 109[7]	-5.4 ± 3	-7.7 ± 4	0.4 ± 4	1.0 ± 3	-10.6 ± 4	-5.0 ± 4	0.83	0.09	0.19
CD8+, Day 0	19.2 ± 2	28.1 ± 2	16.8 ± 2	17.0 ± 2	22.9 ± 2	22.0 ± 2	0.75	0.002	0.30
Day 82	13.2 ± 2	25.4 ± 2	16.5 ± 2	14.1 ± 2	18.3 ± 2	20.5 ± 2	0.87	0.001	0.29
Difference	-6.0 ± 2	-2.7 ± 2	-0.3 ± 2	-2.8 ± 2	-4.5 ± 2	-1.6 ± 2	0.80	0.09	0.17
Day 95	2.9 ± 1	-0.1 ± 1	0.2 ± 1	1.7 ± 1	0.4 ± 1	0.2 ± 1	0.81	0.11	0.76
Day 109	-0.4 ± 1	-5.6 ± 1	-1.6 ± 1	-2.5 ± 1	-4.3 ± 1	-3.8 ± 1	0.32	0.03	0.29
Ratio, Day 0	1.7 ± 0.2	1.2 ± 0.2	1.6 ± 0.2	1.8 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	0.44	0.18	0.42
Day 82	2.1 ± 0.3	1.3 ± 0.3	1.6 ± 0.2	2.4 ± 0.3	1.7 ± 0.3	1.4 ± 0.3	0.47	0.02	0.51
Difference	0.4 ± 0.2	0.1 ± 0.2	-0.01 ± 0.2	0.6 ± 0.2	0.1 ± 0.2	-0.1 ± 0.2	0.77	0.05	0.82
Day 95	-0.3 ± 0.1	-0.02 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	-0.05 ± 0.1	0.03 ± 0.1	0.58	0.33	0.14

Table 16. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on characteristics of T-cell subpopulations in Beagle dogs [1] (Continued)

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Variable	% of + cells								
Day 109	-0.3 ± 0.2	-0.1 ± 0.2	0.3 ± 0.2	0.5 ± 0.2	-0.3 ± 0.2	0.05 ± 0.2	0.44	0.28	0.05
CD4+ IL2R, Day 0	5.6 ± 1	3.8 ± 1	3.9 ± 1	3.5 ± 1	5.1 ± 1	4.0 ± 1	0.82	0.88	0.36
Day 82	-0.1 ± 0.3	-0.3 ± 0.3	-0.3 ± 0.3	0.1 ± 0.3	-0.03 ± 0.3	0.3 ± 0.3	0.21	0.84	0.72
Difference	-5.7 ± 1	-4.0 ± 1	-4.2 ± 1	-3.5 ± 1	-5.2 ± 1	-3.7 ± 1	0.64	0.88	0.48
Day 95	3.5 ± 1	3.0 ± 1	3.7 ± 1	3.4 ± 1	3.4 ± 1	2.7 ± 1	0.61	0.90	0.62
Day 109	-2.4 ± 1	-2.0 ± 1	-1.2 ± 1	1.1 ± 1	-1.0 ± 1	-2.0 ± 1	0.29	0.74	0.29
CD8+ IL2R, Day 0	1.8 ± 1	1.0 ± 1	0.2 ± 1	1.7 ± 1	1.0 ± 1	0.5 ± 1	0.87	0.11	0.94
Day 82	1.7 ± 0.5	-0.02 ± 0.5	0.1 ± 0.5	0.2 ± 0.5	-0.1 ± 0.5	-0.2 ± 0.5	0.15	0.09	0.32
Difference	-0.2 ± 0.4	-0.7 ± 0.5	-0.1 ± 0.5	-1.5 ± 0.4	-0.9 ± 0.5	-0.7 ± 0.5	0.08	0.56	0.50
Day 95	-0.5 ± 0.5	1.2 ± 0.5	1.3 ± 0.5	1.0 ± 0.5	1.2 ± 0.5	0.8 ± 0.5	0.42	0.10	0.11
Day 109	-2.2 ± 1	-0.04 ± 1	-2.0 ± 1	-0.3 ± 1	-0.2 ± 1	-0.2 ± 1	0.19	0.54	0.56
CD4+ CD69+ Day 0	11.9 ± 1	10.0 ± 2	10.2 ± 2	11.6 ± 1	12.9 ± 2	14.3 ± 2	0.09	0.88	0.33
Day 82	9.8 ± 1	7.7 ± 1	7.6 ± 1	9.0 ± 1	8.5 ± 1	8.9 ± 1	0.60	0.37	0.53
Difference	-2.1 ± 1	-2.3 ± 1	-2.6 ± 1	-2.6 ± 1	-4.4 ± 1	-5.4 ± 1	0.08	0.37	0.59

Table 16. Effect of feeding diets that differed in the (n-6):(n-3) fatty acid ratio and level of α-tocopheryl acetate (αT) on characteristics of T-cell subpopulations in Beagle dogs [1] (Continued)

Dietary (n-3)	Low (n-3)			High (n-3)			P values		
Dietary αT[2]	Low αT	Med αT	High αT	Low αT	Med αT	High αT	Ratio[3]	TA[4]	Interaction[5]
Variable	% of + cells								
Day 92[8]	1.9 ± 1	1.6 ± 1	4.3 ± 1	1.3 ± 1	2.5 ± 1	2.3 ± 1	0.61	0.47	0.59
Day 106[9]	2.5 ± 1	1.9 ± 1	3.5 ± 1	1.0 ± 1	1.1 ± 0.6	0.7 ± 0.6	0.27	0.61	0.58
CD8+ CD69+ Day 0	4.1 ± 1	3.9 ± 1	3.7 ± 1	5.0 ± 1	5.6 ± 1	3.4 ± 1	0.40	0.50	0.68
Day 82	2.7 ± 0.6	2.5 ± 0.7	3.5 ± 0.7	3.1 ± 0.6	2.7 ± 0.7	1.6 ± 0.7	0.52	0.82	0.19
Difference	-1.4 ± 0.7	-1.5 ± 0.8	-0.2 ± 0.8	-1.8 ± 0.7	-2.8 ± 0.8	-1.8 ± 0.8	0.10	0.37	0.74
Day 92	1.1 ± 0.5	0.5 ± 0.6	0.8 ± 0.6	-0.02 ± 0.5	1.2 ± 0.6	0.7 ± 0.6	0.62	0.87	0.24
Day 106	1.5 ± 0.6	2.3 ± 0.6	1.0 ± 0.6	0.9 ± 0.6	1.1 ± 0.6	1.4 ± 0.6	0.36	0.62	0.42

[1] Values are means ± SEM.
[2] dl-α-tocopheryl acetate
[3] P value for the (n-6):(n-3) fatty acid ratio factor
[4] P value for the dl-α-tocopheryl acetate factor
[5] P value for the the interaction between the (n-6):(n-3) fatty acid ratio and dl-α-tocopheryl acetate
[6] The difference between day 82 and the 4th day after the first vaccination.
[7] The difference between day 82 and the 4th day after the "booster" vaccination.
[8] The difference between day 82 and the 1st day after the first vaccination.
[9] The difference between day 82 and the 1st day after the "booster" vaccination.